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Deep phenotyping and genotyping of chemotherapy-associated neurotoxicity in children on ALL therapy

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MBBS, MCPS (Clinical Pathology), MPhil (Haematology)

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Abstract

Paediatric ALL is the commonest childhood cancer. Although cure rates are good, treatment is prolonged and toxic. Chemotherapy-induced neurotoxicity remains a significant problem with incidence reported 8-12%. This study broadly aimed to accurately diagnose different forms of neurotoxicity in patients, to be able to identify genetic and environmental risk factors and understand the natural history of different types of neurotoxicity to counsel families, regarding supportive interventions, and decisions on whether to modify treatment.

Exploration of clinical risk-profiling of neurotoxicity and the effects of a neurotoxic event on leukaemia outcome was conducted using SAE reports from the United Kingdom Acute Lymphoblastic Leukaemia (UKALL) 2003 trial. Review of the 276 neurotoxic events identified treatment intensity as the main risk factor for developing neurotoxicity with female sex, increasing age and CNS status having a significant modifying effect.

Next, a large retrospective pooled central neurotoxicity detailed dataset (n=1813) from 14 international study groups (all members of the Ponte di Legno (PdL) consortium) was created and used to deep phenotype and genotype neurotoxic events. The main analysis concentrated on the 3 most common diagnoses: seizures, posterior reversible encephalopathy syndrome (PRES) and stroke-like syndrome (SLS). Initial work identified significant overlap between the diagnostic criteria for SLS and PRES with many cases fulfilling both definitions (i.e., bi-phenotypic). Using multivariate logistic regression analysis odds ratio values for specific clinical and radiology findings were used to construct a diagnostic scoring system for PRES and SLS which successfully separated the cases into distinct entities.

Deep phenotyping of seizure cases reported in the PdL database showed recurrence, Down syndrome, Capizzi methotrexate, Single intrathecal methotrexate, and late leucovorin rescue was associated with an increased risk of seizures compared to the rest of the neurotoxicity cohort. Deep phenotyping of PRES cases showed younger age group (< 10 years), T-cell and CNS involvement, high vincristine dose protocol and use of oral anticonvulsants were significantly associated with PRES. Finally, SLS cases showed older age, consolidation with high dose methotrexate and High-risk treatment regimen allocation to be associated with SLS. Another important finding, with clinical implications, was that patients with Down syndrome ALL showed a significant association with recurrence of neurotoxicity upon re-exposure to the causative agent.

Following deep phenotyping, cases with defined phenotypes were picked to enter a targeted meta-analysis of existing single nucleotide polymorphism (SNPs) data. A list of candidate SNPs of interest was generated and sent to 3 study groups who then compared allele frequencies between cases (with neurotoxicity) and matched controls (without neurotoxicity). Results were combined and analyzed using METAL software. Although a nominally significant association with some candidate SNPs was observed for all the selected phenotypes no individual SNP remained significant after adjustment for multiple testing.

Finally, using novel in silico analysis of two published datasets an attempt was made to correlate changes in genes associated with clinical episodes of neurotoxicity with methylation patterns of genes in response to methotrexate treatment in vivo. Two genes (PDE4B and ASTN2) were that were consistently hypermethylated in response to all three doses (20, 30 and 50 nM) of methotrexate and mapped with significant genetic association in clinical neurotoxicity in paediatric ALL cases post methotrexate in an in-silico analysis. These results may shed some light on potential genetic predispositions to methotrexate induced neurotoxicity.

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List of Abbreviations

ALL	Acute lymphoblastic leukaemia
ADC	Apparent diffusion coefficient
AIEOP	Associazione Italiana Ematologia Oncologia Pediatrica
ANZCHOG	Australian and New Zealand Children's Hematology/Oncology Group
B-ALL	B-cell acute lymphoblastic leukaemia
BBB	Blood brain barrier
BFM	Berlin-Frankfurt-Munster study group
CNS	Central nervous system
COALL	Cooperative Study Group for Childhood ALL (Germany)
COG	Children’s Oncology Group (USA)
CPH	Czech Working Group for Pediatric Hematology
DCOG	Dutch Childhood Oncology Group
DNA	Deoxyribonucleic acid
DWI	Diffusion weighted imaging
EFS	Event free survival
HR	High risk
HSCT	Hematopoietic stem cell transplantation
IV	Intravenous
IT	Intrathecal

MRD	Minimal residual disease
MR	Medium risk
MRI	Magnetic resonance imaging
6 MP	6-mercaptopurines
MTX	Methotrexate
NOPHO	Nordic Society of Paediatric Haematology and Oncology
OS	Overall survival
PdL	Ponte di Legno
PRES	Posterior reversible encephalopathy syndrome
SAE's	Severe adverse events
SIT	Single intrathecal therapy
SLS	Stroke like syndrome
SNP	Single polymorphic nucleotide
SR	Standard risk
T-cell	T cell immunophenotype
TPOG	Taiwan Pediatric Oncology Group
TIT	Triple intrathecal therapy
T2 FLAIR	T2-weighted-Fluid-Attenuated Inversion Recovery
VCR	Vincristine
UK	United Kingdom
UKALL	United Kingdom ALL study group

List of Publications arising from this work:

Abstract publication:

Acute Neurotoxicity during ALL Therapy Is Associated with Treatment Intensity, Age and Female Sex - an Analysis of SAE Reports from the UKALL 2003 Trial

Qurat-ul-Ain Wahid, Lina Hamadeh, Sheena McGowan, Rachael Hough, Ajay Vora, Anthony Moorman, Christina Halsey. (2018) Blood 132 (Supplement 1), 1379-1379 (Poster presentation in American Society of Haematology Conference 2018 (1-4th December) held in San Diego, United States of America).

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Author’s declaration

The work presented in this thesis was carried out by the author, except where otherwise acknowledged. This thesis has not been previously submitted for any degree at this or any other institution.

Qurat-ul-Ain Wahid

May 2022

1 Introduction

1.1 Acute Lymphoblastic Leukaemia (ALL)

Childhood Acute Lymphoblastic Leukaemia (ALL) is the most common cancer in children, accounting for almost 20% of all childhood cancer and it is the leading cause of cancer deaths in children [1]. In the United Kingdom (UK), approximately 791 children are diagnosed with ALL each year. The peak incidence of ALL is observed between 0-4 years of age and decreases sharply thereafter. The incidence of ALL is slightly higher in males than females, with a ratio of 1.4:1 [2].

1.1.1 Disease introduction:

The leukemogenic transformation in ALL affects hematopoietic precursors especially, white blood cells of the lymphoid lineage of the immune system and developing B cells and T cells. As a result, these cells are transformed into immature, malignant, lymphoid stem cells capable of replicating uncontrollably and avoiding natural cell death mechanisms. ALL is a systemic disease that originates from – and accumulates within – the bone marrow and that is known to infiltrate various extramedullary sites around the body including the spleen, kidney, liver, testes, and the central nervous system (CNS) [3].

Over the past sixty years there has been a dramatic increase in the efficacy of ALL treatment, and it is now widely regarded as one of the most successful treatment regimens for cancer, with some countries achieving overall survival rates of more than 90% (Figure 1.1) [4]. This can be attributed to the introduction of multiagent chemotherapy in the 1960's, laying the foundation for the modern approaches to ALL therapy [5]. Several developments have contributed to this achievement, via international collaborative groups, such as the incremental intensifying treatment strategies applied with each clinical trial as well as accurate and refined risk stratification for patients [5,6].

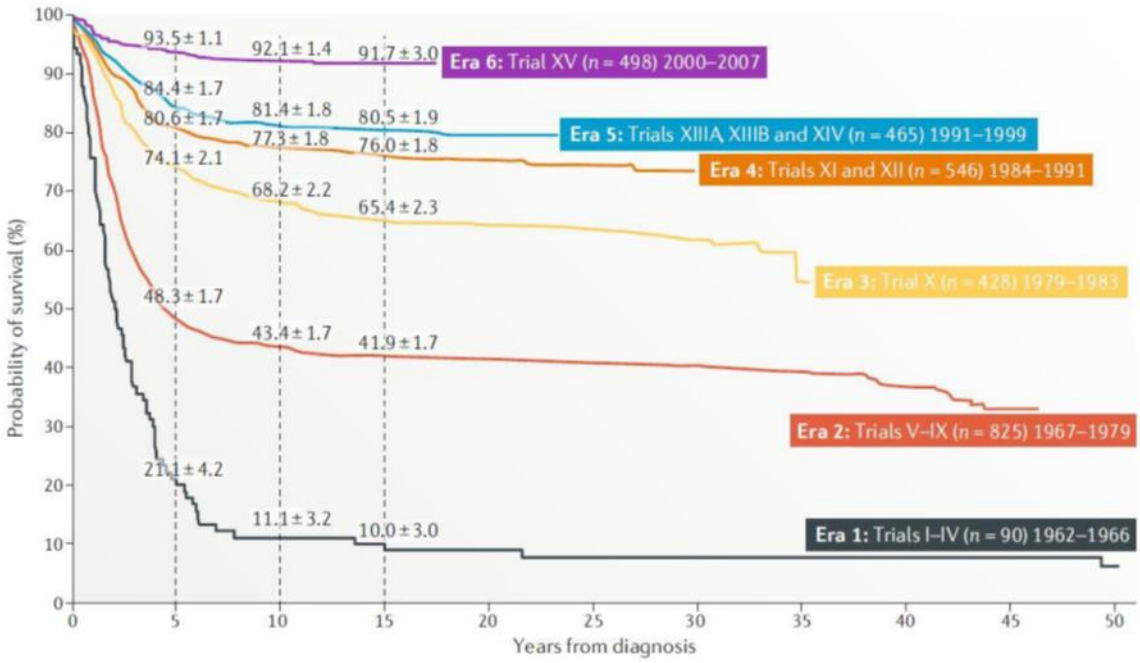


Figure 1.1: Improvements in the overall survival (OS) with the evolution of treatment of paediatric patients with ALL over time.

OS curves range across 6 eras defined by the introduction of novel treatment strategies for a total of 2,852 children with newly-diagnosed ALL who were enrolled in 15 consecutive Total Therapy studies (I-XV) conducted at St. Jude Clinical Research Hospital from 1962 to 2007 [4].

1.1.2 Broad overview of the management:

The present-day treatment protocols for ALL cure about 90% of children, although there are significant competing effects of disease relapses and treatment-related mortality that can occur during the course of treatment [4,6,7]. The treatment includes combined systemic and intrathecal chemotherapy based on risk stratification, with intensified treatment for high-risk patients as per protocol. Risk stratification is mainly based on the molecular response, Minimal Residual Disease (MRD), to the therapy. MRD measured by Polymerase Chain Reaction (PCR) and/or flow cytometry is the main prognostic marker for both B-ALL and T-ALL [8,9].

Risk-group stratification based on the molecular response is fundamental in ALL protocols. Broadly, the objective of the risk stratified ALL therapies is to achieve molecular remission (defined as MRD $<1x10^{-4}$) and then to maintain patients in the longer duration of remission through administration of risk-adapted systemic and intrathecal chemotherapy regimens [10,11]. High-risk patients are allocated more intensified chemotherapy and, in some cases, as per

respective protocol, require cranial irradiation and hematopoietic stem cell transplantation (HSCT) [12-14].

1.1.3 Prognostic factors and risk-stratification:

The standard risk group stratification and important prognostic indicators for paediatric ALL are outlined in Table 1., adapted from [4].

Table 1. Important Prognostic Factors in Acute Lymphoblastic Leukemia (ALL) in Children.*

Variable	Favorable Factor	Adverse Factor	Use in Risk Stratification
Demographic and clinical features			
Age	1 to <10 yr	<1 yr or ≥10 yr	This feature is a part of NCI risk group definition
Sex	Female	Male	No
Race or ethnic group	White, Asian	Black, Native American, Hispanic	No
Initial white-cell count	Lower (<50,000/mm^3)	Higher (≥50,000/mm^3)	Part of NCI risk group definition
Biologic or genetic features of leukemia cells			
Immunophenotype	B-cell lineage	T-cell lineage	Often used to select therapy backbone
Cytogenetic features	*ETV6-RUNX1*, hyperdiploidy, favorable chromosome trisomies	*BCR-ABL1*, *MLL* rearrangements, hypodiploidy	Often used to select treatment intensity, assign the patient to HSCT, or both; some features (e.g., *BCR-ABL1*) can be used to select targeted therapy
Genomic features	*ERG* deletions	*IKZF1* deletions or mutations; Philadelphia chromosome–like ALL with kinase gene alterations	Some research groups use *IKZF1* deletions to assign patients to more intensive therapy; kinase gene mutations may be used to assign patients to targeted therapy, but this is not yet part of routine care
Early response to treatment			
Response to 1 wk of glucocorticoid therapy	Good response to prednisone (<1000 blasts/mm^3)	Poor response to prednisone (≥1000 blasts/mm^3)	Easy to measure and used by many groups; may be supplanted by MRD
Marrow blasts after 1–2 wk of multiagent therapy	M1 marrow (<5% blasts) by day 8 or 15	No M1 marrow (≥5% blasts) by day 8 or 15	Easy to measure and used previously by many groups; now being supplanted by MRD
MRD quantitation during or at end of induction	Reaching low (<0.01%) or undetectable MRD by specific time points	Persistence of MRD ≥0.01% at specific time points; the higher it is, the worse the prognosis	Most important single prognostic factor for contemporary therapy; critical for modern risk stratification
MRD at 3–4 mo	Low (<0.01%), preferably undetectable	Persistence of MRD ≥0.01%	May help select patients for HSCT or new therapies in first remission

* HSCT denotes hematopoietic stem-cell transplantation, MRD minimal residual disease, and NCI National Cancer Institute.

Table 1.1: Important prognostic factors in Acute Lymphoblastic Leukaemia in children

Table and caption adapted from [4].

1.1.4 Treatment protocols:

Current paediatric ALL protocols are based on the risk- stratification and risk-modified therapies approach. The sequential remarkable improvements in overall survival and reduction in relapse in paediatric ALL has been possible with the development of international collaborative consortia [6]. A few examples of these consortia are stated here: Children's Oncology Group (COG) [15], Medical Research Council United Kingdom ALL Study group (MRC-UKALL) [13], Dana-Farber Consortium (DFCI) [16], Berlin-Frankfurt-Munster (BFM) [17], Nordic Society

for Paediatric Haematology Oncology (NOPHO) [18], and the Australian and New Zealand Children's Haematology Oncology Group Study 8 (ANZCHOG) [7].

1.1.5 Treatment:

ALL treatment protocols follow phases of treatment divided into induction remission, consolidation, CNS-directed therapy, re-induction/reconsolidation/delayed intensification /interim maintenance followed by maintenance therapy.

1.1.5.1 Remission Induction:

In order to achieve clinical and molecular remission, by eradicating the bulk of leukaemic blasts particularly in the bone marrow, patients are treated with dexamethasone/prednisolone, vincristine, daunorubicin, asparaginase, intrathecal methotrexate/ cytarabine/hydrocortisone and mercaptopurine for four to six weeks.

1.1.5.2 Consolidation/Intensification:

Combination therapies used to target any residual disease after remission induction are usually administered between ten- or twelve-weeks following remission induction. The combination and intensity are dependent upon clinical risk-status and MRD results. The drug combinations vary in different protocols but include methotrexate, mercaptopurine, asparaginase, and often cytarabine. CNS-directed therapy is administered to eliminate CNS disease.

1.1.5.3 Delayed Intensification/re-induction/interim maintenance:

This phase of therapy occurs towards the end of consolidation, typically of 3-8 weeks duration as per protocol, and is a shorter combination of the remission induction and consolidation with frequent pulses of vincristine and corticosteroids.

1.1.5.4 Maintenance:

Depending on the protocol, gender is the main determinant of duration of maintenance chemotherapy that lasts for two to three years if the leukaemia remained in remission after the previous stages. Typically, patients receive 6-mercaptopurine, methotrexate, vincristine, and either dexamethasone or prednisone as their treatment. During this stage, any residual/quiescent blasts are targeted, and CNS-prophylaxis continues.

1.1.6 CNS-directed therapy:

1.1.6.1 CNS disease:

In order to diagnose and monitor CNS disease, microscopic analysis of the CSF is carried out. CSF samples are obtained through lumbar puncture and examined under a microscope. The number of leukemic and non-leukemic cells in CSF is determined using automated cell counters, while morphological examination of CSF cytospin slides is done to identify and count leukaemic cells. CNS disease is classified based on these cyto-morphological results (Table 1.).

Category	Characterization
CNS-1	no blast cells in a sample of cerebrospinal fluid
CNS-2	< 5 WBC/mm^3 with blasts in a sample with < 10 erythrocytes/mm^3
CNS-3	> 5 WBC/mm^3 with blast cells in a sample with <10 erythrocytes/mm^3
TLP with blasts	> 10 erythrocytes/mm^3 with blast cells
TLP without blasts	> 10 erythrocytes/mm^3 without blast cells

Table 1.2: Diagnostic criteria for CNS disease

At the time of diagnosis, approximately three percent of patients have CNS-3, and fifteen percent have CNS-2, and inferior outcomes were reported in patients with CNS-3 disease, making CNS disease prognostically significant [19-21].

1.1.6.2 CNS relapse:

The term CNS relapse is used to describe a relapse where the CNS is involved. In the case of “isolated CNS relapse” patients may develop no evidence of relapsed ALL in the bone marrow, or they may develop the condition with concurrent evidence of relapsed ALL in the bone marrow, called "combined CNS relapse”.

1.1.6.3 Evolution of CNS-directed therapy:

There was an increase in the rate of Central Nervous System (CNS) relapse reported after the treatment of systemic ALL with chemotherapy improved with time [22]. The incidence of CNS leukaemia rose from 3% to 40% between 1947-60, during which time the median life expectancy of patients increased from four weeks to twelve months [23].

The early descriptions of meningeal ALL suggest that despite the treatment used at the time being effective, only limited penetration of the CNS was occurring, allowing a reservoir of leukaemic cells to survive the therapy [24]. It was then concluded that the CNS acted as a "sanctuary" for leukaemia cells within the body. The increasing incidence of CNS disease was postulated as a result of poor penetration of anti-leukaemic agents into the central nervous system. In order to achieve an overall improvement in survival rates, it was realized that only a CNS directed disease clearing therapy for the CNS could be effective. Prophylactic CNS therapy quickly reduced the frequency of CNS infiltration and greatly contributed to improved survival rates [25]. Initially this was done by cranial irradiation, which had great success in reducing the number of CNS relapses, from 60% to less than 10%, among children in the Total Therapy V-VI studies [26]. However, unfortunately this treatment came at the cost of extremely toxic side-effects occurring in almost two-thirds of long-term ALL survivors [27]. The side effects noted were cognitive impairment [28,29], endocrinopathies, particularly manifesting as stunted growth [30,31], and increased risk in secondary CNS malignancy [32,33]. Children younger than five who have received cranial irradiation are particularly susceptible to these adverse effects [33,34]. Replacement of cranial irradiation with high-dose intravenous methotrexate and regular intrathecal administration of methotrexate even in high-risk patients showed promising results in UKALL XI clinical trial [35]. It was further demonstrated, by the Children's Cancer Study Group, that the cranial irradiation could be safely avoided in patients with low-risk ALL with additional vincristine-prednisolone pulses in maintenance phase [36]. More recently, Total Therapy XV study demonstrated that prophylactic cranial irradiation can be totally omitted when systemic and intrathecal chemotherapy are intensified, with improved outcomes [37].

The CNS involvement is observed in about one-third of ALL relapses, despite universal CNS-directed treatment historically with cranial irradiation [26] replaced later on now by typically high-dose intrathecal therapy and systemic therapy [38], while isolated CNS relapse accounts for

approximately one-fifth (18%) of ALL relapses [39]. Hence, despite of the exposure to the likely side-effects, a significant proportion of ALL patients might suffer CNS relapse.

The current therapies result in Overall Survival (OS) and Event Free Survival (EFS) rates exceeding 90% for most children with ALL, due to the dosage of the treatments increased and intensified, including CNS directed therapy. A major concern in the past few decades has been the chemotherapy-induced toxicity caused by intense by intensified treatment, due to a shift in focus from improving survival rates to quality of life.

1.1.7 Chemotherapy-associated toxicities:

The improved survival rates, even for relapsed ALL, with the intensified chemotherapy and CNS prophylaxis, comes at the cost of severe and potentially life-threatening toxicities during their course of treatment or later in their survivorship [40]. The combined therapeutic agents may result in treatment-related acute adverse events, that are managed routinely with directed supportive care, such as severe neutropenia, infection and mucositis or as less frequent but potentially severe and/or consequential, such as neurotoxicity [41], venous thromboembolism [42,43], osteonecrosis [44,45] and acute pancreatitis [46]. Due to the complicated balance between cure and toxicity, there has been a greater focus on defining and identifying risk factors for toxicity [40]. The incidence and impact of these toxicities may be reduced or eliminated if these toxicities are better characterised, and prophylactic therapy can be potentially directed toward the children at highest risk of toxicities.

In randomised controlled trials, multiple factors can influence severity, incidence, and timing of chemotherapy-induced toxicity, such as the type, dosage and timing of the chemotherapeutic agents used, as well as the definitions and data capture methods used [40].

1.2 Neurotoxicity:

As the cure rates for ALL have improved, the exploration of the balance between the chemotherapeutic agents efficacy and their side effects has become a major research target. Neurotoxicity remains a devastating complication and can be broadly divided into central and peripheral neurotoxicity.

Chemotherapy-induced central neurotoxicity during treatment for childhood acute lymphoblastic leukaemia (ALL) remains a significant problem [13]. The incidence varies between the treatment protocols, different arms of the treatment protocols, Severe Adverse Events (SAE's) reporting systems, and the sub-types of neurotoxicity. On the UKALL 2003 study, the incidence of acute central neurotoxicity (encephalopathy) was reported as 8% on the low-risk protocol and 12% on the augmented therapy for high-risk patients [13,47], whereas 13% was reported in the NOPHO 2008 study [41].

Acute neurotoxicity presentations include Stroke-Like Syndrome (SLS) [48], Posterior Reversible Encephalopathy Syndrome (PRES) [49], and seizures [50]. Symptomatic and asymptomatic neurotoxicity can also affect long-term neurocognitive outcomes (e.g., attention, executive function) [29,51]. Also, findings of a meta-analysis of long-term neurocognitive deficits in children with ALL treated on contemporary (without radiotherapy) protocols indicated IQ deficits of 6 to 8 points, as well as deficits in other cognitive abilities, such as working memory, data processing speed, and fine motor skills, as compared to healthy controls [52].

These conditions can be diagnosed on the basis of clinical symptoms, associated radiological findings and other supportive paraclinical findings such as Magnetic resonance imaging (MRI), computerized tomography (CT) scan and electroencephalographs (EEG) [40,53].

Worryingly, studies in patients 20-30 years post treatment suggest accelerated CNS ageing and the burden of neurological late effects may worsen over the next few decades [54,55].

There is a lack of understanding of which clinical risk factors are associated with central neurotoxicity and they may vary according to the sub-type of central neurotoxicity. Previous reports suggest that treatment intensity, (particularly methotrexate exposure), age greater than 10 years and number of intrathecal may predispose to neurotoxicity [51,56-58], however, these studies may be confounded by the fact that the older age group (more than 10 years) is allocated to medium risk or high risk group and receives more methotrexate. Also, none of the studies have been sufficiently large to identify independent risk factors in multivariable analysis. Inferior leukaemia outcomes after neurotoxic event were reported by a study which might owe to the use of anticonvulsant drugs and high doses of vincristine used during their treatment [59]. As use of enzyme-inducing anticonvulsants has shown increased metabolism of anticancer drugs, increasing clearance and thus increasing relapse rates [60].

Thus, there is an urgent need to better understand the pathophysiology of neurotoxicity and develop ways of identifying children at risk.

1.3 Types of neurotoxicity and incidence:

The focus of this thesis is on more acute events and are related to chemotherapeutic agents, given below

1) Stroke-like syndrome (SLS) – methotrexate-induced
2) Posterior reversible encephalopathy syndrome (PRES)- vincristine & steroids
3) Seizures – Mix of drugs

Other types are

4) CVST or haemorrhage -asparaginase
5) Steroid psychosis- dexamethasone
6) Metabolic imbalances
7) Guillan-Barré syndrome

1.3.1 Stroke-like syndrome (SLS):

SLS (Stroke-Like Syndrome), also described as subacute methotrexate encephalopathy, is a well-recognised methotrexate-associated condition. The reported incidence of SLS varies from <1%-3% [61,62] and varies depending on the schedule and intensity of methotrexate as well as the coadministration of other drugs, such as cyclophosphamide and cytarabine [61].However, the effect of co-administration of cyclophosphamide and cytarabine along with methotrexate needs to be proven in a larger study.

SLS, defined in consensus definitions publications, as neurotoxic episode occurring within 21 days of intravenous or intrathecal methotrexate with three characteristics that all need to be fulfilled:

1) New onset of one or more of paresis or paralysis; movement disorder or bilateral weakness; aphasia or dysarthria; altered mental status including disturbed consciousness (e.g.,

somnolence, confusion, disorientation, and emotional lability); and/or seizures with at least one of the other symptoms.

2) Either typical, but often transient, white matter changes indicating leukoencephalopathy on MRI or a characteristic clinical course with waxing and waning symptoms usually leading to complete (sometimes partial)

resolution within a week.

3) There is no other identifiable cause [40].

Radiological features typical of SLS are that the CT scan is usually normal; however, MRI shows periventricular white matter changes on DWI and FLAIR [63-65].

Pathophysiology:

The summary and figure, Fig 3, adapted showing complex folate metabolism and Vit B12 the probable biochemical reactions when co-administered [66].

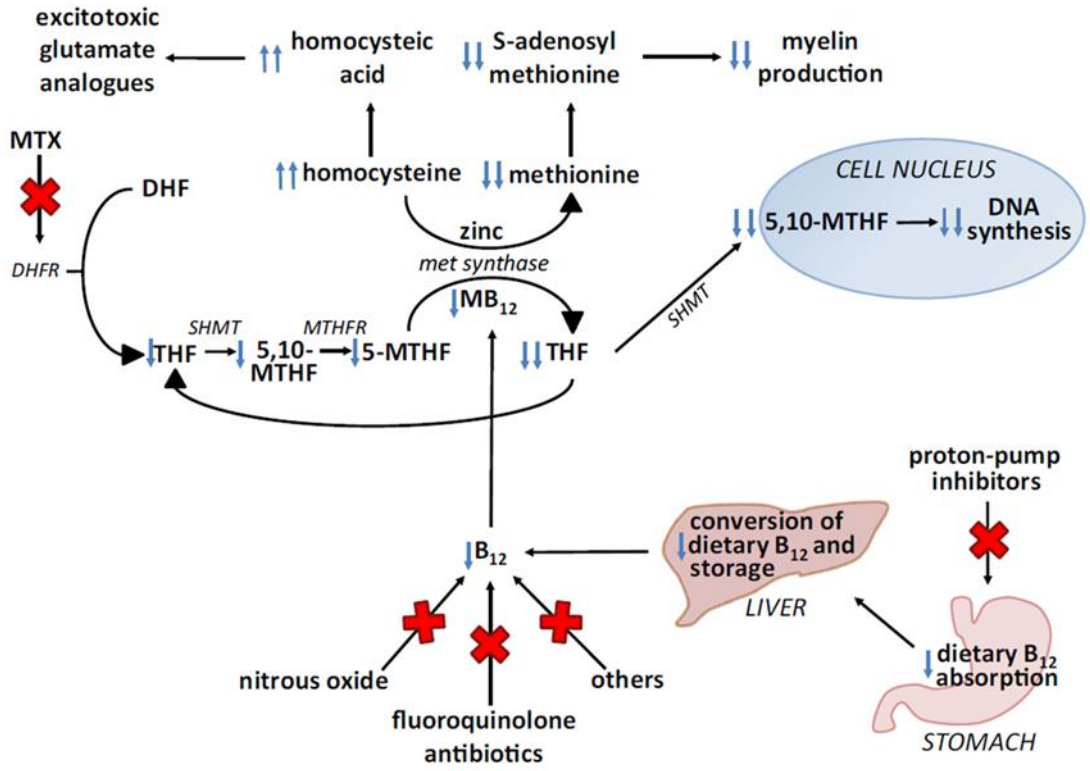


Figure 1.2: A summary of the biochemical reactions involving folate metabolism and drug interactions

The summary shows folate metabolism and vitamin B12 inside an oligodendrocyte and proposed inhibition of myelin production by co-administration of methotrexate (MTX) and drugs affecting vitamin B12.

Abbreviations: 5-MTHF (5-methyltetrahydrofolate, levomefolic acid), MB12 (methyl B12), THF (tetrahydrofolate, tetrahydrofolic acid), 5,10-MTHF (5,10-methylene THF), DHF (dihydrofolate, dihydrofolic acid), DHFR (dihydrofolate reductase), MB12 (methyl-vitamin B12), MTHFR (methylenetetrafolate reductase), MTX (methotrexate), met synthase (methionine synthase), SHMT (serine hydroxyl-methyltransferase).

MTHF participates in the production of methionine from homocysteine by methionine synthase, catalyzed by MB12 and zinc, creating THF and methionine. THF participates in the production of purines and pyrimidines for DNA synthesis. Methionine is a vital amino acid involved in myelin production via its conversion to S-adenosyl methionine (SAM). SAM is involved in the methylation of many proteins and intermediates ultimately involved in myelin production, such as phosphatidylcholine, which is important in the production of sphingomyelin, a major component of the myelin sheath. Homocysteine can be converted to homocysteic acid and homocysteine sulfinic acid which are excitotoxic glutamate analogues acting at the N-methyl-d-aspartate (NMDA) receptor, which may be a factor in acute methotrexate induced neurotoxicity. Methotrexate inhibits the function of DHFR, preventing the conversion of DHF to MTHF. Active vitamin B12 contains reduced cobalt (Co+), but nitrous oxide (N2O) produces irreversible oxidation to Co++ and Co+++, rendering vitamin B12 inactive. Any simultaneous compromise of folate and vitamin B12 via co-administration of methotrexate and agents known to deplete active vitamin B12, such as N2O could result in increased homocysteine and reduced methionine levels both of which may contribute to the neurotoxic effects of methotrexate treatment. Other yet unidentified compounds may also reduce bioavailable vitamin B12 levels. Blue arrows indicate proposed increase or reduction in various relevant pathway metabolites.

SLS occurs within three weeks of methotrexate administration and in most of the cases tends to resolve completely, although long-term deficits in some may occur [38]. One of the potentially intervenable mechanisms to reverse the neurotoxic effect of methotrexate is the accumulation of homocysteine and its metabolites, which has an excitatory effect on the N-methyl-D-aspartate receptor (NMDA) (Fig 3). These changes can be managed and used for prevention of recurrence by dextromethorphan, a non-competitive antagonist to NMDA receptor [67-69], or aminophylline (an adenosine antagonist) [70]. Both drugs have shown promise in small case series, but larger clinical studies are needed. Older age group (more than 10 years) has been identified as a risk predictor, but only in small case series [61,62]. Safety to methotrexate re-exposure post-event without any neurological consequences is unknown. The leukaemia outcomes if the methotrexate dose is modified or skipped are largely unknown, especially in the light of a recent study that reported higher CNS relapse rates in methotrexate-induced neurotoxicity after modification in the ALL treatment [57]. Effect of heterogeneity in methotrexate

administration schedule and intensity in different protocols for occurrence, severity, disease pattern and impact on neurological outcomes are largely unknown.

1.3.2 Posterior Reversible Encephalopathy Syndrome (PRES):

Posterior Reversible Encephalopathy Syndrome (PRES) was first described as a radio-oncological entity [71] with incidence variably reported between 3.8%-4.5% [49,59] making it one of the common types of chemotherapy-associated neurotoxicity in paediatric acute lymphoblastic leukaemia (ALL).

PRES, as defined in consensus definitions, is a clinical diagnosis based on combinations of transient headaches, confusion, seizures and visual disturbances in conjunction with characteristic, but transient, findings on contrast enhanced and diffusion weighted magnetic resonance imaging (MRI) studies [40].

Pathophysiology:

It is postulated to be an interplay between different factors (Fig 1.4). One possible explanation is that PRES may be caused by focal breakdown of the blood-brain barrier (BBB) endothelium because of inadequate autoregulation of cerebral blood flow, with fluid movement from the intravascular to extravascular compartments because of this breakdown. The cytotoxic drugs, in addition to causing axonal swelling and an increase in white matter water content, also cause damage to the cytoplasm.

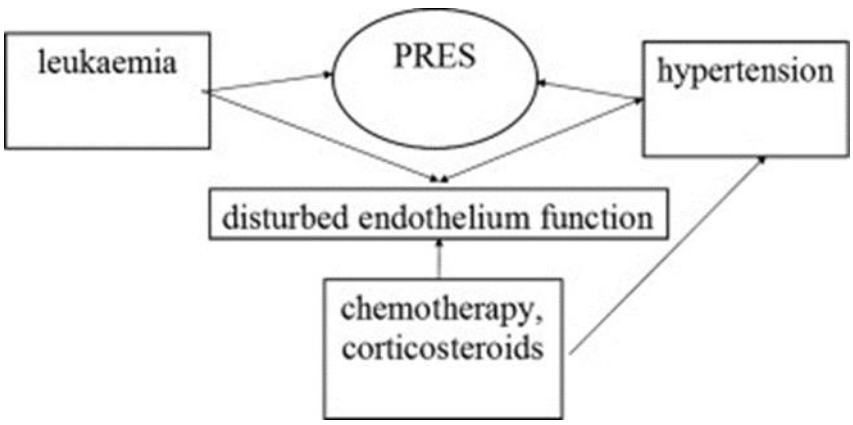


Figure 1.3: Hypotheses of factors leading to PRES.

There is also a possibility that PRES may result from a sudden and severe increase in blood pressure (BP), causing a disruption in the normal autoregulation of cerebral blood flow in the brain. This may lead to dilatation of cerebral arterioles in conjunction with opening of endothelial tight junctions causing leakage of plasma and erythrocytes into the extracellular space, resulting in vasogenic oedema. Additionally, an acute rise in BP may cause vasospasm and ischemia in brain tissue, resulting in vasogenic oedema and extracellular oedema.

PRES has been reported usually during the induction phase of ALL chemotherapy. [2, 72-74.] Older age, T- cell immunophenotype, CNS involvement, high-risk treatment regimen have been reported as risk predictors but this is based on limited numbers [49]. In therapy-related factors, more than 14 days of urinary hydration and alkalinization alongside methotrexate administration is reported as associated with PRES by Banerjee at al. [59]. Inferior leukaemia outcomes, in terms of increased leukaemia relapse, have been shown by Banerjee *at el*. after a PRES event, [59] but this was not observed in the other cohorts [3], making it interesting to test this possibility in comparison with other types of neurotoxicity in larger cohorts. Despite being reported as a reversible episode, there have been few reports of worse neurological outcomes post event and risk predictors of neurological outcomes are largely unknown [75].

1.3.3 Seizures:

Seizures are the most common type of presentation of chemotherapy-induced neurotoxicity and may be reported in up to 10% of children on ALL treatment [50], however recent trials have reported lower incidence (4.7%-7.6%), in the absence of cranial radiation therapy [37,58].

An episode of seizures is characterized by a transient occurrence of signs and/or symptoms owing to abnormal uncontrolled or synchronous neuronal activity in the brain [76]. Depending on

the presentation, according to the working classification by International League Against Epilepsy (ILAE), seizures can be classified as focal, generalized, or unknown onset seizures [77].

Chemotherapy-induced seizures in ALL might occur in isolation (MTX-induced reported in 60%), or as part of another recognized toxicity such as intracranial haemorrhage (reported in 10%), CNS thromboembolism event (reported in 8%) , CNS infection (in 6%), PRES and hypertensive seizures (in 4.5% and 0.9%), metabolic disturbances including hyponatraemic seizures (3.7%) , hypoglycaemic seizures (2.5%) and Syndrome of Inappropriate anti-diuretic hormone SIADH, in different reports [41,78] [79]. Furthermore, risk predictors for recurrence in patients with neurological deficit on ALL therapy were reported earlier [80] but St Jude Children's Research Hospital (SJCRH) paediatric ALL/NHL study were not able to show the same [78]. However, female sex and age less than 3 years have been identified for worse long-term outcomes [78]. According to the SJCRH series, seizures were managed with antiseizure medicine (ASM) in a majority of patients and almost 30% of patients had uncontrollable seizures, with 25% had seizure relapse with the trial of antiseizure medicine withdrawal [78].

Severity of seizures is graded according to the CTCAE where the presentations were

Grade 1 - brief focal seizure

Grade 2 - brief generalised seizure, whereas the more severe forms are

Grade 3 - multiple seizures despite medical intervention,

Grade 4 - life-threatening, prolonged or repetitive seizures

Grade 5 – death resulting from seizures [81].

It would be interesting to characterise seizure cases, especially after PRES and SLS are clearly diagnosed, and to identify the differences and similarities in demographic and clinical features of Seizures with the other types of neurotoxicity. A comparison between different current ALL treatment protocols to see the effect of heterogeneity in the different treatment protocols on the occurrence and timing of seizures is required. Although there are some reports regarding uncontrollable chemotherapy-induced seizures and use of anticonvulsants, the neurological outcomes in these cases are largely unknown.

1.3.4 Pre-existing neurological conditions & neurotoxicity (Down syndrome)

Down syndrome is a condition in which a child is born with an extra copy of chromosome 21 [82]. This not only causes physical and mental developmental delays and disabilities but also predisposes to higher incidence of acute lymphoblastic leukaemia with distinct biological characteristics [83,84]. Furthermore, research has shown inferior outcomes compared to Non Down syndrome ALL because of increased relapse rate and treatment related mortality (TRM) [85]. Down ALL patients also have increased vulnerability to chemotherapy-associated toxicities, particularly MTX and anthracyclines, includes a high risk of toxic death [86-88]. The reason for this increased susceptibility to methotrexate-induced toxicity in Down syndrome ALL patients is most likely due to altered MTX pharmacokinetics [89].

Although neurotoxicity in Down syndrome with ALL has been reported, only 2 isolated chemotherapy-induced seizures cases were reported from, a randomised controlled trial based on the MRD-based risk stratified adapted chemotherapy in UKALL 2003, also reporting DS-ALL outcomes as a sub study [90]. There is lack of detailed clinical description and there is no information about the severity of these events, extent of recovery and their risk of recurrence. Moreover, the risk predictors and their implications on neurological and leukaemic outcomes are unknown, as MTX-dose modifications are frequent and their impact on leukemic outcomes are uncertain. Also, there is a lack of clarity about clinical patterns of neurotoxicity and their effect on clinical outcomes in the presence of an existing neurological condition such as Down syndrome. This highlights the reason to investigate Down syndrome ALL neurotoxicity in a larger pooled cases dataset.

1.2 Severe adverse events (SAE's reporting) and its inaccuracies:

Improved cure rates in the paediatric leukaemia have been possible because of the vigilant severe adverse events reporting systems, by administering supportive care promptly where the patients are exposed to intensified chemotherapy likely to cause substantial treatment-related morbidity. It is well known and accepted that in the conduct of clinical trials, notification of severe adverse effects of cancer treatment has also long been an essential activity for effective

analysis of the outcomes [91]. Research into the severe adverse events reports has highlighted the variation in the severe adverse events reporting systems leading to inaccuracy in the paediatric acute myeloid leukaemia outcomes reporting [92]. The National Cancer Institute (NCI) created Common Terminology Criteria for Adverse Events (CTCAE) in an attempt to define a consistent grading system, which gets regularly updated and including toxicity-associated severe adverse events [93]. Studies report a wide range of toxic effects frequencies, in part due to different definitions of toxic effects, recognising this variation consensus definitions for the most prominent chemotherapy-induced toxicities were published [40]. In the studies relying on the reports from the local centres for reporting these chemotherapy-induced toxic effects/SAEs, it is important to acknowledge likely underreporting and misreporting of cases.

1.3.5 Identifying the problem of inaccurately classified cases

SLS and PRES are well reported, and distinct entities that have a range of clinical presenting features.

Earlier on, the variation in clinical definitions and diagnostic criterion was recognised and applying a Delphi consensus method, diagnostic criterion for SLS and PRES was published in Lancet oncology [40].

Even so, there appears to be some overlap in symptoms between PRES and SLS, and it is still unclear whether radiological criteria can effectively be used to distinguish between these two presentations. A comparative table adapted from the diagnostic criterion for SLS and PRES and to appreciate that there are more similarities than differences, the similarities are colour coded green and differences are colour coded red, this high degree of overlapping symptoms can lead to misdiagnosis.

PRES	SLS
Association with Vincristine & Steroid	Within 21 days of Methotrexate
Headache, confusion, visual disturbances	Confusion, mental status altered affect, paresis/paralysis, aphasia/dysarthria
Seizures,	Seizures,
Spontaneous resolution	Spontaneous resolution
Hypertension prominent	Waxing and waning pattern
Early-onset – first 3-4 months	Usually consolidation/intensification
CT can be normal	CT often normal
MRI T2- hyperintense cortical / subcortical lesions	MRI T2- hyperintense subcortical lesions
DWI –normal or hyperintense	DWI – hyperintense
ADC (classically) increased	ADC decreased (low signal)

Figure 1.4: Difference and similarities in PRES and SLS clinical and radiological features colour coded.

SLS occurs within twenty-one days of administration of methotrexate, the underlying pathophysiology is cytotoxic oedema whereas, PRES is associated with vincristine and steroids, underlying pathophysiology is possibly hypertension-induced vasogenic oedema.

Apparent diffusion coefficient (ADC) values are quantitative measure of the impedance of the water molecule diffusion, calculated automatically by the software and displayed as a parametric map, using MRI with diffusion weighted imaging (DWI) [94,95]. It has recently been suggested that SLS and PRES can be distinguished by their contrasting effects on the ADC values, with PRES showing increased values (due to vasogenic oedema) and SLS showing decreased ADC values (due to cytotoxic oedema), however more studies will be needed to confirm these results. It is important to understand that seizures, while thought being a common feature of SLS, are not only a chemotherapy-induced phenomenon of their own but also can occur as part of the PRES presentation and may or may not share the same pathophysiology. Assessing the degree of the overlapping of SLS and PRES and developing a diagnostic criterion of these entities will result in having access to important information that will prove valuable to the treating

clinicians, patients, and their parents, when confronted with difficult, distressing, and rare clinical scenarios. It may also prove useful for determining preventative strategies.

1.3.6 Ponte di Legno Consortium:

Although neurotoxicity is reported in 8-12% of children undergoing treatment for ALL [13,47], every individual ALL trial group's reports of neurotoxic events are too small to be able to draw a meaningful phenotypic-genetic correlation. Furthermore, it is impossible for a single group to examine the effects of treatment protocol or other exposures independently. There is an annual workshop conducted by the international leukaemia community at which representatives of all the national trial groups participate [96].

Figure 1.5: Participating trial groups in the Ponte di Legno study.

COG (Children's Oncology Group (USA), BFM (Berlin-Frankfurt-Munster study group), UKALL (United Kingdom ALL study group), NOPHO (Nordic Society of Paediatric Haematology and Oncology), AIEOP (Associazione Italiana Ematologia Oncologia Pediatrica), Hungary (Hungarian Paediatric Oncology

Network), ANZCHOG (Australian and New Zealand Children's Haematology/Oncology Group), DCOG (Dutch Childhood Oncology Group),CPH (Czech Working Group for Pediatric Hematology) , Israel (Israel Society of Paediatric Oncology and Hematology) , COALL (Cooperative Study Group for Childhood ALL (Germany) , Spanish (Working group of paediatric hematology), Austria-BFM (Austrian group for paediatric hemato-oncology) , TPOG (Taiwan Pediatric Oncology Group).

This consortium is called the Ponte di Legno (PdL) group. This group aims to address the relatively rare but significant difficulties encountered in childhood leukaemia that necessitate a large number of collaborative efforts between different international groups [97]. A Ponte di Legno Acute Toxicity Working Group was recently established in order to address this issue. It was identified that neurotoxicity is one of the primary toxicities that needs to be addressed through international collaborative efforts. As a first step the neurotoxicity working group (headed by Prof Halsey) has developed consensus definitions for PRES, SLS and seizures, applying a Delphi consensus method, to address the variation in definitions and diagnostic criteria in these neurotoxicity presentations between the different treatment protocols [40].

As neurotoxicity cases reported from single centres were few, the Ponte di legno neurotoxicity working group was established and neurotoxicity cases from 14 study groups and 23 countries were pooled (Figure 1.1) and the results from this dataset are shown in the results chapters.

1.3.7 Treatment protocols and their heterogeneity

There are a lot of different ways to treat leukaemia all with similar outcomes and that different trial groups have adopted different protocols, although published comparative studies examining the similarities and differences are lacking.

Although the current published international ALL protocols have similar overall and leukaemia survival outcomes, but the protocols vary considerably in risk group stratification, duration and type of treatment phases, timing and doses of chemotherapeutic agents administered, and timing of MRD assessment, although published comparative studies examining the similarities and differences are lacking.

As the information regarding the overall treatment was not available from the PdL neurotoxicity database, so to be able to assess the degree of variation in the treatments across the different

protocols and its effects on the occurrence of the neurotoxic events and neurological outcomes, “Ponte di legno toxicity protocols database” was utilised. This “Ponte di legno toxicity protocols database” is another collaborative project, where study groups running the ALL-treatment trials and are part of the Ponte di Legno consortium, pooled the ALL-treatment protocols, under the chairpersonship of Dr Halsey and Dr Linda Vrooman. The database was compiled by a volunteer medical student, Ms Gemma Swan, and helped by the author.

The differences of interest, regarding neurotoxicity, in the ALL-treatment protocols included in the Ponte di Legno Neurotoxicity Working Group are given in the **Error! Reference source not found.**.

	Treatment protocols	Induction	Consolidation	CNS directed	Reinduction/Reconsolidation	Maintenance
BFM like	**ALL BFM 95** **ALLIC BFM 2002** **ALLIC BFM 2009** **AIEOP-BFM ALL 2009** **DCOG ALL 10** **DCOG ALL 11** **ALL BFM 2000** **ANZCHOG Study 7** **ANZCHOG Study 8** **COALL 08-09** **COG 0232 (PH & DH)** **COG 0434 (Arm C & D)** **SEHOP PETHEMA** **CCG 1961** **INS 2009/10** **TPOG 2002**	•Steroid pre-phase •4 (steroid, vincristine , asparaginase & anthracycline drug induction in all patients	Protocol 1 B	•Protocol M/3rd Block •High dose IV MTX 5000 mg/m2	Protocol 2 (2A,2B)	•6-MP and MTX •2 years for all
COG like	**UKALL 2003** **COG 0232 (PC & DC)** **COG 0434 (Arm A & B)** **COG 0331**	3 (steroid, vincristine & asparaginase) drugs-induction in SR only	Consolidation	•Interim maintenance •low dose escalating IV MTX	Delayed intensification	•Use of steroids, vincristine and IT MTX with 6-MP and oral MTX •2 years for females and 3 years for males
Other	**NOPHO 2008**	Vincristine 2 mg/m2- higher doses in induction	Consolidation	Delayed intensification	maintenance 1	maintenance 2 •6 MP & MTX till 2.5(130 weeks total) years for both males and females

Table 1.2: Heterogeneity of the protocols included in the PdL study

Abbreviations: Children's Oncology Group (COG); Berlin-Frankfurt-Munster (BFM); MTX, methotrexate, Intravenous methotrexate (IV MTX), 6-mercaptopurines (6 MP), Standard risk group (SR).

The main variations in the ALL-treatment protocols for the doses and timing of the chemotherapy, phase of the treatment and the variation in duration of different phases of therapy, are shown in the **Error! Reference source not found.** The ALL-treatment protocols can be broadly divided into "BFM-like" group, "COG-like" and the "Other group".

Induction: Additional daunorubicin (fourth drug) is administered in induction in BFM-like group with a steroid pre-phase, where fourth drug is administered in the high-risk patients only in COG-like protocols. "Other group" administered higher dose of vincristine during the induction phase of the treatment.

CNS-directed therapy: BFM-like group administered high-dose methotrexate IV starting from 3-5 gm/m^2 with leucovorin rescue, whereas the COG-like group administered escalating Capizzi methotrexate dose starting from 150 mg/m^2, without leucovorin rescue, then adding 50 mg/m^2 every week.

Maintenance: Same duration of maintenance phase of therapy in the BFM-like group for both genders, whereas in the COG-like group boys were administered chemotherapy for 3 years and girls for 2 years.

1.3.9 Heterogeneity in leucovorin rescue:

Leucovorin rescue, where it was administered, had a variation in the dose administered in different protocols. Two main groups: are a) low dose (5-10 mg/m2) b) high dose (15 mg/m2). The treatment protocols included in this thesis are stratified in the low leucovorin dose and high leucovorin groups as shown below in Table 1.1.

Leucovorin dose (mg/m2)	Treatment protocols
Low dose (5 mg/m2) administered	CCG-1916 AALL0331 AALL0232
High dose (10-15 mg/m2) administered	ALL-BFM 95 ANZCHOG ALL STUDY 8

	ALL BFM 2000 ALL IC-BFM 2002 CoALL 07-03 DCOG ALL10 LAL/SHOP-2005 LAL-SEHOP-PETHEMA 2013 CoALL 08-09 AIEOP-BFM ALL 2009 DCOG ALL 11 NOPHO 2008 INS ALL 2010 ANZCHOG ALL STUDY 7 ALL 0434

Table 1.1: Heterogeneity in leucovorin rescue dose administered

1.3.8 Heterogeneity in timing of leucovorin administered:

As leucovorin, a folate analogue, effectively neutralizes methotrexate's effects, the timing for administrating leucovorin after methotrexate administration is crucial. The leucovorin window cannot be too short as it would, on one hand, reduce the chances of toxicity, but on the other hand it can also affect the desired anticancer results.

ALL treatment protocols could be stratified in two groups depending on the time from start of methotrexate to first leucovorin dose (hours), as per protocols, 1) Early leucovorin rescue, where leucovorin is administered 24-36 hrs post methotrexate administration 2) Late leucovorin rescue, where leucovorin is administered 42-60 hrs after methotrexate administration. The treatment protocols administering either early leucovorin or late leucovorin rescue included are shown below in Table 1.2:

Timing of Leucovorin administered (Early vs late administration)	Protocols
Early (24-36 hrs) leucovorin administered post methotrexate dose	CCG-1916 LAL/SHOP-2005 NOPHO 2008 ANZCHOG ALL STUDY 7
Late (42-60 hrs) leucovorin administered post methotrexate dose	ALL-BFM 95 ANZCHOG ALL STUDY 8 ALL IC-BFM 2002 AALL0331 CoALL 07-03 AALL0232 DCOG ALL10 LAL-SEHOP-PETHEMA 2013 CoALL 08-09 AIEOP-BFM ALL 2009 DCOG ALL 11 INS ALL 2010 ALL 0434

Table 1.2: Heterogeneity in timing of leucovorin administered post methotrexate dose administration

1.3.9 Heterogeneity in intrathecal CNS-directed therapy

To intensify the CNS-directed therapy, protocols have varied approaches in administering intrathecal therapy during the maintenance phase. One approach was to give a single drug intrathecally (IT MTX), methotrexate only, whereas the other was to administer intrathecally

triple therapy (ITT), methotrexate, cytarabine and hydrocortisone combined. The protocols included in the PdL cohort administrating either IT MTX or TIT are shown in Table 1.3 below.

Intrathecal CNS directed therapy (Single drug vs Triple drug)	Protocols
Triple intrathecal therapy (Methotrexate, cytarabine and hydrocortisone)	DCOG ALL10 LAL/SHOP-2005 LAL-SEHOP-PETHEMA 2013 DCOG ALL 11 CoALL 08-09 (HR) NOPHO 2008 (HR) ANZCHOG ALL STUDY 7 (HR) ALL-BFM 95 (HR) ANZCHOG ALL STUDY 8 (HR) ALL IC-BFM 2002 (HR) ALLIC BFM 2009 (HR)
Single intrathecal drug administered (Methotrexate only)	CCG-1916 UKALL 2003 AALL0331 CoALL 07-03 AALL0232 CoALL 08-09 (SR, MR) AIEOP-BFM ALL 2009 INS ALL 2010 ALL 0434 CoALL 08-09 (SR & MR) NOPHO 2008 (SR & MR) ANZCHOG ALL STUDY7 (SR & MR) ALL-BFM 95 (SR & MR) ANZCHOG ALL STUDY8 (SR & MR) ALL IC-BFM 2002 (SR & MR)

	ALLIC BFM 2009 (SR & MR)

Table 1.3: Heterogeneity in intrathecal CNS directed therapy

Abbreviations: High risk (HR); Standard risk (SR); Medium risk (MR)

Although there are variations in the ALL-treatment protocols for MTX dose administration, leucovorin dose and timing, and CNS- directed therapy, it is important to acknowledge here that additional modifications in the treatment protocols depending on individual patients, clinician's preference or centre-based scenarios might also occur.

1.4 Deep phenotyping and genotyping:

Deep phenotyping can be defined as the precise and comprehensive analysis of phenotypic abnormalities in which the individual components of the phenotype are observed and described.

Genotyping is a technology that detects small genetic differences that allows to do a genotype-phenotype correlation, which is the relation between specific germline mutations (genotype) and their effects (phenotype) and has the power to help in understanding why some individuals are susceptible to a certain disease and others aren't.

1.4.1 Genome wise association study (GWAS) technology:

Genome-wide association studies are carried out to identify novel susceptibility loci, single nucleotide polymorphisms (SNP's) that are found associated with the phenotype of interest. A well-defined case phenotype and control group are the pre-requisites for the case-control GWAS design [98]. To carry out genotype-phenotype association studies, SNP array chips are commonly used, pooling data for all the SNP's tested across the genome. Logistic regression or Cox-regression analysis is then conducted on the cohort. SNP's that reach genome-wide significance level p-value $<0.05^{-8}$ (Bonferroni adjustment for multiple SNP's testing: p= 0.05/1,00,000= $5x10^{-8}$) in the discovery cohort are then validated in a different cohort for credible genome-wide association results [98]. Further functional exploration of the significant SNP's may be done with determining the linked genes, gene expression, epigenomic expression, and be determining tissue-specific expressions of encoded proteins [38].

1.4.2 Candidate gene approach:

The candidate-gene association approach is a hypothesis-driven approach based on the previous knowledge that allows for picking a specific set of genes, and SNP's in these genes, for association with a phenotype [99].

1.4.3 Meta-analysis:

Meta-analysis is the approach to combine GWAS on several different study populations, analyse them together and obtain combined results. Meta-analysis is typically done when analysis was performed on different chips and/or when results from different investigators need to be combined and raw data cannot be exchanged for confidentiality reasons. Meta-analysis of GWAS results from different consortia are progressively being conducted to boost study power and to be able to detect rare disease-associated variants. However, consideration needs to be given to the potential sources of heterogeneity, including phenotype definitions, population ethnicity [38].

The meta-analysis of an association between the candidate SNP list and their GWAS data, comparing cases with matched controls for the phenotypes from different collaborators allows to further strengthen the associations of polymorphisms involving pathophysiologic pathways with the phenotype but on the other hand, this also limits the possibility of finding novel associations.

1.4.4 Imputation:

There may not be much overlap between SNPs when studies are genotyped on different chips. Therefore, direct SNP-by-SNP meta-analysis is difficult when genotyping studies on different chips. For example, the overlap between Illumina Infinium Omni 2.5 exome-8- Beadchip array (with 2.5 million SNPs) and Illumina Infinium Onco-array 530-K Beadchip array (with 530,000 SNPs) is only approximately 300,000 SNPs [100,101]. It is standard to impute the genotypes of all the SNPs from all samples to resolve this problem. A variety of good methods are currently available to accomplish this task [102].

1.1.7 GWAS meta-analysis

Although neurotoxicity is reported in 13% of children undergoing treatment for ALL [41], the individual numbers of patients with PRES/SLS etc. in each trial group are too small to allow genotype-phenotype correlations. There have been some SNP array-based studies that have reported genetic polymorphisms associated with Methotrexate-induced neurotoxicity, both acute and long-term manifestations, related to neuronal development, MTX clearance or folate metabolism by-products namely homocysteine [103,104]. GWAS studies for MTX associated neurotoxicity have shown an association with polymorphisms with a potential role in neurodevelopment, drug transport, developmental delays or with phenotypes such as attention deficit hyperactivity disorder and autism [51,57], but they were looking at the polymorphism associated with overall methotrexate-associated neurotoxicity without stratifying them in types of neurotoxicity. Smaller studies have identified vincristine-related neurotoxicity but none of these were able to show significance at GWAS level [105]. Genetic variations depicting the severity of the neurotoxic event, as measured by whether there was a full recovery or persistent neurological deficits, and the recurrence frequency are unknown.

1.1.8 Overlapping the differently methylated genes post-methotrexate exposure with the genes shown significant association with methotrexate-induced neurotoxicity- understanding the mechanisms of neurotoxicity

Improving cure rates in paediatric ALL came at a cost of chemotherapy-induced neurotoxicity reported in 8-12% of cases on different arms of the UKALL 2003 study [13,47].

DNA methylation, one of the epigenetic markers that can modulate gene expression, is altered more easily by a variety of more subtle exposures than DNA sequence itself [106,107]. Evidence suggests that DNA methylation may play an important role in chronic neurotoxicity such as cognitive impairments [108]. Altered DNA methylation of brain/central nervous system cells could be one mechanism involved in methotrexate treatment-related neurotoxicity and late neurocognitive effects in ALL survivors [109]. Moreover, epigenetic reprogramming is the process by which an organism's genotype interacts with the environment to produce its phenotype [110,111]. A recent study has also focused on identifying the possible mechanisms between drugs interaction, environmental factors and neurotoxicity [66].

It is clear that a complete mechanism explaining the link between genomic factors (SNPs), epigenetics (DNA methylation status) and neurotoxicity is largely missing. A study on developing rats showed post-methotrexate exposure epigenetic changes affecting neurogenesis and myelination processes [112]. It is not known how DNA methylation affects genetic polymorphisms and how these affect the outcome (neurotoxic events). Therefore, mapping methylation changes post-exposure to MTX with genetic variations associated with neurotoxicity may shed some light on the interplay that leads to the expression of neurotoxicity. It would be interesting to investigate if genes that showed altered methylation in neuronal cells post-exposure to methotrexate may have overlapped with genes identified using GWAS analysis of patients with methotrexate-induced neurotoxicity.

1.1.8 Aims and objectives:

Increasingly improved cure rates of ALL made the exploration of the balance between efficacy and side effects a major challenge in research. Chemotherapy-induced neurotoxicity remains a significant problem with incidence reported 8-12% [13,47].

1) To be able to accurately diagnose patients – this will help with studies looking at causes and potential preventative measures

2) To be able to identify risk factors – both genetic and environmental

3) To understand the natural history of each condition – to help counselling of families, supportive interventions, and decisions on whether to modify treatment

In order to achieve this there is a need for large studies. This thesis investigates chemotherapy-related neurotoxicity in large clinical cohorts and by analysis of genomic and transcriptomic data sets.

In chapter 3 – To explore clinical risk-profiling of neurotoxicity and the effects of a neurotoxic event on leukaemia outcome.

In chapter 4 - To deep phenotype SLS and PRES cases based on their clinical and radiological findings that can help in the re-classification of the cases to be able to identify true cases for genotyping

In chapter 5, 6, 7, 8 - To clinically characterise the SLS, PRES and seizures and to identify the differences and similarities in comparison with the other types of neurotoxicity. To explore the effects of heterogeneity of the protocols on the neurological outcomes in SLS, PRES and seizures. The exploration of the effects of neurotoxic events, SLS, PRES and seizures, on the leukaemia and neurological outcomes compared with the rest of the neurotoxicity. To study clinical patterns of neurotoxicity in Down syndrome ALL and to identify the risk predictors of worse neurological outcomes in Down syndrome ALL.

In chapter 9 - Exploration of the significance level of the genotypic candidate genes of interest by running a meta-analysis of the GWAS association results against the neurotoxicity candidate SNPs list for the true identified phenotypes.

In chapter 10 - To better understand the mechanisms of neurotoxicity by overlapping the differently methylated genes post-methotrexate exposure with the genes shown significant association with methotrexate-induced neurotoxicity.

2 Materials and methods

The broad aims of this project were to be able to accurately diagnose the neurotoxicity cases (SLS and PRES), to identify clinical and genetic risk predictors, and to investigate possible mechanisms of neurotoxicity. The details given in this methodology chapter give an overview on the data collection and tools utilised for analysis. As different chapters belong to work done on different studies/datasets, the details are broken down chapter-wise.

2.1 Materials and methods for chapter 3

In order to investigate clinical risk factors for neurotoxicity and its effect on outcomes in a large cohort of patients, a review of all neurotoxic serious adverse events (SAEs) reported, compared to no neurotoxicity cases as controls, for the UKALL2003 trial was carried out.

2.1.1 Patient cohort and methods:

Between Oct 1, 2003, and June 30, 2011, consecutive children and young adults (aged 1–24 years) with ALL from the UK and Ireland (3113 cases) were recruited to the UKALL 2003 trial.

The central trials database was interrogated for SAEs under the category neurotoxicity. There were 301 SAE reports of neurotoxicity in 276 patients (8.8% of all trial participants). Five patients with miscellaneous encephalopathy secondary to systemic disorders and seventeen with steroid psychosis were excluded from this analysis based on the difference in the underlying aetiologies. The remaining 254 patients (159 with encephalopathy, 86 with seizures and 31 with SLS) were compared to 2837 controls without any reported neurotoxicity. (**Error! Reference source not found.**)

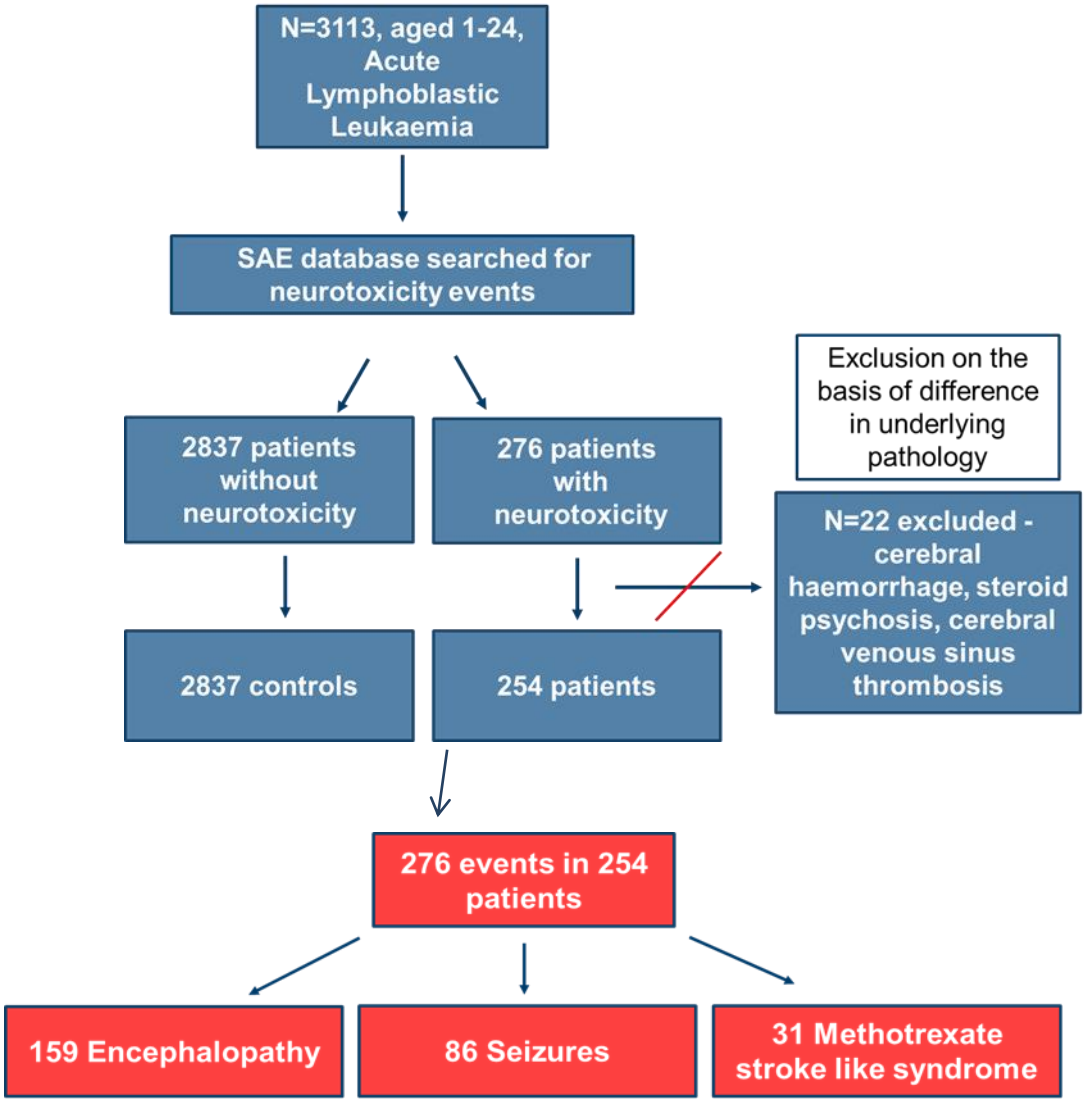


Table 2.1: Consort diagram of neurotoxic events from UKALL2003 trial

The trial SAE (Severe Adverse Event) database was interrogated for the central neurotoxicity events reported by the centres and the cases reported as seizures, encephalopathy and SLS were included. The cases reported as steroid psychosis, cerebral haemorrhage and cerebral sinus venous thrombosis were excluded from this analysis based on different underlying aetiologies and were included in a separate analysis that had the same underlying aetiology e.g. the neurotoxicity with cerebral sinus venous thrombosis were included in a PdL study deciphering the thrombotic chemotherapy-induced toxicity.

2.1.2 Analytical tools

Survival rates were calculated and compared using the Kaplan-Meier method and log-rank tests. The association between neurotoxicity event and risk factors was investigated using univariate chi-squared test and multivariate logistic regressions. All tests were conducted at the 5% significance level, and the analyses were performed using Intercooled Stata. Statistical analysis was performed by a biostatistician, Dr Lina Hamadeh, at Leukaemia research cytogenetic group in Newcastle University. Graphics and all interpretation of results in this thesis were done by the author.

2.2 Materials and methods for chapters 4, 5, 6, 7, 8, 9

As neurotoxicity cases reported from single centers were few, the Ponte di legno neurotoxicity working group was established and neurotoxicity cases were collected from 14 study groups and 23 countries. All patients were treated on frontline paediatric ALL protocols from 2000 – 2017.

Neurotoxicity Working Group

List of the participating study groups and national PIs taking collaborating in this study are given below:

1) Christina Halsey (UKALL) (Chair) Rachael Hough,
2) Kjeld Schmiegelow & Arja Harila-Saari (NOPHO),
3) Deepa Bhojwani & Naomi Winick (COG),
4) Shlomit Barzilai (Israel),
5) Mary Relling & Hiroto Inaba (SJCRH),
6) Der-Cherng Liang (TPOG),
7) Gabriele Escherich (COALL),
8) Daniel Erdelyi & Judit Sagi (Hungary/BFM),
9) Caterina Putti & Maria-Grazia Valsecchi (AIEOP),
10) Glenn Marshall, Toby Trahair, & Marion Mateos (ANZCHOG),
11) Andishe Attarbaschi (Austria-iBFM),

12) Inge van der Sluis (DCOG),
13) Iveta Janotova & Ester Zapotocka (CPH),
14) Anja Moricke (BFM),

The study was approved by the NHS Health research authority, London-Westminster Research Ethics committee, Reference number 17/LO/1258. (Attached in appendix)

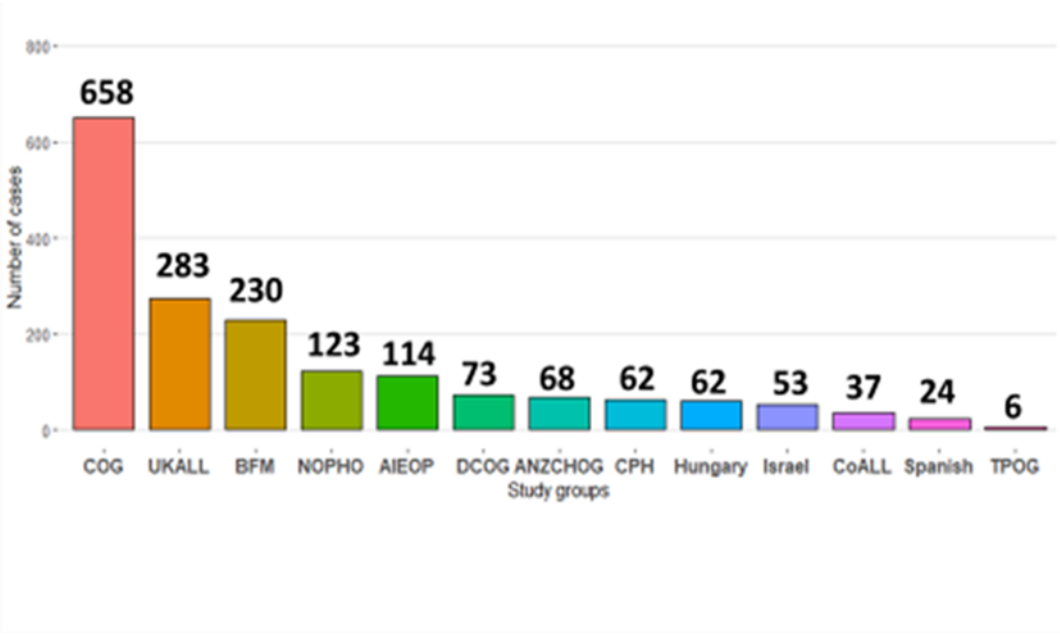


Table 2.2: Cases contributed by the study groups

2.2.1 Questionnaire

A questionnaire was designed with more than 200 variables to capture detailed information about clinical aspects of chemotherapy-induced central neurotoxicity and its different types. The variables to be collected were generated by the members of the PdL Neurotoxicity working party during a number of face-to-face meetings between 2015 and 2016. The subsequent survey was piloted by ANZCHOG and Finnish (NOPHO) study groups. On the basis of inputs from the study groups involved and feedback from the piloting the questionnaire was finalized. Adobe pro 9 software was used so that data can be electronically entered and transferred. (Attached in appendix). To make it easier for the study groups according to their preference the questionnaire was shared with the study groups in Excel and PDF formats.

2.2.2 Patient cohort:

Patients having neurotoxicity event reported to the database that were confirmed to fulfil the inclusion and exclusion criteria for the study, i.e.:

Inclusion criteria:

1) Central neurotoxicity events occurring in patients (any age) treated on front- line paediatric ALL protocols, for the first episode of ALL or LBL

2) Patients diagnosed with ALL/LBL from 1/1/2000 to present

Exclusion criteria:

1) Neurotoxicity events occurring post-HSCT

2) Patients whose neurotoxicity is due to peripheral neuropathy

3) Patients whose event occurs at diagnosis before administration of any chemotherapy

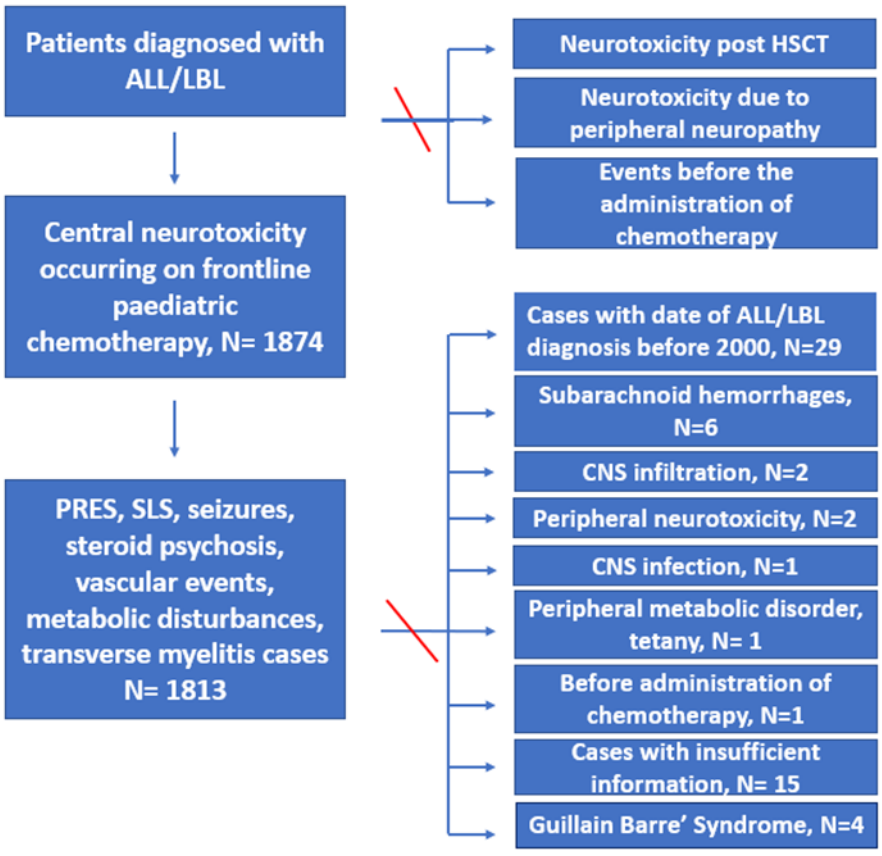


Figure 2.3: Consort diagram for the selection of neurotoxicity cases in Ponte di Legno study.

2.2.3 Individual-level data collection and extraction:

Data was collected for eight cases by the author of this thesis. These cases came from the Royal Hospital for children, Glasgow, for which detailed clinical notes of these patients were accessed. To access the detailed clinical notes from the hospital, Good clinical practice certification and a research passport were obtained (attached in appendix). In the rest of the centers in the UK the data was collected, fill in the questionnaire in the either the PDF format or in the excel sheet format, by the research nurses assigned by the respective PIs.

Data collected from the study groups in the form of questionnaire, excel sheet or selected neurotoxicity cases from the SAE's trial database (for COG and UKALL study group) required individual review of each patient's clinical and para-clinical information. The study group national PIs selected the cases from their respective trial databases or from the clinical notes. This detailed patient records review was performed to ensure that patients were correctly included as cases and all the information available was extracted. (Checked by the chair of the study Prof. Halsey).

Cases that were sent by the study groups were manually inspected to ensure they fulfilled the inclusion/exclusion criteria. Reasons for exclusion of cases included: cases with ALL/LBL diagnosis before 2000 (due to difference in cranial irradiation administration in earlier protocols), tetany, Guillain Barre' syndrome, peripheral neurotoxicity, leukaemic infiltration, and insufficient information to assess. This resulted in n = 1813 neurotoxicity cases. (Figure 2.3)

2.2.4 Sources of data collection:

The dataset collected for this Ponte di Legno (PdL) study was pooled from three sources: 1) Clinical notes of the event 2) Severe adverse events (SAE's) trial databases 3) Combined clinical notes and SAE's trial databases. Due to different sources, almost 40% of data combined for the PdL neurotoxicity cases was just from the SAE's trial databases; on the one hand, this allowed us to obtain a larger sample size for these relatively rare cases and to be able to find novel findings, but on the other hand, there were a few limitations such as limited ability to carry

out meaningful analysis on all cases, due to them not always being well-characterised and thus containing missing data. There was variation observed in the response rate for demographic and clinical sections of the dataset, and it was observed that the data missing was intermittent and missing at random for the cases. To account for the missing data, it was decided to analyse every category/variable for the total number of responses obtained for the category without considering the missing information.

One other limitation of the PdL dataset was the lack of "no neurotoxicity" cohort. For the purpose of analysis, the different types of neurotoxicity were compared with the rest of the neurotoxicity cohort (RONC). This on one hand allowed us to have some interesting findings, but on the other hand the results need to be interpreted carefully because they cannot always be extrapolated to a clinical scenario. However, where possible, the comparison of the different types of neurotoxicity was also carried out with UKALL 2003 cohort to explore if different types of neurotoxicity had distinct demographic or clinical variables compared to children presenting with ALL. UKALL 2003 was a randomised trial for children with acute lymphoblastic leukaemia that included the patients aged one year up to 25 years and recruitment levels were high (>90%), therefore the patient characteristics can be considered "typical" of childhood ALL [13,47].

2.2.5 Statistical courses taken and analytic tools used:

The author of this thesis undertook following courses to learn applied statistics and R software.

1) Applied Statistics for Postgraduate Students

2) Advanced Medical Statistics

3) What is R?

4) More Advanced Use of R

All the analysis was run by the author and then cross-checked by the second supervisor, Dr Robin Young (Independent Biostatistician, Robertson Centre for Biostatistics, University of Glasgow).

Analysis to investigate the association with demographic and clinical parameters was carried out with R software (version 3.6.1); chi-squared and Fisher exact tests were used to assess associations between categorical variables. Unpaired two-tailed t-tests were applied for assessing the differences between continuous measures. For binary outcomes, logistic regression was used in both adjusted and unadjusted analyses. No adjustments for multiple testing were made, and a p-value <0.05 was considered statistically significant.

2.3 Materials and methods for chapter 9

2.3.1 Genotyping cohort

After re-classifying the SLS and PRES cases in the PdL Neurotoxicity database with the help of a differential diagnostic scoring system for SLS and PRES (see chapter 4 for details), the rest of the cases were manually reviewed. These above-mentioned steps lead to identification of defined phenotypes for which genetic polymorphisms associated were explored.

The neurotoxicity-associated candidate SNPs list was compiled by finding the reported SNPs of interest, that had shown an association with a) methotrexate-associated neurotoxicity b) Vincristine-associated neurotoxicity, by conducting a thorough literature search (Neurotoxicity candidate SNPs list attached in appendix).

The study groups from the PdL cohort with GWAS data available ran the list of neurotoxicity-associated candidate SNPs mentioned above against the GWAS data available from their group, including identified cases and matched controls; subsequently, a meta-analysis of the combined GWAS data was performed by the author.

Phenotypes included are given below:

2.3.2 Phenotype 1: All neurotoxicity cases:

This comprises all cases reported to the database that were confirmed to fulfil the inclusion and exclusion criteria for the study, i.e.:

Inclusion criteria:

1) Central neurotoxicity events occurring in patients (any age) treated on front- line paediatric ALL protocols, for the first episode of ALL or LBL

2) Patients diagnosed with ALL/LBL from 1/1/2000 to present

Exclusion criteria:

1) Neurotoxicity events occurring post-HSCT

2) Patients whose neurotoxicity is due to peripheral neuropathy

3) Patients whose event occurs at diagnosis before administration of any chemotherapy

2.3.3 Phenotypes 2-5: Classification method for SLS/PRES cases:

Firstly, an R-based algorithm was designed to test SLS/PRES cases (as reported by the referring clinicians) against the diagnostic criteria published in The Lancet Oncology consensus definitions paper [40].

Although about two-thirds of reported SLS/PRES cases fulfil the diagnostic criteria, there was a high level of "bi-phenotypic" (i.e., that fulfil criteria for both SLS AND PRES) cases and "missed" cases, and the consensus definition-based algorithm was ineffective in differentiating between SLS and PRES.

To overcome the problems of overlapping SLS and PRES cases (bi-phenotypic cases) and of the amount of missing data for clinical and radiological findings, a Differential Diagnostic Scoring System (DDSS) for SLS and PRES was developed by running a multivariate logistic regression analysis, on the cases reported as SLS and PRES, and identifying the clinical and radiological features that are significantly associated with these types of neurotoxicity. The development of the DDSS is explained in detail in chapter 4).

2.3.4 Phenotype 2 & 3: Stroke-like Syndrome:

Methotrexate-associated Stroke-Like-Syndrome is characterized by focal neurological deficits or hemiparesis, often accompanied by disturbances in speech and/or affect, which can wax and wane over the course of hours to days [40]. Stroke is a clinical potentially life threatening syndrome of presumed vascular origin characterized by rapidly developing signs of cerebral dysfunctions that can be focal or central.

Differential Diagnostic Scoring System DDSS for SLS:

To enter the scoring system, two conditions were a must:

1) The event occurring within 21 days of MTX administered

2) Other reasons ruled out (on the basis of MRI reports including cerebral infection CVST, leukaemia infiltrate and other non-specific findings consistent with other diagnosis)

Scoring based on the results from the multivariant analysis:

The range of scores was (1-3) where scoring 3, 2 and 1 indicated Definite SLS, probable SLS and possible SLS respectively.

After running the DDSS for SLS on the complete PdL dataset, there were two groups identified:

a) **Phenotype 2:** SLS definite (cases with a score of 3).

b) **Phenotype 3:** SLS definite + probable + possible SLS (SLS-DPP) - where probable and possible cases had some clinical features consistent with SLS. (Cases with a score of 1 and 2)

<u>Phenotypes 4 & 5: Posterior Reversible Encephalopathy Syndrome:</u>

PRES is defined as a clinico-radiological entity, often associated with vincristine and/or steroid therapy, clinically characterised by headache, hypertension, confusion (altered mental status), seizures and visual disturbances [40].

DDSS for PRES:

1) Initially, to enter the scoring system, a radiological finding consistent with PRES (MRI: T2WI-hyperintense, DWI-normal/ hyperintense, white matter or MRI provisional diagnosis consistent with PRES) was a must.

Scoring based on the results from the multivariate analysis:

The range of scores was from 2-5.5, where scoring 3.5 or higher with PRES-like MRI findings classified as having definite PRES, while cases scoring between 2-3 were classified as probable radiological PRES.

There were many cases with a clinician diagnosis of PRES but missing radiology due to the nature of the database, so it was decided to include an additional subset of cases that relaxed the requirement to have a radiological diagnosis provided on the database and further classified as detailed below:

The cases with missing radiological findings but scoring more than 3.5 based on their clinical findings were taken as probable clinical PRES and cases scoring 2-3 were taken as possible clinical PRES signifying that there were some PRES-like clinical features.

Therefore, there were two groups of cases picked from the whole PdL dataset with the score for PRES:

a) **Phenotype 4:** PRES definite (cases that fulfilled the score).

b) **Phenotype 5:** PRES definite + probable (radiological or clinical) + possible PRES (PRES -DPP) - where probable and possible cases had some clinical features like PRES.

2.3.5 Phenotype 6: Seizures NOS (Not Otherwise Specified):

Inclusion criteria:

Seizure during ALL therapy

Exclusion criteria:

1) Fulfil diagnostic criteria for PRES and SLS (Definite/probable/possible),

2) Due to cerebral bleed or CVST

3) Secondary to meningitis or cerebral infection

The cases that had seizures due to cerebral bleed or CVST and cerebral infection/meningitis were excluded because of a different aetiology, and it would be very unlikely to have the same genetic predisposition as a chemotherapy induced seizure.

2.3.6 Phenotype 7: Encephalopathy Not Otherwise specified (NOS):

Inclusion criteria:

same as for Phenotype 1

Exclusion criteria:

1) Classified as SLS (Phenotype 2 & 3)

2) Classified as PRES (Phenotype 4 & 5)

3) Classified as Seizures (Phenotype 6)

4) Classified as steroid psychosis.

5) Encephalopathy due to identified causes i.e., hepatic encephalopathy, leukaemic infiltrates, cerebral bleed, CVST, infection, radiological evidence of non-chemotherapy associated cause for symptoms e.g., vascular/cyst/anatomical malformation.

2.3.7 Phenotype 8: Recurrent neurotoxicity:

A recurrent neurotoxicity episode upon re-exposure to the presumed drug, of the same type of neurotoxicity (or similar type with the same presumed drug e.g., where the initial event was SLS and the recurrent episode was encephalopathy, as the presumed drug for both is MTX) of

definite PRES, PRES-DPP, definite SLS, SLS -DPP, Seizures NOS, and MTX-associated encephalopathy.

2.3.8 Phenotype 9: Persistence of symptoms:

Persistence of neurological symptoms includes cases that satisfy at least one of the following:

1) Clinician selected “no” resolution of symptoms after the first event
2) The patient had delayed resolution of neurotoxic symptoms (symptomatic more than one month after the primary event)
3) A record of ongoing neurological symptoms at the last follow-up for definite PRES, PRES-DPP, definite SLS, SLS -DPP, Seizures NOS, Seizures OA and MTX-associated encephalopathy.

2.3.9 GWAS results obtained from the study groups for Meta-analysis:

Our aim was to run a Genome-wide association meta-analysis to identify any single nucleotide polymorphisms (SNPs) associated with the risk of neurotoxicity, SLS, PRES, Seizures NOS, and encephalopathy NOS (phenotypes 1-9 mentioned in detail earlier) for children treated on ALL protocols. The following international study groups, that had run the GWAS on their respective cohorts, collaborated: 1) The Children's Oncology Group (COG), 2) Nordic Society of Paediatric Haematology and Oncology (NOPHO) and 3) Australian and New Zealand Children's Haematology/Oncology Group (ANZCHOG).

The platforms used by the study groups collaborating are given below:

Study group	Data	Array used
ANZHCOG	GWAS	Illumina Infinium Onco-array 530-K Beadchip
NOPHO	GWAS	Illumina Infinium Omni 2.5 exome-8- Beadchip

COG	GWAS	Affymetrix Genome-wide Human SNP 6.0

Table 2.3: Shows the GWAS array used by the collaborating study groups for the meta-analysis

Cases for each phenotype were identified by the author. The associated trial numbers were provided to the study groups COG, NOPHO and ANZCHOG. These study groups, COG, NOPHO and ANZCHOG, shared the results of a GWAS (i.e., SNP name and p-values) for the cases (of well-defined phenotypes as described above) identified on the PdL dataset, against controls for the neurotoxicity candidate SNPs list. The controls were defined as "no neurotoxicity" for each case, matched where possible for age, gender, race, ALL immunophenotype and treatment regimen.

Two of the study groups collaborating (COG and ANZCHOG) had ethnically varied patients enrolled on their respective treatment trials. Therefore, for ethnicity quality control the groups were requested to run the PCA for ethnicity to correct for population stratification in cases and controls for GWAS results.

Imputation of the results was conducted by the study groups; for the quality control of imputation, the rate of imputation was restricted to 0.8

2.3.10 COG cases:

The patient selection for the COG group is given in the **Error! Reference source not found.**.

There was no case-to-control matching of controls for each case and each phenotype, but each phenotype was analysed against the whole control group.

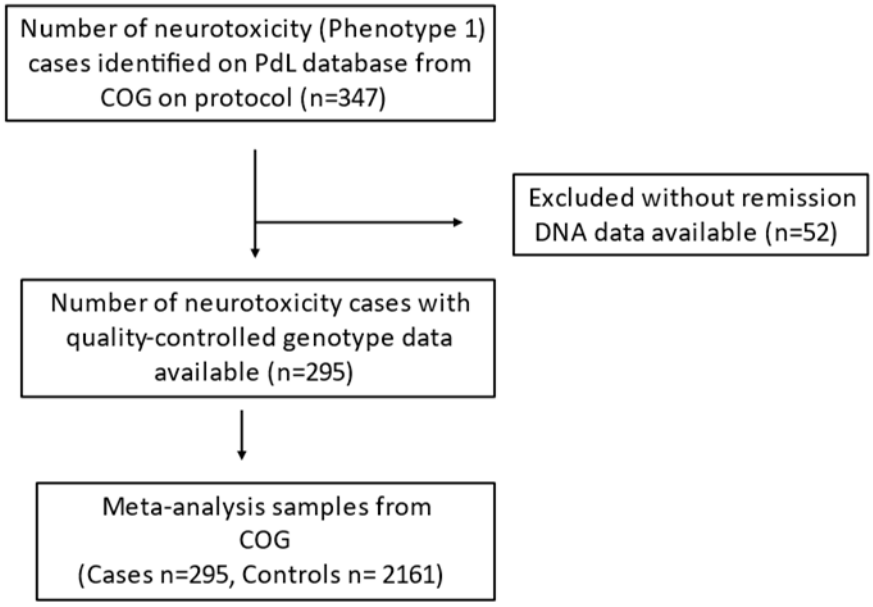


Figure 2.4: Consort diagram of COG neurotoxicity cohort for genotyping

There were 2456 samples (Cases=295, (From which the cases from trial AALL0232 n= 219 + trial AALL0434 n=76) and Control= 518, (From which the controls from the trial AALL0232 n=1636 + AALL0434 n= 525)) in The Children's Oncology Group (COG) contributed for the meta-analysis. The final COG cohort contributed symptomatic n= 295 cases (those who experienced neurotoxicity (Phenotype 1) during first line ALL treatment) and n= 2161 controls (those who did not experience symptomatic neurotoxicity during first line ALL treatment).

2.3.11 NOPHO cases:

There were 592 samples (cases=74 and controls= 518) contributed by the NOPHO study group. Details are shown in the Figure 2.5.

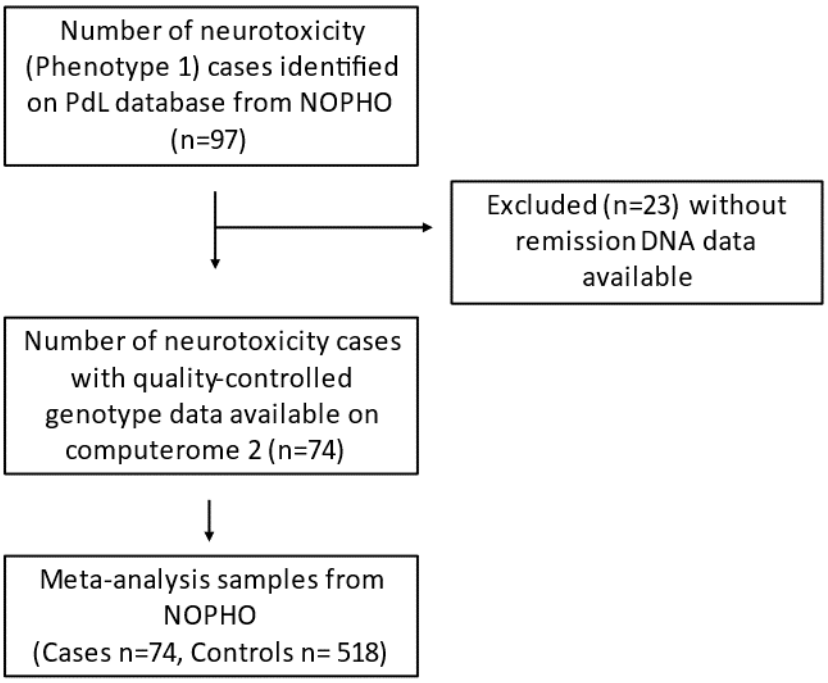


Figure 2.5: Consort diagram of NOPHO neurotoxicity cohort for genotyping

There were 592 samples (Cases=74, Control= 518) in the Nordic Society of Paediatric Haematology and Oncology (NOPHO) contributed to the meta-analysis. The final NOPHO cohort contributed symptomatic n= 74 cases (those who experienced neurotoxicity during first line ALL treatment) and n= 518 controls (those who did not experience symptomatic neurotoxicity during first line ALL treatment).

2.3.12 The Demographics of cases and controls:

Summary of the distribution of cases and their matched controls, at a ratio of (1:7), across age, sex, immunophenotype and treatment stratification on day 79 of the therapy is given below in **Error! Reference source not found.**.

	Controls (n=518)	P1 (n=74)	*P3 (n=15)	P4 (n=30)	P5 (n=46)	P6 (n=12)	*P8 (n=10)	P9 (n=39)
Age	8.0 -/+ 7.7	8.2 -/+ 4.4	10.3 -/+ 4.6	8.3 -/+3.2	8.13 -/3.7	7.4 -/+4.3	7.1 -/+3.7	8.0 -/+ 4.6
Male Female	283 235	37 37	6 9	16 14	23 23	7 5	6 4	15 24
Insufficient data SR IR HR (+HSCT) Excluded	22 221 196 51+28 -	1 28 27 11+7 -	0 6 8 0+1 -	0 12 10 4+4 -	0 19 16 6+5 -	0 4 5 2+1 -	0 3 5 2 -	1 18 7 8+5 -
B-precursor T-ALL Bi-lineage ALL mature B-cell missing data	442 65 7 2 2	57 17 0 0 0	12 3 0 0 0	23 7 0 0 0	36 10 0 0 0	9 3 0 0 0	9 1 0 0 0	29 10 0 0 0

Table 2.4: Summary of the cases and controls contributed by the NOPHO group showing the demographic distribution.

*PHENOTYPES 2 (n=1) and 7 (n=4) are excluded from table due to <10 patients.

SR (standard risk), IR (intermediate risk), HR (high risk), HSCT (haematopoietic stem cell transplant), B-precursor (immature B cell lymphoblasts leukaemia), T-ALL (T- lymphoblastic leukaemia), Bi-lineage ALL (bi-phenotypic myeloid and lymphoid leukaemia).

The information regarding demographics and clinical data for the COG and ANZCHOG study groups was not available at the time of writing this thesis.

2.3.13 ANZCHOG cases:

Consortium diagram (Figure 2.) shows the cases contributed by the ANZCHOG group.

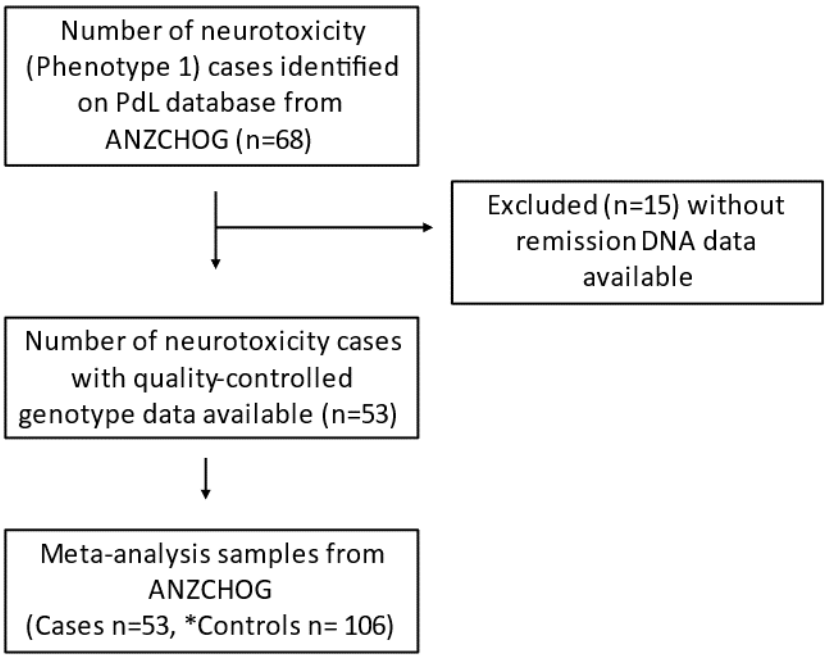


Figure 2.6: Consort diagram of ANZCHOG neurotoxicity cohort for genotyping

There were 159 samples (Cases=53, Control=106) in the Australian and New Zealand Children's Haematology/Oncology Group (ANZCHOG) contributed to the meta-analysis. The final ANZCHOG cohort contributed symptomatic n= 53 cases (those who experienced neurotoxicity during first line ALL treatment) and n= 106 controls (those who did not experience symptomatic neurotoxicity during first line ALL treatment) *out of which 97 controls were matched on all factors; sex, age, study protocol, diagnosis, and risk group. 9 controls were matched prioritising study protocol, diagnosis, and risk group.

2.4 Materials and methods for chapter 10

In order to be able to investigate possible mechanisms of neurotoxicity, an in-silico analysis was conducted to overlap the differentially methylated genes post-methotrexate exposure with the genes that have been shown to be significantly associated with neurotoxicity after methotrexate exposure.

For this purpose, two data sets were obtained:

2.4.1 Methylation changes post methotrexate exposure (in-vitro): Dataset 1

The first dataset was obtained from a collaborator Dr Vicky Foster at Newcastle University. In this model, human oligodendrocyte hybrid cell lines, created by fusing primary oligodendrocytes with the rhabdomyosarcoma cell line M03.13, were exposed to different concentrations of methotrexate (20, 30 and 50 nM), and methylation status was assessed with bisulphite conversion followed by pyrosequencing. (Figure 2.7)

Dr Forster collated data has CpG loci nearby which are similarly hypo or hypermethylated consistently in all three doses (20, 30 and 50 nM) of methotrexate used. A total of 340 probes were hypermethylated with significant delta-beta value, and 17 were found hypomethylated. Delta beta represents the change in methylation rate, measured in % change, at a particular CpG in the treated group compared to the control, e.g., a delta-beta value of 0.1747 indicates an increase of 17.47% methylation rate at that CpG loci in the treated cells compared to the control. The 340 hypermethylated CpG targets were annotated to 181 genes. The 17 hypomethylated loci were annotated to 13 genes, the rest were non-annotated. Part of this study is already published [109].

For the purposes of this investigation a delta beta threshold value of more than 5% for all the three doses (20 nM, 30 nM, and 50 nM) of methotrexate, was considered as differential methylation response post-methotrexate exposure.

2.4.2 Genetic variations from paediatric ALL neurotoxicity cases (in-vivo): Dataset 2

The second dataset was collected from a collaborator Dr Deepa Bhojwani and her group from St. Jude's Children Research Hospital, Memphis, USA. Genotyping data was collected from 369 children with ALL (including 14 cases with neurotoxicity and 86 cases with leukoencephalopathy) treated in a contemporary chemotherapy regimen that included five courses of high-dose MTX [51].

Neurotoxicity-enriched data yielded SNPs associated with neurotoxicity and leukoencephalopathy on the Affymetrix 500K/6.0 array sets (Santa Clara, CA) and custom Illumina Golden Gate array (San Diego, CA) designed on candidate SNPs as published previously. To expand the chances of hits while trying to map the methylation variation dataset with the SNPs dataset, raw data for all the custom 1,321 candidate SNPs of the Illumina Golden gate array was requested.

Significant value for association with clinical neurotoxicity and leukoencephalopathy was considered at p-value <0.05.

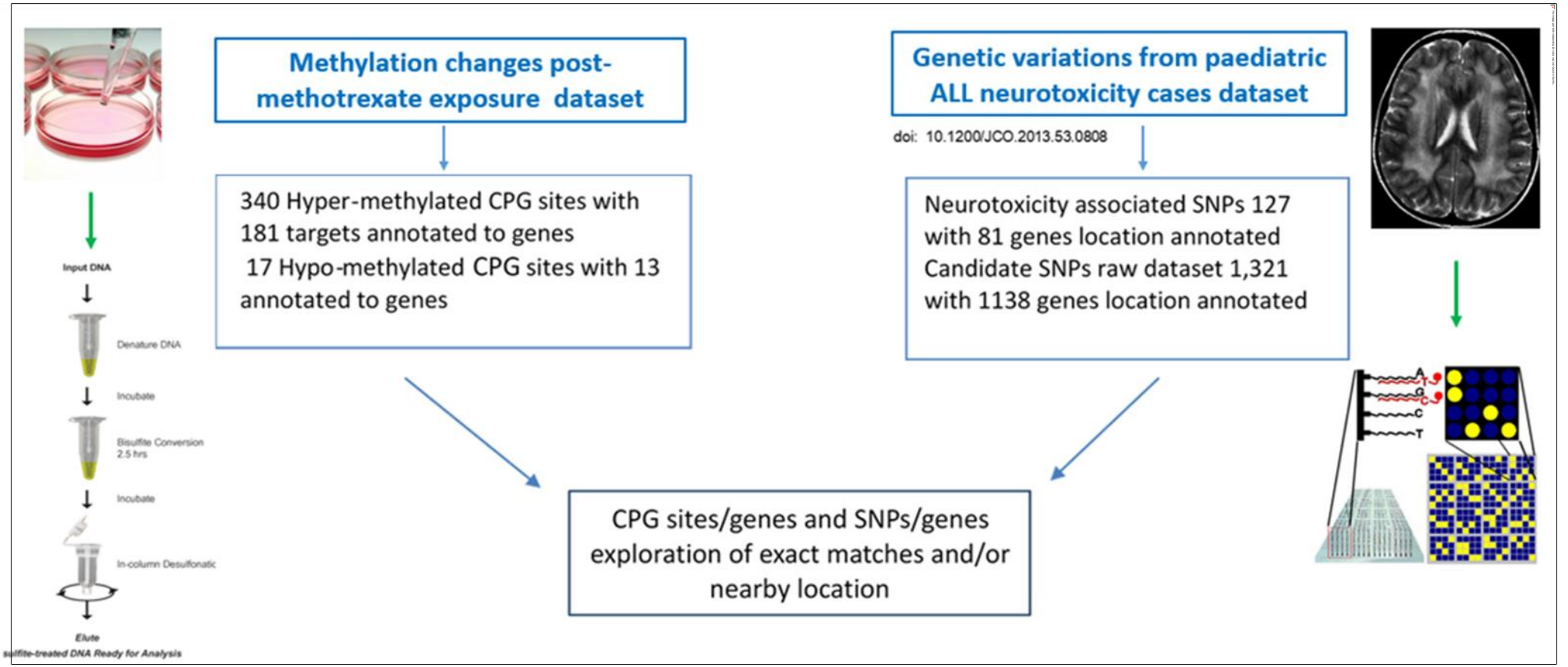


Figure 2.7:Flow chart showing datasets used for the in-silico analysis

To compile a list of SNPs associated with neurotoxicity two data sets were obtained (Figure 2.7):

a) Neurotoxicity and leukoencephalopathy-enriched SNPs published dataset with 127 SNPs with positions annotated to 81 genes evaluated on both Affymetrix and Illumina Golden gate analyses (Table 3 and supplemental Table 3 from the publication referenced) [51].
b) A raw dataset of 1,321 candidate SNPs evaluated on a customised Illumina Golden gate array, with 1138 positions annotated to genes, for these neurotoxicity and leukoencephalopathy cases [51].

2.4.3 Data analysis

2.4.3.1 Target position-based (SNPs/ CPGs) mapping:

Chromosome location-based coding:

In order to compare the large SNP genotyping and DNA methylation datasets, statistical software R version 3.5.1 was used. For comparison between these two datasets, there was a need to format the datasets to make them comparable. As the methylation sites were tagged with CpG target ID, e.g., cg_1224456, and SNPs sites were tagged with reference SNP cluster ID, e.g., rs-1445578. For this purpose, chromosome locations were determined on the UCSC genome browser (reference genome selected – Human GRCh37/hg19 assembly) for both CPG target IDs and SNP variation IDs and a unique chromosome-position identifier code was created that was comparable between the two datasets.

Search for nearby positions/regions in the datasets:

Following the coding of Methylation and SNPs datasets. Multiple search criteria were applied to identify matches between the methylation and SNPs datasets.

1) First, analysis was performed to identify exact matches of the two datasets (methylation and SNPs) using the chromosome-position identifiers.

2) Next, analysis was performed to identify positions/regions in close proximity to each other. For this purpose, a search within 50 base pair distance was performed.

3) Lastly, expanding the search, the nearest location between the target positions of CpGs from dataset 1 and SNPs from dataset 2 was carried out.

Genes annotated to SNPs and CpG targets-based mapping between the two datasets:

After no identification of matches between the methylation change and genetic variation datasets (by CpGs/SNPs), a candidate gene approach was adapted.

2.4.3.2 Annotation of the missing gene places:

The Methylation dataset (hyper-methylated and hypo-methylated CpG targets) had annotated genes for 181 of the hypermethylated and 13 of the hypo-methylated CpG targets, but there were 163 CpG targets, on both hypermethylated and hypo-methylated probes, with no genes annotated. To increase the chances of matches between methylation and SNPs datasets, a model for genes annotation in relation to methylation sites was adapted.

Model adapted:

This distance-based model for CpG annotation with genes described five gene-centric regions: the inter-genic region (>10 kb from the nearest Transcription start site ((TSS)), the distal promoter (−10 kb to 1.5 kb from the nearest TSS), the proximal promoter (−1.5 kb to +500 bp from the nearest TSS), the gene body (+500 bp to 3' end of the gene) and the downstream region (3' end to +5 kb from 3' end) [113].

Still, 113 CpG target sites on hyper-methylated probes and 4 CpG targets sites on hypo-methylated sites remained un-annotated in the methylation dataset.

Gene to gene matches

Gene to gene matches between the methylation and genetic variation datasets were investigated manually, as there were relatively fewer sites to look for. There were genes that

were already annotated in the two datasets (Dataset 1 and Dataset 2) for methylation and genetic variation [51]. For, the genes that were not annotated to any gene in the methylation dataset (Dataset 1) an attempt was made to annotate them by locating nearby genes up to 500 bp with the help of UCSC genome browser.

3 Acute neurotoxicity risk predictors – UKALL 2003 trial SAE's report analysis

3.1: Introduction

Chemotherapy-induced central neurotoxicity during treatment for childhood acute lymphoblastic leukaemia (ALL) remains a significant problem [13]. The incidence varies between the treatment protocols, different arms of the treatment protocols, Severe Adverse Events (SAE's) reporting systems, and the sub-types of neurotoxicity. On the UKALL 2003 study, the incidence of acute central neurotoxicity (encephalopathy) was reported as 8% on the low-risk protocol and 12% on the augmented therapy for high-risk patients [13,47], whereas 13% was reported in the NOPHO 2008 study [41].

Acute neurotoxicity presentations include Stroke-Like Syndrome (SLS) [48], Posterior Reversible Encephalopathy Syndrome (PRES) [49], and seizures [50].

There is a lack of understanding of which clinical risk factors are associated with central neurotoxicity and how they may vary according to sub-type. Previous reports suggest that treatment intensity, (particularly methotrexate exposure), age greater than 10 years and number of intrathecal may predispose to neurotoxicity [51,56-58], however, these studies may be confounded by the fact that the older age group (more than 10 years) is allocated to medium risk or high risk group and receives more methotrexate. Also, none of the studies have been sufficiently large to identify independent risk factors in multivariable analysis. Inferior leukaemia outcomes after neurotoxic events were reported by a study which might owe to the use of anticonvulsant drugs, used during their treatment [59]. More recently, a study reported increased CNS leukaemia relapses in methotrexate-induced neurotoxicity, but this finding was limited only to the patients with chemotherapy modifications [57].

Thus, there is an urgent need to better understand the risk factors and natural history of neurotoxicity and develop ways of identifying children at risk. In order to investigate clinical risk factors for neurotoxicity and its effect on outcomes in a large cohort of patients, a review and case-control study was carried out of all neurotoxic serious adverse events (SAEs) reported for UKALL2003.

UKALL 2003, a randomised controlled trial, running from 1st October 2003 to 30th June 2011, to determine whether treatment intensity could be adjusted for children and young adults with MRD-based risk stratification.

Severe adverse events (SAE's) reporting to ensure patients safety on the trial based on the comprehensive National Cancer Institute (NCI) created Common Terminology Criteria for Adverse Events (CTCAE) was used. Although it has its beneficial merits in patient safety but has limitations such as its time consuming and lacks detail of the incident.

Treatment regimen allocation in UKALL 2003 trial

A simplified version of the criteria for treatment regimen allocation (on the basis of the demographic and clinical features at the time of ALL diagnosis, the bone marrow minimal residual disease (MRD) response to the chemotherapy) and a description of the treatment regimens are given below.

1) **Regimen A** - standard risk, allocated to age less than 10 years, white blood cell counts $\leq 50x10^9/l$ at the time of ALL diagnosis

   Treatment allocated - three drugs (Vincristine, dexamethasone and asparaginase) induction phase (weeks 1-5), CNS-directed IT MTX with dexamethasone and vincristine pulses (3 weeks), Interim maintenance I with dexamethasone and 6-mercaptopurine (8 weeks), Delayed intensification I (8 weeks), and shorter duration maintenance phase with dexamethasone and mercaptopurine (weeks 39-112 for girls and weeks 39-164 for boys)

2) **Regimen B** – medium risk, ≥ 10 years, white blood cell counts ≥ 50x109/l at the time of ALL diagnosis

   Treatment allocated – additional daunorubicin, four drug induction (week 1-5), additional Interim maintenance II for the cases (8 weeks) and relatively longer maintenance phase (weeks 41-114 for girls and 41-166 for boys)

3)Regimen C - high risk, BCR-ABL, MLL rearrangements, hypodiploidy, AML1 amplification, and inadequate bone marrow response to chemotherapy based on MRD on day 8/15 of Regimen A & B.

Treatment allocated – additional four drug induction (weeks 1-5), Interim-Capizzi I with escalating doses of IV methotrexate (8 weeks), additional Delayed intensification II (8 weeks) and longer than maintenance phase (week 47-118 for girls and week 47-170 for boys)

3.1 Aims:

1) To identify demographic and clinical risk predictors of susceptibility for the occurrence of chemotherapy-induced neurotoxicity.
2) To investigate whether having a reported neurotoxic SAE had an impact on 10 year event-free survival, overall survival, and relapse rates.

3.2 Results

3.2.1 Aim 1: To identify demographic and clinical risk predictors of susceptibility for the occurrence of chemotherapy-induced neurotoxicity

3.2.1.1 The timing of Neurotoxicity events

It was of interest to explore the timing of neurotoxic events from the start of the chemotherapy and to observe any difference in the timing of occurrence depending on the treatment regimen allocated.

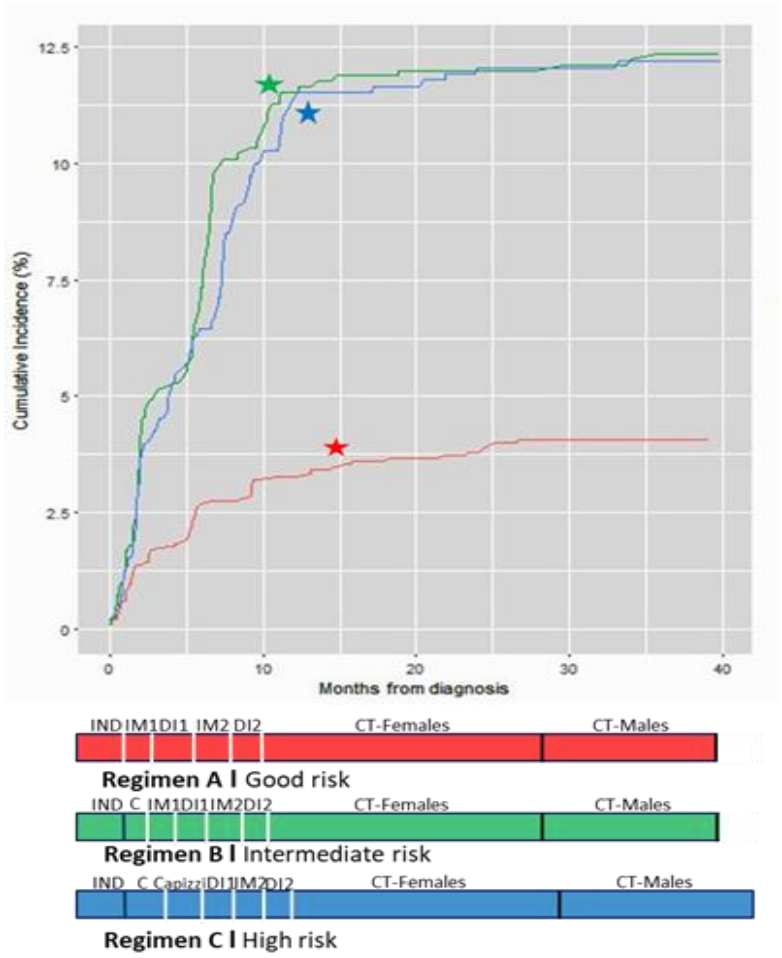


Figure 3.1: Shows the cumulative incidence of neurotoxicity events during the treatment regimen A, B and C.

Time to neurotoxicity event was defined in months from the start of ALL treatment to the day of neurotoxicity, censoring for the occurrence of neurotoxic events. The coloured stars on the plot correspond with the treatment regimen block shown at the base of the plot. (Treatment Regimen A ★ , Treatment Regimen B ★, and Treatment Regimen C ★)

It was observed that the cumulative incidence of neurotoxicity events during all three treatment regimens (A, B and C) were more common in the initial 220 days of treatment (induction, consolidation and delayed intensification time point in treatment) than in the later part of the treatment. The cumulative incidence was peaking at the 15 month time point in treatment regimen A (less intense therapy), which is later than the treatment regimen B & C (more intense therapy), which peaked at 12 months and 13 months respectively. (Figure 3.)

3.2.1.2 Risk predictors of central neurotoxicity - Univariate analysis

To identify risk predictors of central neurotoxicity in this cohort, the demographic and clinical features were explored. The features of interest were

1) Age at the time of ALL diagnosis

a) 1-10 years b) 10 -14 years c) more than 15 years

2) Gender a) Male b) Female

3) White blood cell counts at the time of ALL diagnosis a) ≤ $50x10^9$/l b) ≥$50x10^9$/l

4) Treatment Regimen allocation a) Regimen A, b) Regimen B, c) Regimen C

5) CNS status at the time of diagnosis

a) CNS status 1 (no blast cells on CSF sample)

b) CNS status 2 (< 5 WBC/mm3 with blasts in a sample with < 10 erythrocytes/mm3)

c) CNS status 3 (> 5 WBC/mm3 with blast cells in a sample with <10 erythrocytes/mm3)

d) TLP (> 10 erythrocytes/mm3 with blast cells) (Table 1 Introduction chapter)

6) Race a) white ethnicity b) non-white ethnicity

7) Cytogenetics a) Good risk (High-hyperdiploidy, ETV6-RUNX1) b) Intermediate risk (all other) c) poor prognosis (hypodiploidy, BCR-ABL, MLL rearrangement)

8) Immunophenotype a) B-cell immunophenotype b) T-cell immunophenotype

Results of the univariate chi-squared analysis of these above-mentioned demographic and clinical variables to explore the association with neurotoxicity are shown in the (**Error! Reference source not found.**).

Variable	Category	Total	with NT	No NT	% with NT	P*
Total		3091 (100)	254 (100)	2837 (100)	8	
Sex						
	Female	1335 (43)	126 (50)	1209 (43)	9	0.03
	Male	1756 (57)	128 (50)	1628 (57)	7	
Age						
	1-9	2265 (73)	133 (52)	2132 (75)	6	<.001
	10-14	499 (16)	81 (32)	418 (15)	16	
	>=15	327 (11)	40 (16)	287 (10)	12	
WCC						
	<50	2412 (78)	181 (71)	2231 (79)	8	0.006
	>=50	679 (22)	73 (29)	606 (21)	11	
Treatment						
	A	1528 (49)	62 (24)	1466 (52)	4	<.001
	B	833 (27)	103 (41)	730 (26)	12	
	C	730 (24)	89 (35)	641 (23)	12	
CNS						
	CNS1	2695 (87)	204 (80)	2491 (88)	8	0.005
	CNS2	156 (5)	22 (9)	134 (5)	14	
	CNS3	34(1)	3 (1)	31 (1)	9	
	TLP	206 (7)	25 (10)	181 (6)	12	
Race						
	White	2636 (86)	227 (89)	2409 (85)	9	0.06
	Non-white	455 (15)	27 (11)	428 (15)	6	
Cytogenetics						
	Good risk	1579 (51)	109 (43)	1470 (52)	7	0.05
	Intermediate risk	852 (28)	77 (30)	774 (27)	9	
	High risk	121 (4)	14 (5)	107 (4)	12	
Lineage						
	B	2711 (88)	211 (83)	2500 (88)	8	0.02
	T	380 (12)	43 (17)	337 (12)	11	

Table 3.1: Association of demographic and clinical variables with neurotoxicity

Results of the chi-squared test are shown in the table.

In the univariate analysis, neurotoxicity was found significantly associated with treatment intensity (p-value <.001), age (p-value <.001), female sex (p-value .03), CNS status (p-value .005), immuno-phenotype (p-value .02) and white cell count (p-value .006). However, it was observed that race did not show significant association with neurotoxicity (occurrence of neurotoxicity in overall UKALL 2008 cohort without stratifying into low-risk and augmented risk groups).

3.2.1.3 Univariate logistic regression analysis:

Univariate logistic regression analysis was run on the variables that showed significant association with neurotoxicity to explore the likelihood of the neurotoxicity as an outcome, with these demographic and clinical features.

Table 3.2: Univariate logistic regression analysis of the demographic and clinical significantly associated features

Variable	Comparison	OR	95% CI	p
Sex	Male vs Female	0.76	0.58-0.98	0.03
Age	>=10 vs <10	2.75	2.12-3.56	<.001
WCC	>50 vs <50	1.49	1.12-1.98	0.006
Immunophenotype	T-cell vs B-cell	1.52	1.07-2.15	0.02
Treatment	non-A(B&C) vs A	3.27	2.43-4.40	<.001
Race	non white vs white	0.67	0.44-1.00	0.05
CNS	non CNS1 vs CNS1	1.77	1.27-2.46	<.001

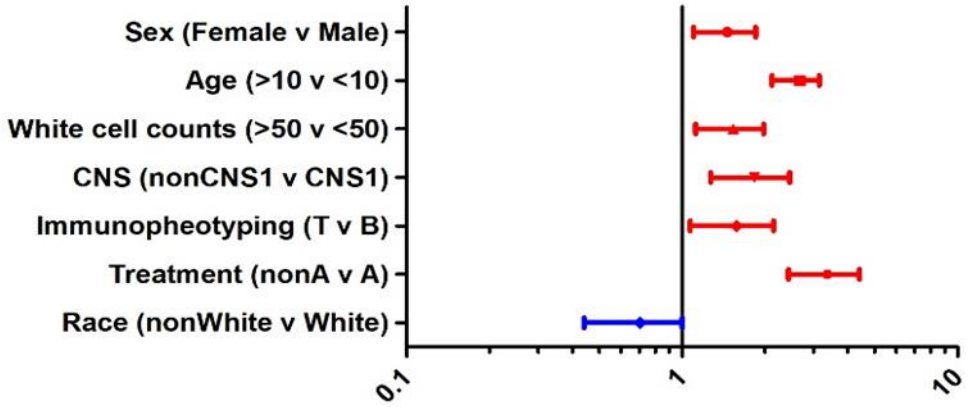


Figure 3.2: Forest plot for association of clinical and demographical risk factors with neurotoxicity event.

The odds ratio values, shown with 95 % CI, found significantly associated are coded in red and the one not found associated is coded in blue.

Univariate logistic analysis model showed that female gender, older age group, CNS involvement at the time of diagnosis, treatment intensity B & C and T-cell immunophenotype showed significant association with neurotoxicity. (

Table **3.2**Error! Reference source not found. & Figure 3.)

Age (>=10 years vs <10 years) and treatment intensity (Treatment regimen B&C vs Treatment regimen A) had the highest odds ratio, showing that the older age was 2.75 times more likely and treatment regimen B and C are 3.27 times more likely to get a neurotoxic event.

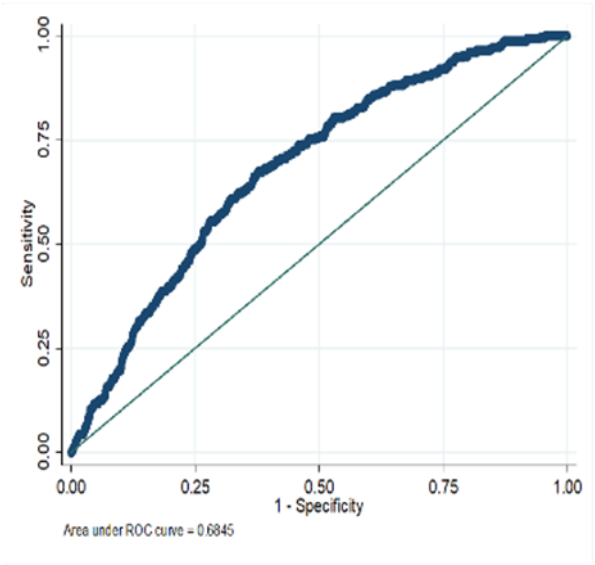
3.2.1.4 Multivariate logistic regression analysis:

The variables significantly associated with neurotoxicity were fitted in a multivariate logistic regression analysis model. The results of the model with the highest predictive value are shown below in the **Error! Reference source not found.** & Figure 3..

Error! Reference source not found.**: Shows the multivariate analysis model with the highest s tatistically significant predictive value**

Variable	Comparison	OR	95% CI	p-value

Sex	Female vs Male	1.42	1.10-1.85	0.008
Age	continuous	1.04	1.01-1.07	0.003
CNS	not CNS1 vs CNS1	1.60	1.14-2.24	0.006
Treatment regimen	B/C vs A	2.61	1.86-3.65	<.001



Sensitivity 64.17%

Specificity 63.89%

Figure 3.3: ROC curve with the best predictive value

Model sensitivity: is the probability of being predicted to have neurotoxicity given that the patient has neurotoxicity

Model specificity: is the probability of being predicted not to have neurotoxicity given that the patient does not have neurotoxicity

Multivariate analysis revealed that treatment allocation (regimen B/C vs A, odds ratio 2.61 (95% CI 1.86-3.65), female sex (1.42 (1.10-1.85), CNS status (CNS2/3/TLP vs CNS1, 1.60 (1.14-2.24)) and increasing age (1.04 (1.01-1.07) per year) remained significant independent predictors of neurotoxicity.

3.2.1.5 Increasing age as a risk predictor

The finding that older age is significantly associated with neurotoxicity may be confounded by the fact that the older age group is allocated to the more intense treatment regimen group. To separate the effect of age from the effect of treatment group, a focussed analysis on the younger patient group only was conducted. Confining analysis to the patients that were treated on Treatment regimen A (age <10 years), age as a continuous variable was analysed; results are shown in **Error! Reference source not found.**.

Error! Reference source not found.: Multivariate analysis of patients on Reg A for age and C NS status at the time of diagnosis as variables.

Patients originally treated on A and continued treatment A				
Variable	**Comparison**	**OR**	**95% CI**	**p-value**
Age (per year)	continuous	1.15	1.03-1.29	0.02
CNS	not CNS1 vs CNS1	2.08	1.08-4.00	0.03

In the age group <10 years, age as a continuous variable remained significant, in patients treated on the same lower intensity treatment regimen, so the association with age is not due to differences in treatment intensity in this cohort. Increasing age with OR 1.15 showed a higher chance of having neurotoxicity, raised by 15% per year.

3.2.2 Aim 2: Survival rates: Ten-year event-free survival, overall survival, and relapse rate

3.2.2.1 Survival analysis

It was of interest to run survival analysis in the neurotoxicity cases in comparison with the "no neurotoxicity" cases to be able to see if there are any effects on the leukaemia outcome.

1) Event-free survival (EFS) - the length of time after treatment for ALL ends and that the patient remains free of certain complications or events.
2) Relapse rate (RR) - the length of time after treatment return of ALL in patients who have already undergone treatment for the disease.
3) Overall survival (OS) - length of time, from the date of diagnosis to the point of analysis, that patients diagnosed with the disease are still alive.

The overall and relapse-free survivals of the patients with and without neurotoxicity are shown in the Kaplan Meier (KM) plots below: (Figure 3., Figure 3. , Figure 3.**Error! Reference source not found.**)

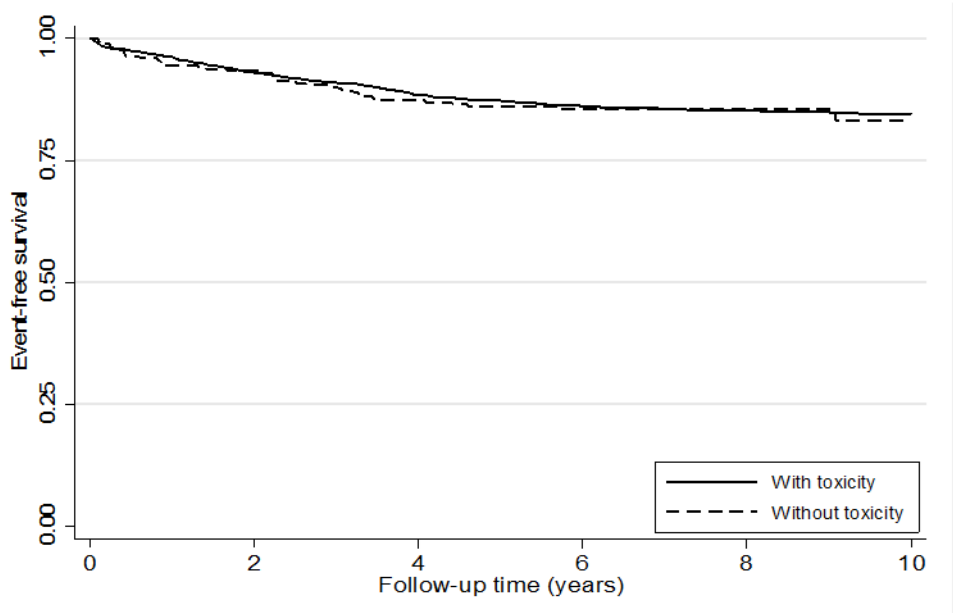


Figure 3.4: Ten-year event-free survival between groups with and without neurotoxicity

Kaplan Meier calculated the event-free survival probabilities of each patient of two different groups, neurotoxicity and without neurotoxicity group, with censoring for death, relapse, other relevant complications, or last follow-up, whichever occurred first. There was no significant difference observed between the two groups.

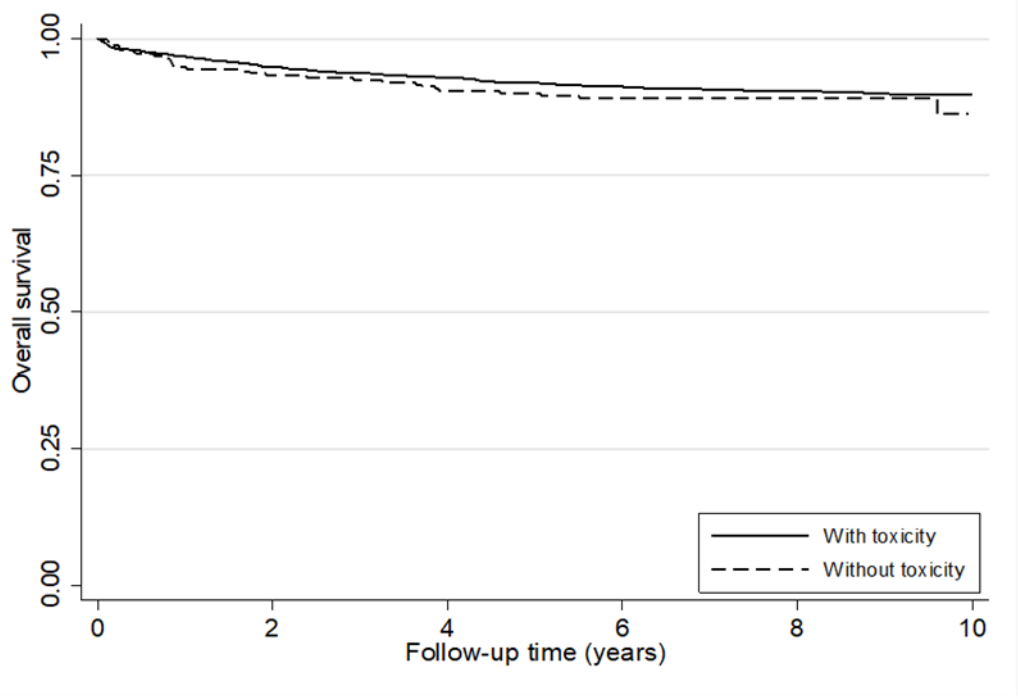


Figure 3.5: Ten-year overall survival comparison for the two groups, with and without neurotoxicity

Kaplan Meier calculated the survival probabilities of each patient of two different groups, neurotoxicity and without neurotoxicity group, with censoring for death, relapse or last follow-up whichever occurred first. There was no significant difference observed between the two groups.

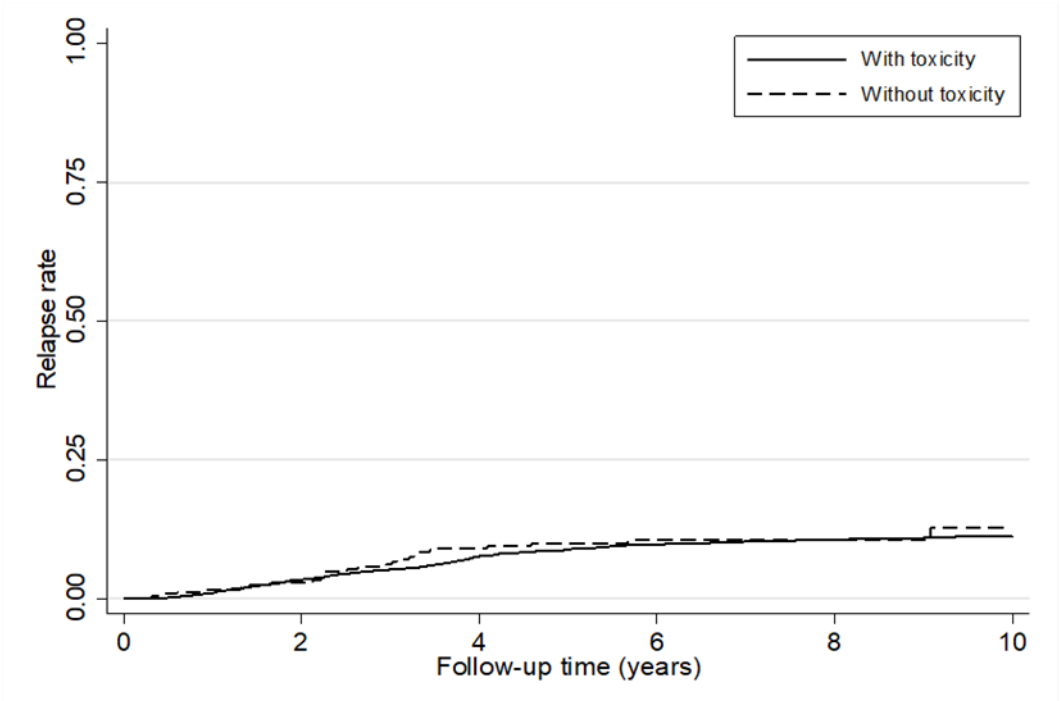


Figure 3.6: Ten-year relapse rate comparison between the two groups, with and without neurotoxicity

Kaplan Meier showing the calculated relapse probabilities of each patient of two groups, neurotoxicity and without neurotoxicity, with censoring for relapse or last follow-up, whichever occurred first. There is no significant difference observed between the two groups.

There was no statistically significant difference in ten-year event-free survival (p-value 0.8), overall survival (p-value 0.3) and relapse risk (p-value 0.7) between the two groups. The number of deaths reported in the neurotoxicity group were 28 (11% vs 9% in no neurotoxicity group) but to establish that the cause of death was due to the neurotoxicity or other causes was not possible.

3.3 Discussion

To the best of our knowledge, the present study is the largest case series of neurotoxicity cases, and it reports interesting novel findings. Analysis of the UKALL 2003 trial dataset, with 3091 patients enrolled (276 neurotoxicity reports in 254 patients and 2837 controls), identified independent risk predictors. Treatment intensity was the main risk factor for developing acute neurotoxicity, with female sex, increasing age and CNS status having a significant modifying effect. (**Error! Reference source not found.**)

Analysis showed that events were frequent in the first 220 days of therapy, which goes in accordance with a study that showed SLS is more likely in the consolidation and delayed intensification part of the therapy [114].

In the age group <10 years, age remained significant, independent of the treatment regimen with OR 1.15, p-value 0.002 (**Error! Reference source not found.**). In a previous study, age w as shown as a predictor in the above 10 years age group, but this finding is confounded by the fact that age group above 10 years age are given more intense treatment and this finding was not observed in multivariate analysis, which might be due to small patient numbers [51]. More recently, a study showed age more than 10 years as an independent risk predictor, but the report included only methotrexate-associated neurotoxicity [57].

Females are more vulnerable to chemotherapy-induced neurotoxicity on contemporary chemotherapy treatment protocols. The chances of late cognitive dysfunction in female patients are higher than those in male patients, particularly if given CNS-prophylaxis with a combination of cranial irradiation and high doses of methotrexate [115,116]. Another report regarding neurocognitive effects in long-term paediatric ALL survivors, St. Jude Total XV treatment protocol (chemotherapy only), suggested that, in comparison to male survivors, female survivors are more likely to have impairments to the working memory network, contributing to continuing neurocognitive difficulties [117]. The one possible explanation can be the developmental gender-based differences reported previously in relative white matter and parenchymal volume between male and female children, where delay in development was observed in female children [118] but requires further research. This finding of female patients showing significant association with central neurotoxicity in our study is surprising because male patients get a lot more therapy than females (this difference is in maintenance whereas a lot of the acute neurotoxicity occurs earlier) but it still might impact on the chronic neurocognitive outcomes as discussed above.

CNS status may reflect increased intrathecal therapy during the therapy given to non-CNS-1 patients where CNS-1 (treatment regimen A) were administered twenty-three (in boys – as the duration of maintenance phase of the therapy was more in al treatment regimens for boys as per protocol) and nineteen- in girls IT MTX but in non-CNS-1 (treatment regimen B and C) were administered twenty-six and twenty-eight (in boys) and same twenty-three in girls in both treatment regimen B and C, IT MTX during the therapy showing that CNS-1 were receiving less IT MTX overall as compared with non-CNS-1. Previously, intrathecal methotrexate neurotoxicity has been linked with increased levels of methotrexate in the central nervous system following

an intrathecal methotrexate administration, where they found that the amount of the drug in the central nervous system after 48 hours was on average 13 times higher in those who had experienced neurotoxicity (one-quarter of patients) than those who did not have neurotoxicity [119]. There is also evidence that patients with CNS leukaemia may be more susceptible to PRES, a type of neurotoxicity, owing to inflammation and endothelial dysfunction related to the presence of bulky CNS disease [49,120].

Treatment intensity (more intense Treatment Regimen B and C) was an independent risk factor in our study, which might owe to more intense chemotherapy allocated. Bhojwani *et al.* reported standard risk/high risk treatment regimen allocated as a risk predictor for methotrexate-induced neurotoxicity but did not demonstrate it in a multivariate analysis [51].

Survival analysis of UKALL 2003 neurotoxicity SAE's dataset showed that there was no difference in 5-year event-free survival, overall survival and relapse risk (Figure 3., Figure 3.,**Figure 3.**). Although studies published showed that leukaemia relapse rate is increased after the neurotoxic event, the results might be confounded by the adjuvant use of anticonvulsant drugs, especially phenytoin, used during therapy [59]. Enzyme-inducing anticonvulsants, phenytoin, phenobarbital, and carbamazepine, have been shown to increase metabolism of anti-leukaemic drugs, increase the clearance and reduce drug levels thus increasing relapse risk in paediatric ALL [60].

3.3.1 Conclusion:

The results from this study provide an important benchmark for studies focused on deep phenotyping and studies of chemotherapy-associated neurotoxicity. The strengths of this study being that the comparative group was “no neurotoxicity”. As this analysis was carried out on SAE reports of UKALL 2003, limited information about clinical aspects of these neurotoxicity events made it impossible to carry out deep phenotyping. Also, as there was a reliance on local centres for reporting these SAE’s, it is important to acknowledge underreporting and misreporting as there is a lack of accurate understanding to report and classify these cases.

4 Classification of PRES and SLS cases to address bi-phenotypic cases.

4.1 Introduction

SLS and PRES are well reported and thought to be aetiologically distinct entities that have a range of clinical presenting features.

Earlier on, the variation in clinical definitions and diagnostic criteria was recognised and applying a Delphi consensus method, diagnostic criteria for SLS and PRES were published in Lancet oncology [40].

Even so, there appears to be some overlap in symptoms between PRES and SLS, and it is still unclear whether radiological criteria can effectively be used to distinguish between these two presentations.

SLS occurs within twenty-one days of administration of methotrexate, the underlying pathophysiology is cytotoxic oedema whereas, PRES is associated with vincristine and steroids, underlying pathophysiology is possibly hypertension-induced vasogenic oedema.

More recently, Apparent diffusion coefficient (ADC) values has been suggested to discriminate between SLS and PRES, where ADC values are the quantitative measure of the impedance of the water molecule diffusion, calculated automatically by the software and displayed as a parametric map, using MRI with diffusion weighted imaging (DWI) [94,95]. ADC values have a contrasting effect for SLS and PRES, with PRES showing increased values (due to vasogenic oedema) and SLS showing decreased ADC values (due to cytotoxic oedema), however more studies will be needed to confirm these results. It is important to understand that seizures, while thought being a feature of SLS, are not only a chemotherapy-induced phenomenon of their own but also can occur as part of the PRES presentation and may or may not share the same pathophysiology. Assessing the degree of the overlapping of SLS and PRES and developing a diagnostic criterion of these entities will result in having access to important information that will prove valuable to the treating clinicians, patients, and their parents, when confronted with difficult, distressing, and rare clinical scenarios. It may also prove useful for determining preventative strategies.

Also, to accurately classify PRES and SLS was important for the analysis of this thesis, as there appeared to be apparent under-reporting of SLS from some countries despite widespread use

of methotrexate. This means to identify cases for phenotyping cannot rely on self-reports from the centres on the questionnaire.

4.2 Aims

1. To test consensus definition diagnostic criteria for PRES and SLS on cases reported in the PdL neurotoxicity study.
2. To identify any areas in the diagnostic criteria that need improvement with findings from literature review.
3. To create a differential diagnostic score to accurately assign cases as SLS and PRES with minimal overlap and to assign cases as definite, probable or possible dependent on the amount of information available.

4.3 Results

Cases:

For analysis, the cases reported as PRES, n=231 were the test group, and the Rest of the Neurotoxicity Cohort for PRES (RONC-P, n=1582) was taken as the comparative group.

For the cases reported as SLS, n=248, the rest of the neurotoxicity cohort for SLS RONC-S, n=1565, was taken as the comparative group.

4.3.1 Aim 1: To test consensus definition diagnostic criteria on clinical cases from PdL with the ability to do a comparison for PRES and SLS criterion.

Recognizing the problem of overlapping SLS and PRES symptoms and due to the need to identify the true cases of SLS and PRES for further analysis, an R-based algorithm was designed based on the consensus definitions diagnostic criterion already published [40]. The diagnostic criterion for PRES and SLS is given below:

The diagnostic criterion for PRES is composed of two categories

1) Essential criteria with

Clinical features any combination of; a) Headaches b) encephalopathy/confusion c) seizures d) visual disturbances and

Radiological features a) MRI DWI and b) MRI T2 FLAIR findings.

2) Additional supportive findings: a) Timing during the first3-4 months of therapy

b) presence of arterial hypertension c) complete resolution of symptoms.

The diagnostic criteria for SLS,

Within 21 days of MTX therapy (intrathecal/intravenous): an essential condition

The cases must fulfil all 3 of the following criteria:

1) New onset of one or more of the following neurological symptoms/signs

a) Paresis/paralysis b) Aphasia/dysarthria c) Altered mental status- somnolence, confusion, disorientation, emotionally lability etc.

d) Movement disorder e) Loss of consciousness f) Bilateral weakness

g) Seizures (isolated seizures without accompanying features from the list above and without criteria 2 and 3 below are excluded)

2) EITHER: Findings of characteristic white matter changes of leukoencephalopathy on MRI

AND/OR: Characteristic waxing and waning of symptoms usually with complete resolution within a week

3) No other identifiable cause

The R-based consensus definitions diagnostic criteria-based algorithm was designed replicating the exact conditions for PRES and SLS.

There were four different conditions for which the cases on the PdL database were tested

1) The cases reported as PRES and SLS were tested against their diagnostic criteria

2) Reverse testing of the algorithm i.e., testing the diagnostic criteria of PRES on SLS cases and vice versa to identify any overlapping cases.

3) The complete neurotoxicity dataset was tested with the algorithm in order to identify additional probable, possible SLS and PRES cases and encephalopathy cases.

4) The SLS and PRES cases that were picked from the whole dataset with the algorithm were reverse tested on the opposite diagnostic criteria algorithm i.e., SLS whole dataset cases were tested with PRES algorithm and vice versa.

The results for these four different conditions are given below:

	SLS (cases/total)	**PRES (cases/total)**
Cases as reported by the study groups	248	231
Cases true to algorithm	155/248	206/231
Cases tested on reverse algorithm	154/231	83/248
Cases picked from the whole dataset using the algorithm	418/1813	481/1813
Whole dataset cases tested on reverse algorithm (i.e., SLS cases identified as PRES and vice-versa)	368/481	121/418

Table 4.1: Testing of reported PRES and SLS cases on an R-based algorithm

1) About two-thirds of the reported SLS cases were true to SLS consensus definitions diagnostic criteria algorithm. In case of PRES, more cases were true to their diagnostic criteria algorithm.
2) The reverse testing revealed many bi-phenotypic cases, i.e., about two-thirds of reported SLS and PRES fulfilled criteria for both conditions.
3) Testing the complete dataset with the diagnostic criteria algorithm to pick SLS and PRES revealed likely "missed cases".
4) Reverse testing of the SLS and PRES cases picked from the whole dataset showed that algorithm is ineffective in discriminating between SLS and PRES clearly.

4.3.2 Aim 2: To identify any areas in the diagnostic criteria that need improvement with findings from literature review.

The next step taken was to do a literature review to be able to identify any areas in the clinical and radiological features in the consensus definitions diagnostic criteria that can be improved on, or if there are any features that are not frequently reported in SLS and PRES cases. The PubMed search terms are given below:

1) PRES were - "Posterior reversible encephalopathy syndrome" OR "reversible encephalopathy syndrome" AND "acute lymphoblastic leukaemia" from 2000 onwards, on 17 -01-2019

2) SLS were - "stroke like syndrome" OR "methotrexate encephalopathy" OR "subacute encephalopathy" AND "acute lymphoblastic leukaemia" from 2000 onwards, on 17 -01-2019

PRES: The results were filtered and the studies that were excluded were a) aetiologies other than chemotherapy used in frontline ALL protocols for PRES b) insufficient MRI radiology findings reported and 3) studies of adult patients. The observations made from the literature review (**Error! Reference source not found.** & **Error! Reference source not found.**) for the publications included are given below:

Author	Year publi-shed	Age	Clinical symptoms	MRI - T2	DWI	ADC
Norman JK et al [72]	2007	4-13 years	Severe headache, episodes of confusion, seizures, eye twitching, **Hypertension**	abnormalities	normal	hyperintense
Gupta A et al [74]	2008	9-11 years	Seizure, visual impairment, paralytic ileus, lower motor neuron weakness of the left lower leg (1/2)	hyperdense		Increased values
Dicuonzo F et al [121]	2009	15 years	Multiple seizures	hyperintense	hypointense	high values
Shokichi T et al [122]	2012	28 years	**Hypertension**, generalized seizures, loss of consciousness, visual acuity, and muscle weakness in the legs.	hyperintensity	hyperintensity	elevated intensities
Shimizu Y et al [123]	2013	10 years	Headache, repeated with vomiting, nausea, drowsy, **Hypertension**, visual disturbances	hyperintensity	Isointense	increased values

Papayannidis C et al [124]	2014	18 years	Generalized tonic- clonic seizures, unconsciousness **Hypertension**, confused, strong-force defect involving the left arm(1 patient)	Intensities	Intensities	showed elevated signal

Table 4.2: Literature review of PRES for clinical and radiological findings

1) Seizures were the most common clinical feature presented. However, three-quarters of the studies reported hypertension as part of the clinical presentation of the PRES cases that had MRI presentations reported consistent with PRES.
2) It was also observed that there were a couple of instances of focal pyramidal weakness, a clinical feature characteristic of SLS.
3) The studies included had ADC mapping findings (hyperintense or increased values) that are consistent with radiological ADC findings for PRES.
4) The MRI T2 Flair findings are consistent with PRES.
5) The terminologies that are used to report the radiological findings vary, which can potentially lead to misinterpretation and/or misdiagnosis.

Author	**Year**	**Age**	**Clinical symptoms**	**MRI - T2**	**DWI**	**ADC**
Haykin et al [125]	2006	13-20 years	Hemiparesis, confusion, ataxia, aphasia, ataxia, dysarthria		hyperintense	hypointense
Balin J, Parmar H,	2008	24 years	Right-sided paralysis, aphasia, facial asymmetry,	unremarkable	restriction diffusion	

Kujawski L [65]			swallowing impaired, paresis of right upper & lower limb			
Tufekci O et al [126]	2011	11 years	Headaches, dysarthria, blurred vision, hemiparesis, with 4/5muscle strength in right upper & lower extremities	minimal increased	restricted diffusion	
Iwatani K, Fujii N, Deguchi S, Tanimoto M [127]	2012	20 years	Right hemiplegia, waxing and waning of symptoms, inability to vocalize, & dysphagia	unremarkable	high signal intensity	low signal intensity
Bond J et al. [114]	2013	4-18 years	focal weakness or hemiparesis, accompanied by disturbances in speech and/or affect, may wax and wane, **Seizures** (2/31)	diffuse white matter changes		
Millan NC et al [128]	2018	0.1-16 years	Upper-limb weakness & lower-limb paraesthesia, flaccid quadriparesis & visual impairment		hyperintense	hypointense

Table 4.3: Literature review of SLS for clinical and radiological features

1) Focal pyramidal weakness was the most common clinical feature reported in all the studies with varying degree of other clinical symptoms that constitute the SLS, such as ataxia/movement disorders, dysphasia/aphasia, and waxing and waning pattern of neurological deficits.
2) It was observed that seizures were very rarely reported in SLS cases in just one study (2/31 patients).
3) The publications included had DWI typical of SLS (hyperintense) and there were only three studies reporting ADC (hypointense) findings. ADC is not very frequently reported, especially in the older studies.
4) The use of different terminologies to report MRI findings and not being consistent in reporting might cause confusion in interpretation.

Observations that were made from the literature review for the clinical and radiological features of PRES and SLS were clearly some differences from the consensus definitions criterion. It was decided to test the cases reported to us SLS and PRES to identify the features that were enriched in the cases reported.

4.3.3 Aim 3: To create a differential diagnostic criterion for classification of PRES and SLS cases to address bi-phenotypic cases.

It was aimed to explore the clinical and radiological features that are enriched in the cases reported as PRES and SLS and fit them in a multivariate model to be able to improve existing diagnostic criteria. For this purpose, univariate analysis was run on the clinical and radiological findings reported in PRES and SLS and each of these two groups was compared with its respective RONC. The clinical and radiological features that show significant association in a univariate analysis will be fitted in the multivariate model later. The analysis results are shown below:

Essential criteria:

Clinical features:

Headaches:

Out of the responses obtained for clinical symptom “Headaches”, total 590 cases, 200 (33.8%) experienced headaches as part of their clinical presentation of the neurotoxicity. In the cases reported as PRES there were 158 responses for the “Headache” variable, out of which 36% answered “yes” and therefore were known to have experienced headaches.

Table 4.4: Univariate logistic regression of Headache with PRES

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-cient	OR (CI)
Total	590		158		432				
Headache – Yes	200	33.8	58	36.7	142	32.8	0.383	0.1	1.1* (0.8-1.7)
Headache -No	390	66.1	100	63.2	290	67.1			

*Logistic regression analysis

It was observed that there was no statistically significant difference in the prevalence of headaches between PRES and other neurotoxicity (RONC-P). (Table 4.4)

Seizures:

Information about seizures as a clinical symptom was reported in 1469 neurotoxicity cases. Out of these, 1091 (74.2%) cases reported having a seizure and 378 (25.7%) reported no seizures.

Table 4.5: Univariate logistic regression of Seizures with PRES.

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-cient	OR (CI)
Total	1469		226		1243				
Seizures – Yes	1091	74.2	193	85.3	898	72.2	4.63e-05	0.8	2.2 (1.5-3.3) *
Seizures - No	378	25.7	33	14.6	345	27.7			

*Logistic regression analysis

There is a statistically significant difference (p value 4.63e-05) in the prevalence of seizures with PRES compared to other neurotoxicity cases, with the odds being 2.2 times higher in PRES (95% CI 1.5-3.3). (Table 4.5)

Encephalopathy/confusion/altered mental status:

Data was collected about confusion, altered mental status, or encephalopathy, as a clinical symptom in 813 neurotoxicity cases during neurotoxicity event. Out of those, more than half of the cases reported to have confusion: 455 (55.9%), reported experiencing confusion/ altered mental status.

Table 4.6: Univariate logistic regression of Encephalopathy with PRES

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-cient	OR (CI)
Total	813		204		609				
Encephalopathy – Yes	455	55.9	151	74	304	50	4.47 e-09	1.05	2.8 (2.02-4.08) *
Encephalopathy -No	358	44	53	26	305	50			

*Logistic regression analysis

There is a statistically significant difference (p value 4.47e-09) in the prevalence of encephalopathy with PRES compared to other neurotoxicity cases, with the odds being 2.8 times higher in PRES (95% CI 2.8-4.08).

Visual disturbances:

Information about having visual disturbances, as part of their neurotoxic event, was obtained in 630 cases. Out of those, 112 (17.7%) experienced visual disturbances during their neurotoxicity event.

Table 4.7: Univariate logistic regression of visual disturbances with PRES

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-cient	OR (CI)
Total	636		180		456				
Visual disturbances - Yes	119	18.7	59	32.7	60	13.1	2.91e-08	1.1	3.2 (2.1-4.8) *
Visual disturbances - No	517	81.2	121	67.2	396	86.8			

*Logistic regression analysis

There is a statistically significant difference (p value 2.91e-08) in the prevalence of visual disturbances with PRES compared RONC-P cases, with the odds being 3.2 times higher in PRES. (Table 4.7)

Radiological Findings

Radiological findings consistent with PRES:

The PRES MRI-radiology findings consistent with PRES include 1) white matter change, 2) on T2WI hyperintense findings or 3) on the DWI normal or hyperintense reports. Due to large amount of missing data for the radiology reports, a composite variable including all these presentations was made and the findings opposite to the above-mentioned (made in a composite variable) were analyzed in comparison with the RONC-P group.

The total responses obtained for white matter change + MRI T2 WI (hyperintense) + DWI (normal or hyperintense) were 367, out of which 294 (80%) had these consistent with PRES MRI findings reported. (Table 4.8)

Table 4.8: Univariate logistic regression of PRES consistent MRI findings with PRES

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-cient	OR (CI)
Total	367		103		264				
MRI: T2WI hyperintense) and/or DWI: (normal or hyperintense) and/or white matter change - Yes	294	80	86	83.4	208	79	0.3	0.3	1.3 (0.7-2.5) *
MRI: T2WI (hypointense or normal) and/or DWI:(hypointense) and/or No white matter change	73	20	17	16.5	56	21			

*Logistic regression analysis

MRI changes consistent with PRES showed no statistically significant association with PRES.

Additional Findings

In the consensus definition diagnostic criteria of PRES there were a few additional findings stated to help in diagnosing these cases.1) occurrence during the first 3-4 months initial phase of therapy 2) hypertension 3) Complete resolution of symptoms

Timing of neurotoxicity event: During Induction phase of treatment

First 90 days of therapy:

As the consensus definitions additional criteria for PRES included timing of these events between 3-4 months (0-90 or 120 days) from the start of ALL chemotherapy, information was gathered about timing of neurotoxic events during the therapy for 1774 cases. (Table 4.9) For the purpose of this analysis only first 3 months or 90 days were taken as a variable as PRES is more frequent early on the therapy.

Out of these 1774 cases, 791 cases had a neurotoxic event in less than 90 days after starting chemotherapy, as per protocol.

Table 4.9: Univariate logistic regression of first 90 days of therapy with PRES

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-	OR (CI)

								cient	
Total	1774		231		1543				
First 90 days of the treatment - Yes	791	44.5	141	61	650	42	1.11e-07	0.7	2.1 (1.6-2.8) *
First 90 days of the treatment - No	983	55.4	90	39	893	58			

*Logistic regression analysis

The patient's group having a neurotoxic event in early phase of chemotherapy (first 90 days) showed a statistically significant association (p value 1.11e-07) with PRES.

Hypertension:

Out of the responses obtained for hypertension, more than 95th percentile- tested at centres and just the presence or absence of hypertension was reported to us. Hypertension as a clinical finding as part of their neurotoxic event, was observed in 616 cases in total, there were 214 (214/616- 34.7%) cases had hypertension. (Table 4.10)

Table 4.10: Univariate logistic regression of hypertension with PRES

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-cient	OR (CI)
Total	616		173		443				
Hypertension - Yes	214	34.7	142	82	72	16.2	<2e-16	3.1	23.6 (15.03-38.06) *
Hypertension - No	402	65.2	31	17.9	371	83.7			

*Logistic regression analysis

There is a statistically significant difference (p value <2e-16) in the prevalence of hypertension with PRES compared to other neurotoxicity cases, with the odds being 23.6 times higher in PRES.

Complete resolution of symptoms:

Information was collected about neurotoxic events with complete resolution of symptoms within a week after the first event. There were 989 responses obtained, out of which 852 (852/989- 86.1%) cases reported that their symptoms completely resolved, and the rest had persistence of neurotoxic symptoms beyond one week. (Table 4.11)

Table 4.11: Univariate logistic regression of complete resolution of symptoms with PRES

Variable	Total	%	PRES	%	RONC-P	%	p-value	Coeffi-cient	OR (CI)
Total	989		208		781				
Complete resolution of symptoms after first event - Yes	852	86.1	177	85	675	87	0.6	0.1	1.1 (0.7-1.7) *
Complete resolution of symptoms after first event - No	137	13.8	31	14.9	106	13			

*Logistic regression analysis

It was observed that there was no statistically significant difference between the PRES and RONC-P for complete resolution of symptoms after the first event.

PRES:

Summary of the clinical features that showed significant association with PRES with the highest significance shown on univariate analysis: (Table 4.12)

Table 4.12: Summary of variables showing significant association on univariate logistic regression analysis with PRES

Variable	p value	OR	CI
Hypertension	<2e-16	25.8	16.4-41.9
Encephalopathy/confusion	6.8e-11	3.23	2.28-4.6
Visual disturbances	1.96e-09	3.65	2.39-5.59
Within 90 days of therapy	7.81e-08	2.21	1.66-2.97
Seizures	1.25e-07	2.87	1.96-4.30

Multivariate logistic regression analysis:

The variables combined from the essential and additional diagnostic criteria for PRES, that showed significant association with PRES, were fitted in a multivariate logistic regression model. (Table 4.13)

Table 4.13: Multivariate logistic regression analysis of PRES

Variable	p value	Co-efficient	OR	CI	Suggested score
Hypertension	< 2e-16	2.6	14.4	8.29 - 25.8	2.5
Seizures	0.000198	1.18	3.25	1.76 - 6.161	1
Encephalopathy/confusion	0.003286	0.85	2.35	1.33 - 4.18	1
Visual disturbances	0.018237	0.82	2.27	1.15 - 4.53	1
First 90 days of therapy	0.574640	0.15	1.17	0.66 - 2.03	-

Hypertension, seizures, encephalopathy/confusion, and visual disturbances remained independently statistically significantly associated with PRES as compared to the RONC-P group.

To formulate a differential diagnostic scoring system, the coefficient values rounded off (as shown in the Table 4.13) of the variables showing significant association were taken and given a suggested score, adapting a model from a previous study [129].

Suggested score for the differential diagnostic criteria for PRES from the multivariate logistic regression results:

PRES differential scoring system was developed, on the basis of scores taken from the multivariate logistic regression analysis on the basis of suggested score from the coefficient value (Table 4.13). The resultant categories are given below

Definite PRES: As for diagnosis of definite PRES, it requires a combination of clinical finding and radiological finding, so the minimum score was decided 3.5 and the maximum score 5.5.

Probable clinical PRES: The cases that had a score above 3.5 but didn't have the supportive MRI finding (missing radiology) were classified as probable clinical PRES.

Probable radiological PRES: The group of the cases that had a score between 2-3 with radiological findings consistent with PRES, were classified as probable radiological PRES.

Possible clinical PRES: The score between 2-3 without radiology were classified as possible clinical PRES.

Cases picked after running the differential diagnostic scoring system:

As PRES is a clinical-radiological entity, after testing for the presence of any of the MRI finding (T2WI - hyperintense or DWI - normal or hyperintense or white matter change or provisional diagnosis) that are consistent with PRES, the differential diagnostic scoring was run, and cases classified as under Table 4.14.

Table 4.14: Definite PRES cases true to differential diagnostic scoring system without overlap with definite SLS

PRES cases	N/total
PRES cases reported by the study groups	231/1813
PRES cases true to differential diagnostic scoring system from the reported as PRES cases group	128/231
PRES cases picked from the complete dataset (definite PRES)	158/1813

SLS cases (definite SLS) true to SLS differential diagnostic score picked by PRES score	0/45
Probable radiology PRES picked with differential diagnostic scoring system	69/1813
Probable clinical PRES picked with differential diagnostic scoring system	31/1813
Possible clinical PRES picked with differential diagnostic scoring system	63/1813
Bi-phenotypic according to the differential diagnostic scoring system	31/1813

Cases reported by study groups classified as PRES:

There were 231 cases reported by the investigators as PRES. When tested with the differential diagnostic scoring system, 128 cases were confirmed as PRES. It will be interesting to look more into the clinical and radiological findings, or lack thereof, in the 103 cases that were reported as PRES but did not fulfill the differential diagnostic scoring system.

PRES cases picked from the complete dataset:

The testing of the complete PdL dataset with a differential diagnostic scoring system resulted in 158 cases. One of the aims of developing a differential diagnostic scoring system was to be able to better classify cases that were initially reported by the study groups either as encephalopathy or mis-classified as having other type of neurotoxicity.

Overlapping with SLS cases:

There were no cases that were true to SLS differential diagnostic score and were also fulfilling the differential diagnostic score for PRES. As one of the aims of developing this differential diagnostic score was to be able to classify definite PRES and SLS cases with minimal or no overlap, this testing of definite SLS cases with PRES differential diagnostic scoring system shows that this problem of overlapping cases has been eliminated. However, there were still a few bi-phenotypic cases from the possible clinical PRES and overlapping with possible SLS. (**Error! Reference source not found.**)

To develop a differential diagnostic criterion for SLS to address the bi-phenotypic cases

To explore the clinical and radiological features that were enriched in the cases reported as SLS in the PdL dataset, a univariate analysis was run based on the consensus definition criteria for SLS [40]. For this purpose, the SLS cases reported were analyzed for the features from the consensus definition criteria:

Univariate logistic regression analysis for SLS diagnostic criteria:

Results of the univariate logistic regression analysis of SLS cases are shown below:

Paresis/paralysis/pyramidal weakness:

Information was gathered about cases experiencing pyramidal weakness, paresis, or paralysis as a symptom during their neurotoxic event. Total responses obtained were 757, out of which 362 (362/757- 47.8%) cases were reported as having pyramidal weakness.

Table 4.15: Univariate logistic regression analysis of pyramidal weakness with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coeffi-cient	OR (CI)
Total	757		229		528				
Pyramidal weakness – Yes	362	48	182	79.4	180	34	<2e-16	2.01	7.4 (5.2-10.9) *
Pyramidal weakness - No	395	52	47	20.5	348	66			

*Logistic regression analysis

Pyramidal weakness showed statistically significant difference (p value <2e-16) with SLS, with the odds being 7.4 times higher in SLS.

Dysphasia/aphasia/dysarthria:

Inquiry was made about dysphasic or aphasic symptom, disturbances in speech due to changes in language centre, as part of the neurotoxic event. Total 713 responses were obtained, out of which 276 responded as having dysphasia.

Table 4.16: Univariate logistic regression analysis of dysphasia with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coefficient	OR (CI)
Total	755		218		537				
Dysphasia – Yes	320	42.3	157	72	163	30.3	<2e-16	1.7	5.9 (4.1-8.4) *
Dysphasia - No	435	57.6	61	27.9	374	69.6			

*Logistic regression analysis

Dysphasia showed statistically significant association with SLS and the odds of having dysphasia in SLS cases were 5.9 times. (Table 4.16)

Altered mental status/encephalopathy/confusion:

Information about 813 cases having confusion/altered mental status/encephalopathy manifesting as part of their neurotoxic event was gathered; out of those, 455 (455/813 – 55.9%) experienced encephalopathy and the rest did not experience it.

Table 4.17: Univariate logistic regression analysis of encephalopathy with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coefficient	OR (CI)
Total	813		218		595				
Encephalopathy – Yes	455	55.9	103	47.2	352	60	0.00254	-0.4	0.6 (0.4-0.8) *
Encephalopathy - No	358	44	115	52.7	243	40			

*Logistic regression analysis

There was a statistically significant protective association between encephalopathy and SLS. (Table 4.17)

Ataxia/movement disorder:

Data was collected for ataxia or movement disorder symptom during neurotoxic event. Out of the 553 responses obtained, 121 (21.8%) cases reported as experiencing ataxia.

Table 4.18: Univariate logistic regression analysis of Ataxia with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coefficient	OR (CI)
Total	553		164		389				
Ataxia – Yes	121	22	49	30	72	18.5	0.0034	0.6	1.8 (1.2-2.8) *
Ataxia - No	432	78	115	70	317	81.4			

*Logistic regression analysis

Ataxia showed a statistically significant difference (p value 0.0034) with SLS compared to other neurotoxicity cases, with the odds being 1.8 times higher in SLS.

Seizures:

Neurotoxicity cases were enquired about having seizures as a clinical symptom experienced during the event. Total responses obtained were 1277, out of which 898 cases were reported as having seizures and the rest did not.

Table 4.19: Univariate logistic regression analysis of seizures with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coefficient	OR (CI)
Total	1469		222		1247				
Seizures – Yes	1091	74.2	52	23.4	1039	83.3	<2e-16	-2.7	0.06 (0.04-0.08) *
Seizures - No	378	25.7	170	76.5	208	16.6			

*Logistic regression analysis

Seizures showed statistically significant protective association with SLS (p value <2e-16). (Table 4.19)

2. Findings of characteristic white matter changes of leukoencephalopathy on MRI

The characteristic radiology findings of SLS reported are 1) MRI white matter change or 2) MRI DWI hyperintense finding or 3) MRI ADC hypointense [130]. As there was missing data for the MRI findings in each separate type of MRI investigation for the cases reported as SLS, a composite variable of all the MRI findings consistent with SLS (MRI white matter change or DWI hyperintense finding or ADC hypointense) was made and analyzed with the opposite radiological findings available on the dataset.

Out of the total 1400 responses for the MRI findings consistent with SLS, 37.3% of cases had SLS-like MRI and the rest did not. (Table 4.20)

Table 4.20: Univariate logistic regression analysis SLS consistent MRI findings with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coefficient	OR (CI)
Total	1400		244		1150				
MRI: white matter change + DWI (Hyperintense) + ADC (Hypointense)	523	37.3	149	60.8	371	32.2	<2e-16	1.1	3.2 (2.4-4.3)
MRI: No White matter changes + DWI (normal+ hypointense) + ADC hyperintense	877	62.6	95	39.2	779	67.7			

Cases with MRI findings consistent with SLS were over-represented in SLS cases (152/250 – 60.8%) as compared to the RONC-S group (371/1150 – 32.2%) and showed statistically significant association (p value <2e-16) with SLS.

Characteristic waxing and waning of symptoms

The characteristic clinical course of waxing and waning pattern of the symptoms usually has complete resolution over 1-7 days. Data collected showed that the neurotoxicity cases having waxing and waning pattern of neurotoxic symptoms, out of the responses obtained n=386, were 129 (33.4%).

Table 4.21: Univariate analysis of waxing and waning pattern of neurological symptoms with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coefficient	OR (CI)
Total	386		119		267				
Waxing and waning of symptoms – Yes	129	33.4	76	63.8	53	20	1.01e-15	1.9	7.1 (4.4-11.6) *
Waxing and waning of symptoms- No	257	66.5	43	36.1	214	80			

*Logistic regression analysis

There is a significant association of waxing and waning pattern of symptoms with SLS (p value 1.01e-15). The odds of having SLS increase by 7.1 if the cases have waxing and waning of symptoms. (Table 4.21)

3. Other reasons ruled out:

For this analysis, MRI provisional diagnosis of leukemic infiltration, infection, vascular causes, normal scans and other (reported as Arnold-Chiari, arachnoidal cyst, hygromas, previous fungal infection) were taken as "other reasons". The rest of the "relevant to SLS MRI findings" reported, such as PRES, SLS, leukoencephalopathy, infarction/inflammation were taken as a group. The total number of cases with MRI provisional finding were 697, out of which 126 cases had "Other MRI findings", while "Relevant MRI findings" were reported in 571 patients.

Table 4.22: Univariate analysis of MRI findings with identifiable cause with SLS

Variable	Total	%	SLS	%	RONC-S	%	p-value	Coefficient	OR (CI)
Total	697		169		528				
Other MRI findings	126	18	18	10.6	108	20.4	0.004	-0.7	0.4 (0.2-0.7) *
Relevant MRI findings	571	81.9	151	89.3	420	79.5			

*Logistic regression analysis

There was a statistically significant (p value 0.0044) negative protective association between "Other MRI findings" and SLS cases.

SLS:

Summary of the univariate logistic regression results for clinical and radiological features of SLS:

Table 4.23: Summary of the univariate logistic regression analysis results for SLS

Variable	p value	Coefficient	OR	CI
Waxing and waning of symptom	1.01e-15	1.9	7.1	(4.4-11.6)
Pyramidal weakness/paresis	<2e-16	2.01	7.4	(5.2-10.9)
Dysphasia	<2e-16	1.7	5.9	(4.1-8.4)
MRI: white matter change + DWI (Hyperintense) + ADC (Hypointense)	<2e-16	1.1	3.2	(2.4-4.3)
Ataxia	0.0034	0.6	1.8	(1.2-2.8)
Protective association				
Seizures	<2e-16	-2.7	0.06	(0.04-0.08)
Other reasons ruled out	0.0044	-0.7	0.4	(0.2- 0.7)
Encephalopathy/ confusion	0.00254	-0.4	0.6	(0.4-0.8)

Multivariate logistic regression analysis:

The variables from the diagnostic criteria for SLS that showed significant association with SLS (Table 4.23) were fitted in a multivariate logistic regression model. (Table 4.24)

Table 4.24: Multivariate logistic regression analysis results for SLS

Variable	p value	Coefficient	OR	CI	Suggested score
Waxing and waning of symptom	0.0006	1.09	2.9	(1.5- 5.6) *	1
Pyramidal weakness/paresis	8.65e-06	1.4	4.3	(2.2- 8.4) *	1
MRI: white matter change + DWI (Hyperintense) + ADC (Hypointense)	0.5	0.19	1.2	(0.6 - 2.3) *	-
Ataxia/dyspraxia/dysphasia	0.004	0.9	2.6	(1.3- 5.1) *	1
Protective association					**-**
Seizures	0.0001	-1.2	0.2	(0.1- 0.5) *	-1
Encephalopathy/ confusion	0.9	0.03	1.03	(0.5 - 1.9) *	-

*Logistic regression analysis

Results from the multivariate analysis:

The waxing and waning pattern of neurological symptoms, pyramidal weakness and ataxia/dyspraxia/dysphasia remained independently significantly associated with SLS. Seizures showed independent protective association with SLS.

Suggested score from the results of multivariate analysis:

The differential diagnostic scoring system was designed on the rounded-off coefficient values (Table 4.24), adapting a model published previously [129].

Keeping within 21 days of MTX administration & no other identifiable cause was considered a must condition for SLS diagnosis:

Definite SLS: Scoring 3 will be definite SLS.

Probable SLS: The cases that scored 2 were classified as probable SLS.

Possible SLS: The cases scoring 1 were classified as possible SLS.

Conditions and differential diagnostic score applied to the cases:

Keeping within 21 days of MTX administration & no other identifiable cause as a pre-condition, the cases picked by SLS differential diagnostic score:

Table 4.25: Definite PRES cases true to differential diagnostic scoring system without overlap with definite SLS

SLS cases	N/total
SLS cases reported by the study groups	248/1813
SLS cases true to differential diagnostic scoring system	37/248
SLS cases picked from the dataset with the differential diagnostic scoring system	45/1813
PRES cases overlapping SLS cases of the cases picked with differential diagnostic scoring system	0/158

Probable SLS diagnosed with differential diagnostic scoring system	102/1813
Possible SLS diagnosed with differential diagnostic scoring system	139/1813
Bi-phenotypic cases with differential diagnostic scoring system	31/1813

Cases reported by study groups classified as SLS:

There were 248 cases reported by the investigators as SLS. When tested with the differential diagnostic scoring system, 37 cases stood true to the scoring system. The cases that didn't were because the complete information for the clinical and radiological findings was not provided.

Overlapping with PRES cases:

There was no overlap between the re-classified definite SLS cases and definite re-classified definite PRES cases. Additionally, there was also no overlapping of probable SLS cases with definite PRES. As one of the aims of developing this differential diagnostic score was to be able to classify definite PRES and SLS cases without any overlap, this testing of definite SLS cases with PRES differential diagnostic scoring system shows that this problem of overlapping cases has been eliminated. However, there were a few bi-phenotypic cases of possible SLS with possible clinical PRES.

4.4 Discussion

To the best of our knowledge, for the first time ever a statistical approach has been used to design a differential diagnostic scoring system (DDSS) to differentiate between SLS and PRES without any overlap for the cases that had more detailed information on the clinical and radiological finding. There was concordance of diagnosis in a fair number of cases that were reported as SLS and PRES between the clinician's diagnosis and DDSS. It has not only improved on the classification of the probable and possible SLS and PRES but also improved the classification of the few cases that were reported as encephalopathy. The differential diagnostic scoring system for SLS, and PRES came from a multivariate logistic regression analysis and the clinical and radiological features that went into it were already identified and published [40]. The structure and conditions set for running for the differential diagnostic criteria are also taken from the existing diagnostic criteria. However, there are some differences from the consensus definition diagnostic criteria for PRES and SLS. In the PdL differential diagnostic scoring system, the main difference consists in one clinical feature (hypertension) switching from additional to becoming "essential" for the diagnosis and "headaches" being excluded. Additionally, one feature that was considered part of the presentation and was included in the consensus definition diagnostic criteria showed, instead, negative association. The varying amount of missing data for each category because the database was collected through two sources (clinical notes and the SAE trial databases, the latter mostly lacking detail of the event), needs to be acknowledged. The use of this scoring system for other similar retrospective studies and its clinical application will be clearer after the validation on the dataset from St Jude's group. This scoring system was developed to differentiate SLS from PRES in trial databases. Its use in a clinical setting for differential diagnosis is unproven. Possibly, for clinical application of this differential diagnostic scoring system, a consideration might also need to be given to include additional features (differentially significantly associated demographic features) / risk predictors shown from the deep phenotyping of these cases in our dataset and/or any other terms that may not be statistically significant in our data but are known to be relevant from literature etc. (e.g., age, gender) however, till now there are no strong differential demographic association shown in the literature for SLS and PRES.

In the differential diagnostic scoring system for definite PRES, one of the main differences from the existing diagnostic scoring system, hypertension, part of the additional criteria, was made essential for the diagnosis of the definite PRES.

Hypertension showed independent significant association, based on the coefficient value, was a must for the diagnosis of definite PRES in the differential scoring system. Hypertension has also shown significant association with PRES previously [59]. The exact role played by hypertension in the pathophysiology leading to PRES has been hypothesized to be that it overrides the autoregulation of cerebral blood flow, which leads to cerebral hyper perfusion, however, this needs confirmation [131]. Additionally, there are a few normotensive PRES cases have also been reported previously and shown here in the literature review, (Table 4.2). For normotensive PRES cases, it should be noted that the measurements might be 1) taken at not the highest point of increased blood pressure and 2) does the 95th percentile reflect the upper limit of self-regulation.

Seizures included in the DDSS for PRES are the most common symptom of the PRES [49], also shown by our literature review results (**Error! Reference source not found.**) especially the hyponatremic seizures that have been previously linked with vincristine-induced toxicity [132],[133]. Encephalopathy and visual field defects, showing independent significant association and part of the differential diagnostic criteria, are one of the common presenting symptoms for PRES and have been frequently reported previously [72,124]. Headaches, which were included in the consensus definition diagnostic criteria, didn't show significant association with PRES. It is plausible that headache is an accompanying symptom due to hypertension and/or seizures.

In SLS differential diagnostic scoring system, the waxing and waning pattern of the neurological symptoms was independently significantly associated with SLS and is a characteristic clinical feature of the methotrexate-induced SLS reported in the cases confirmed on ADC radiology finding [134,127,135].

Pyramidal weakness or hemiparesis, previously reported as the most frequent presenting symptom for SLS, often involving corticospinal tracts and motor cortex, was included in the differential diagnostic scoring system [114,125].

Dysphasia and dyspraxia, involving the language (Broca's) center, is included in the differential diagnostic scoring system and has been reported already [114]. In the previous reports the areas involved in the methotrexate-induced SLS radiologically, on MRI T2 FLAIR, are subcortical and involving the centrum semiovale, which corresponds to the clinical manifestations of the SLS in the form of focal motor deficits and aphasia [63,64]. Characteristic ataxia or movement disorders associated with SLS, is previously reported in studies [125] and ataxia is usually part of stroke presentation.

One of the main differences from the existing diagnostic criteria was seizure showing protective association with SLS. This finding is also favored by the findings from the table of the literature review that seizures prevalence was not very frequent (Table 4.3). In most of the studies reporting SLS (subacute methotrexate related-neurotoxicity), especially when confirmed on the ADC findings, the cases reported did not have seizures, but the numbers were small [125]. Limitation about having seizure as a qualifying neurological symptom for MTX-induced neurotoxicity (SLS) is that isolated seizures occurring within 21 days of methotrexate are very common relative to SLS occurrence. Methotrexate-induced asymptomatic leukoencephalopathy has also been shown in three-quarter of ALL cases [51]. So, if seizure plus leukoencephalopathy on MRI scan fulfils the diagnostic criteria for SLS then it might lead to over-diagnosing.

The gold standard to differentiate between PRES and SLS is ADC mapping on MRI. ADC values have a contrasting effect for SLS and PRES [94,95], but there were only 48 cases on the PdL dataset that were reported with ADC mapping conducted. The reasons for very few reports on the PdL data can be speculated as

1) ADC radiology not done in routine for every neurotoxicity, especially in the older trials

2) One-third of the cases were reported from SAE databases which might lack detailed information on radiological findings of the event.

Validation of DDSS:

It is important to verify the DDSS using an independent dataset. To do this Dr Hiroto Inaba and Dr Raja Khan from the St Jude Children's Research Hospital, USA have agreed to review their records (including primary radiology) of SLS and PRES over the last decades and apply the DDSS. This project is in the data collection phase. (Validation plan sent to the collaborators attached in appendix 4)

5 Deep phenotyping of Chemotherapy-associated seizures from the Ponte di Legno Neurotoxicity study

5.1 Background

Seizures are the most common type of presentation of chemotherapy-induced neurotoxicity and may be reported in up to 10% of children on ALL treatment [50], however recent trials have reported lower incidence (4.7%-7.6%), in the absence of cranial radiation therapy [37,58].

An episode of seizure is characterized by a transient occurrence of signs and/or symptoms owing to abnormal excessive or synchronous neuronal activity in the brain [76]. Depending on the presentation, according to the operational classification by International League Against Epilepsy (ILAE), seizures can be classified as focal, generalized, or unknown onset seizures [77].

Seizures in ALL might occur in isolation (often thought to be methotrexate-induced, reported in 60%), or as part of another recognized treatment related side-effect such as intracranial haemorrhage (reported in 10%), CNS thromboembolism (reported in 8%), CNS infection (in 6%), PRES and hypertensive seizures (in 4.5% and 0.9% respectively), metabolic disturbances including hyponatraemic seizures (3.7%), hypoglycaemic seizures (2.5%) and seizures as part of syndrome of inappropriate anti-diuretic hormone SIADH, in different reports [41,78,79]. Furthermore, risk predictors for recurrence in patients with a neurological deficit on ALL therapy were shown [80] but St Jude Children's Research Hospital (SJCRH) paediatric ALL/NHL study was not able to show the same worse outcome in patients with neurological deficits [78]. However, female sex and age less than 3 years have been identified for worse long-term outcomes [78]. According to the SJCRH series (62 patients), seizures were managed with anticonvulsants in most patients and almost 30% of patients had uncontrollable seizures, with 25% having seizure relapse with the trial of anticonvulsants drug withdrawal [78].

It would be interesting to characterise seizure cases, after PRES and SLS are re-classified and excluded, and to identify the differences and similarities in demographic and clinical features of patients with seizures compared to patients with other types of neurotoxicity. A comparison between different current ALL treatment protocols to see the effect of heterogeneity in the different treatment protocols on the occurrence and timing of seizures is required. Although there are some reports regarding uncontrollable chemotherapy-induced seizures and the use of anticonvulsants, the neurological outcomes for most patients with seizures are largely unknown.

5.2 Aims

1) To deep phenotype the cases with seizures reported in the PdL neurotoxicity study

2) To identify the differences and similarities in demographic and clinical features of patients with seizures with the rest of the neurotoxicity cohort

3) To identify any differences in the occurrence of seizures and effects on neurological outcomes of seizures between different treatment protocols.

4) To investigate the effects of neurotoxicity on the leukaemia outcomes in seizures cases

5) To explore the effects of neurotoxicity on neurological outcomes in the seizures group

Material and methods:

Analysis to investigate the association with demographic and clinical parameters was carried out with R software (version 3.6.1); chi-squared and Fisher exact tests were used to assess associations between categorical variables. Unpaired two-tailed t-tests were applied for assessing the differences between continuous measures. For binary outcomes, logistic regression was used in both adjusted and unadjusted analyses. No adjustments for multiple testing were made, and a p-value <0.05 was considered statistically significant.

5.3 Results

Cases:

Seizures can occur alone or in conjunction with another recognized toxicity (CNS thromboembolism, PRES, metabolic events, etc seizure cases were divided into different groups according to proposed underlying aetiology (**Error! Reference source not found.**).

Table 5.1: Seizures classified according to possible aetiology

Type of event	Criteria	Number of cases
Isolated seizures	normal imaging + no other neurological symptoms+ other aetiologies ruled out	3
Seizures with PRES + probable PRES (radiological & clinical)	Radiology + clinical score	279
Seizures with possible SLS	Within 21 days MTX +Clinical score	32
Seizures with cerebral infection	Reported as seizures with infection +/- MRI consistent with infection	26
Seizures with vascular disorders	Reported as vascular +/- MRI thrombosis/CVST	47
Seizures not otherwise specified (NOS)	Missing data/ unclassifiable otherwise	704

Seizures not otherwise specified (NOS):

Inclusion criteria:

Seizure during ALL therapy

Exclusion criteria:

1) Fulfil diagnostic criteria for PRES (Definite/probable/possible), possible SLS

2) Due to cerebral bleed or CVST

3) Secondary to meningitis or cerebral infection

For analysis, the group with Seizures not otherwise specified (NOS), n=707, cases were taken only. It will be referred to as seizures cases or Seizures PdL (Ponte di Legno) from here onwards. Whereas, due to the lack of a “no neurotoxicity” control group the Rest of the Neurotoxicity Cohort (RONC, n= 1106), even if they had seizures as a symptom along with PRES and others, was taken as the comparative group.

5.3.1 Aim 1: To deep phenotype the Seizures cases

5.3.1.1 Pre-existing neurological conditions:

In terms of exploring whether a pre-existing neurological condition has an association with chemotherapy-induced seizures, data was collected for a few common conditions in children such as Down syndrome, epilepsy, autism, and febrile seizures.

Table 5.2: Comparison of pre-existing neurological conditions in seizures with RONC

Characteristic	Seizures		RONC		p value	OR (CI)
Down syndrome	n	%	n	%		
Total	702		1089			
Yes	24	3.4	18	1.6	0.01*	2.1 (1.1-3.9)
No	678	96.5	1071	98.3		
Epilepsy						
Total	252		578			
Yes	9	3.5	9	1.5	0.11*	-
No	243	96.4	569	98.4		
Autism						
Total	245		576			
Yes	3	1.19	4	0.69	0.7*	-
No	242	98.7	572	99.3		
Febrile seizures						
Total	145		429			
Yes	6	4.13	7	1.6	0.1*	-
No	139	95.8	422	98.3		

*Logistic regression analysis

Out of the pre-existing neurological conditions analysed, only Down syndrome showed a statistically significant association with the seizures where the odds of having a seizure episode were 2.1 (1.1-3.9) times more likely in Down syndrome ALL (DS-ALL) patients when compared with the rest of the neurotoxicity RONC (Table 5.2). However, due to lack of more detailed information to sub stratify the seizures in Down syndrome into three types of seizures seen in Downs syndrome – infantile spasms, focal seizures, late onset myoclonic epilepsy (Alzheimer's like) was not possible.

5.3.1.2 Demographic and clinical features of the Seizures cases:

The deep phenotyping of the seizures cases, to examine the distribution of demographic and clinical features in sub-categories for the seizures cases were examined and the results are shown in the **Error! Reference source not found.**:

Table 5.3: Demographic and clinical features of the seizures group

Demographic feature		n	%
Age groups	Total	707	
	<10 years	407	57.5
	10-15 years	185	26.1
	>15 years	115	16.2
	Median age	8.5	
Gender	Male	394	55.7
	Female	313	44.2
Immunophenotype	Total	705	
	B cell type	584	82.8
	T cell type	121	17.1
White blood cell counts	Total	705	
	Less than 50x109/L	540	81
	More than 50x109/L	165	19
CNS status	Total	686	
	CNS 1	551	80.3
	CNS 2	86	12.5
	CNS 3	44	6.4
	TLP without blasts	5	0.7
Karyotype	Total	290	
	Hyperdiploidy	37	12.7
	TEL-AML1	71	24.4
	MLL	6	2.06
	Other	164	56.5
	Good prognosis (Hyperdiploidy + TEL-AML 1)	12	4.13

As shown in Table 5.3, the Seizures cases were commoner in the younger age group, in males, with B-cell immunophenotype, white blood cell counts less than 50,000, in cases without CNS involvement and more with favourable karyotype (high hyperdiploidy and TEL-AML-1).

However, as paediatric ALL is commoner in younger age group, more in males, white blood cell counts less than 50,000, B- cell immunophenotype and CNS status with no involvement and good prognosis karyotype so these observations in seizures group, reported in more than half of the PdL cases, might be reflective of the demographics of the ALL trials included in this database.

5.3.1.3 The timing of event:

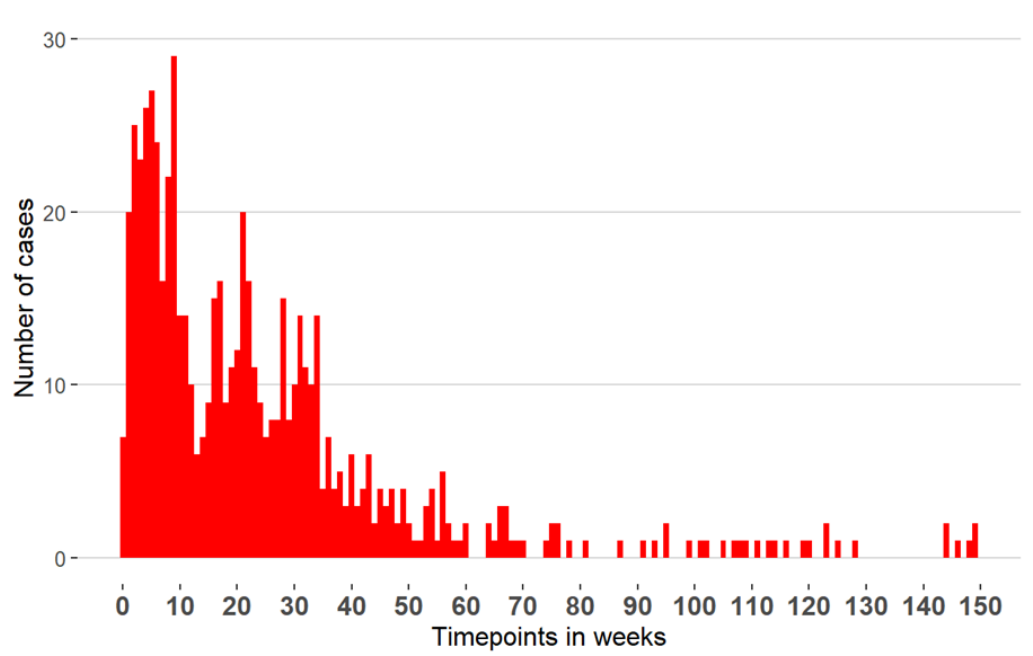


Figure 5.1: Bar-graph of time points of Seizures cases

Bar graph showing time points from the beginning of ALL treatment to the occurrence of Seizures event.

Figure 5. shows the timing of seizures during ALL treatment from the start of the therapy. The cases started having seizures from the induction phase of the therapy, peaking around 10th week of the treatment, continuing throughout the mid-phase (consolidation, delayed intensification I&II and interim maintenance I&II), while a few sporadic cases were occurring late in the treatment phase with the last few cases reported just before and at week 150.

5.3.1.4 Clinical presentation of the Seizures cases:

To characterise the clinical presentation of the seizure cases reported the types, duration and pattern reported were explored. The observations are given in (**Error! Reference source not found.**)

Table 5.4: Patterns of seizures cases with presumed causes

Para-clinical findings	Number of cases	Percentage (Out of the total responses for that category)
Types of seizures		
Generalised seizures	229/538	42.5%
Partial/focal seizures	55/538	10.2%
Focal seizures with impaired awareness (Complex partial seizures)	2/538	0.3%
Partial and then generalised	11/538	2%
Unknown	241/538	44.7%
Duration of seizures		
Less than 5 mins	93/535	17.3%
More than 5 mins	113/535	21.1%
Unknown	329/535	61.4%
Recurrence		
Single seizure	153/260	58.8%
Multiple seizures (Lasting less than 24 hrs)	70/260	27%

Multiple seizures (Lasting less than 1 week)	24/260	9.2%
Multiple seizures (Lasting more than 1 week)	13/260	5%
Presumed cause		
Unknown	47/540	8.7%
Electrolyte imbalance	3/540	0.5%
Low glucose	1/540	0.18%
Infection	26/540	4.8%
CVST	20/540	3.7%
Febrile convulsions	14/540	2.5%
Hypertensive	5/540	0.9%
Other	107/540	19.8%

"Unknown onset" type of seizures was most reported, in 44% of the cases, followed by Generalised type of seizures observed in 42%. Focal seizures were observed in 10%, and 2% of cases had partial seizures that progressed to a generalized type of seizures. Where in 21% of cases the duration of the seizures lasted for more than 5 minutes and in 17% of cases, they lasted for less than 5 minutes, indicating the severity and probably the need of intervention was more likely in the latter group. (For about 60% the duration was unknown)

During the seizures event, thirteen cases (5%) experienced multiple seizures lasting more than one week, twenty-four (9%) cases experienced multiple seizures lasting from one to seven days, seventy (27%) cases experienced multiple seizures lasting up to 24 h and 153 cases, three-fifth of the total, experienced single seizures of varying duration.

The underlying causes of seizures included unknown (8%), infection (4.8%) and metabolic imbalances with seizures reported in 3 cases and hypoglycaemia in 1 case. However, it was observed that in 18% of cases the cause was "Other" than the options provided. In majority of cases though, in 60% of cases, due to missing data they were unclassifiable.

5.3.1.5 Other accompanying neurological symptoms with seizures

Table 5.5: Other neurological symptoms with seizures and paraclinical findings

Other neurological accompanying symptoms	Number of patients	Percentage (Out of the total responses for that category)
Headache	23/84	27.3%
Focal weakness	21/107	19.6%
Altered mental status	19/110	17.2%
Waxing and waning of symptoms	12/85	14.1%
Dysphasia	10/102	9.8%
Hypertension	6/93	6.4%
Sensory disturbances	5/85	5.8%
Constipation	3/71	4.2%
Ataxia	3/75	4%
Visual disturbances	2/92	2.1%
Dyspraxia	1/87	1.1%
Signs		
Infection	26/108	24%
Lab investigations		
Hyponatremia	3/225	1.3%
Hypoglycaemia	1/225	0.4%
Raised CSF protein	5/23	21.7%
CSF low glucose	3/22	13.6%
CSF raised lactate levels	1/6	16.6%
CSF raised homocysteine levels	0/1	0

Of the 707 Seizures cases, out of the responses obtained, the most common symptom was headache (27%), followed by focal pyramidal weaknesses (19.6%), then altered mental status/encephalopathy (17%) and then waxing and waning pattern of neurological symptoms. Waxing and waning of neurological symptoms is described as developing a focal weakness on one side the focal weakness resolves on one side and develops on the other side, over the time of hours to days. The focal weakness and the classical waxing and waning pattern of symptoms is a characteristic of methotrexate-induced neurotoxicity making it plausible that these patients experienced methotrexate-induced seizures.

Hypertensive seizures were observed in 6.4% of cases that had "Other non-specific MRI findings" such as Infection, normal scan, or mixed inconclusive provisional diagnosis, the reason for not classified as definite PRES.

There were 26 cases (24%) reported to have an infection along with the ongoing seizures event.

Out of the responses obtained, based on the lab investigations reported, Raised CSF protein was reported in 5 cases (21.7%), of these two cases reported to have cells in CSF (without specifying if they are blasts or red blood cells or non-leukaemic lymphocytes/neutrophils). However, there was very limited information available regarding hyponatremia and hypoglycaemia at the time of the event and there were only three and one cases reported respectively.

5.3.1.6 Radiological findings in seizures cases:

The radiological features of the seizures cases were of interest, to determine the cases reported with leukoencephalopathy and Electroencephalogram findings of these cases.

Table 5.6: Radiological and EEG features of seizures cases

Radiological findings	Number of cases	Percentage (Out of the total responses for that category)

CT scan findings		
Normal	14/34	41.1%
MTX encephalopathy	13/34	38.2%
MRI findings		
Leukoencephalopathy	93/135	68%
Normal scan	22/135	16.3%
Diffusion Weighted Imaging (DWI)		
DWI hypointense	1/5	20%
DWI normal	4/5	80%
T2 FLAIR MRI findings		
T2 FLAIR normal	1/17	5.8%
T2 FLAIR hyperintense	16/17	94.1%
ADC MRI findings		
ADC hyperintense	1/2	50%
ADC normal	1/2	50%
EEG findings		
Generalised abnormality	47/552	8.5%
Focal abnormality	68/552	12.3%
Other abnormal finding	437/552	79.1%
Persistent radiological findings		
Present	22/95	23.1%

It was observed, out of the responses obtained, that in almost two-fifths of the seizures cases the CT scan findings were reported as normal (41.1%-14/34) whereas almost the same number of cases had CT scan findings consistent with MTX encephalopathy.

Looking at the MRI findings, Leukoencephalopathy (with white matter changes) was the most common type of presentation, reported in 68% (93/135), whereas there were 22 cases with

normal MRI scans. On further investigation, there was only three seizure case with normal MRI and no other neurological symptoms (Isolated seizure).

Out of the responses obtained, almost half of the cases had frontal region MRI changes (56%-13/23), followed by the parietal region (61.1% -11/18), then in the occipital region (57.8%-11/19), then by temporal (47%- 8/17), then in cerebellum region (29.4%-5/17) and then by basal ganglia (14.2%-2/14).

In the cases reported to have Fluid-Attenuated Inversion Recovery Imaging (T2 FLAIR imaging) done, the most common abnormality reported was hyperintense (94%).

Observing the Electroencephalogram (EEG), of the seizures cases, the Other non-specific type of abnormalities (79.1%) was the most common presentation, whereas 12% had focal abnormality and 8% had a generalised abnormality.

Looking at the long-term persistent radiological findings of these seizures cases, 23% (22/95) cases had persistent radiological findings consistent with leukoencephalopathy at their last follow-up. (Table 5.6)

5.3.1.7 Overall treatment strategies post-event:

To explore the interventions given at the time of seizures event or the other drugs that were being administered at the time of event were of interest. Data was collected and the information is shown in **Error! Reference source not found.**.

Table 5.7: Treatment administered after seizures event

Group of the drug administered	Number of patients	Percentage (Out of the total responses for that category)
Treatment for recovery		
Oral antiseizures	87/153	56.8%
Parenteral antiseizures	64/113	56.6%
No other drugs specified	14/60	23.3%

Antibiotics	11/60	18.3%
Benzodiazepines	16/60	26.6%
Leucovorin	6/60	10%
Antivirals	5/60	8.3%
Diuretics	5/60	8.3%
Infusion not specified	3/60	5%
Dextromethorphan	4/87	4.5%
Aminophylline	3/88	3.4%
NSAID	2/60	3.3%
Anticoagulants	2/60	3.3%
Antifungals	2/60	3.3%
Steroids	1/60	1.6%

Out of the responses obtained, the most common drugs administered were oral antiseizures medication in 56.8% and parenteral antiseizures in 56.6%. Benzodiazepines used in almost 26% is probably administered as rescue medication. Also, its noticeable that in 23% no other drug was administered and Leucovorin was administered in only 10% of cases.

In summary, seizures showed a significant association with Down syndrome. Seizure cases commonly had single seizure episode and leukoencephalopathy on MRI.

5.3.2 Aim 2: To identify the differences and similarities in demographic and clinical features of patients with Seizures compared to the rest of the neurotoxicity cohort

5.3.2.1 Comparison of demographic and clinical features of Seizures cases with the RONC group

It was interesting to explore if the seizures cohort was any different or similar demographically or clinically, to the RONC group. The results of comparative analysis are shown in **Error! Reference source not found.**.

Table 5.8: Comparison of Demographic and clinical features of seizures cases with RONC

Characteristic	Seizures n=707		RONC n=1106		p-value	OR(CI)
	n	%	n	%		
Age						
Total	707		1103			
<10 years	407	57.5	638	57.8		1(ref)
10-15 years	185	26.1	297	26.9	0.586	0.9(0.7-1.2) *
>15 years	115	16.2	168	15.2	0.861	1.07(0.8-1.4) *
Age as continuous variable (Mean)	8.5		8.8		0.2	
Gender						
Total	707		1104			

Male	394	55.7	578	52.3	0.145	1(ref)
Female	313	44.2	526	47.6		0.8(0.7-1.04) *
Immunophenotype						
Total	705		1094			
B-cell type	584	82.8	885	80.8	0.3	1(ref)
T-cell type	121	17.1	209	19.1		8.65(0.6-1.1) *
White cell counts						
Total	705		1102			
$<50x10^9/L$	540	81	860	78	0.4	1(ref)
$>50x10^9/L$	165	19	242	21.9		1.08(0.8-1.3) *
White blood cell counts as continuous variable (Mean)	58361.51		54072.36		0.484Ψ	-
CNS involvement						
Total	680		1069			
CNS 1	551	81	859	80	0.7*	1(ref)
CNS involvement (CNS 2 + CNS 3)	129	19	210	20		0.95(0.7-1.2) *

Ψ Two-tailed t-test analysis

* Logistic regression analysis

Age:

For analysis, the cohort was divided into three age categories, less than 10 years old, 10-15 years and more than 15 years old.

There was no statistically significant difference observed between the seizures group when compared with the RONC (Table 5.8).

Age taken as a continuous variable did not show a statistically significant association with seizures compared with the age of the RONC.

Gender:

There was no statistically significant difference observed in the gender distribution of males and females between the seizures group and the RONC (Table 5.8).

Immunophenotype:

Patients with seizures had predominantly B cell immunophenotype, 82%, and there was no statistically significant difference observed in the B-cell and T-cell immunophenotype distribution between seizures and the RONC group.

CNS involvement at the time of ALL diagnosis:

The CNS status at the time of ALL diagnosis was categorized into two categories "No CNS involvement- CNS 1" and "CNS involvement- CNS 2 + CNS 3". There was no statistically significant difference observed in the distribution of cases with CNS involvement and No CNS involvement at the time of ALL diagnosis between seizures group and the RONC (Table 5.8).

5.3.2.2 Comparison of treatment characteristics of seizures cases with the RONC

It was of interest to see if the treatment features were any different from the RONC group. The NCI risk allocation, treatment risk allocation and phase of treatment between the two groups, seizures and RONC were analysed, and the results are shown in Table 5.9 below:

Table 5.9: Comparison of treatment characteristics of seizures with the RONC group

Characteristic	Seizures (n=707)		RONC (n=1103)		p-value	OR(CI)
	n	%	n	%		
NCI risk group allocation						
Total	704		1099		0.3	
Low risk	306	43.4	501	45.5		1(ref)
Intermediate/high risk	398	56.6	598	54.5		1.09(0.9-1.3) *

Treatment risk group allocation						
Total	625		1013			
Standard risk group	228	36.4	348	34.3	0.01	1.4(1.0-1.9) *
Medium risk group	296	47.3	441	43.5	0.005	1.48(1.1-1.9) *
High risk group	101	16.3	224	22.1	-	1(ref)
Phase of treatment						
Total	668		1016			
Induction	199	29.7	267	26.2	1.67e-06	2.6(1.7-4)
Consolidation	111	16.5	157	15.4	2.56e-05	2.5(1.6-3.9)
Consolidation with HDMTX	39	5.8	139	13.6		1(ref)
Maintenance	64	9.5	90	8.8	0.000139	2.5(1.5-4.1)
Other	255	38.2	363	35.7	4.15e-06	2.4(1.7-3.7)

*Logistic regression analysis

NCI risk allocation:

There was no statistically significant association observed between the seizures group and the RONC cases for the NCI-risk group allocation (Table 5.9).

Risk group allocation:

The cases allocated medium risk treatment regimen (47%-296/625) were identified as significantly associated (p-value 0.005) with having seizures as compared with the RONC. The odds of having seizures in the medium risk allocated group were 1.48(1.1-1.9) higher, followed by odds of standard risk 1.4(1.0-1.9), times higher than the RONC, as an outcome when compared with the standard risk regimen allocated. (**Error! Reference source not found.**)

The phase of treatment:

Seizures were most common, 38.2% (255/668) in the "Other treatment phase" including delayed intensification I &II, interim-maintenance I &II, and re-induction phase.

It was identified that Seizures cases in comparison with the RONC group, the Induction phase of therapy showed the most significant association with Seizures, 29.7% (199/668) vs 26% (267/1016), p-value 1.67e-06. The odds of having seizures episode during the Induction phase were 2.6 times higher than the RONC. Followed by the consolidation phase and maintenance phase of treatment showed significant association with seizure cases, where cases were 2.5(1.6-3.9) and 2.5(1.5-4.1) respectively, times more likely to have a seizure as compared with the RONC.

In the cases that were in the "Other" phase of therapy, which comprises delayed intensification I &II and interim maintenance I&II phases of therapy depending on the protocol, the odds of having a seizures episode were 2.4(1.7-3.7) times more when compared with the RONC group. (**Error! Reference source not found.**)

5.3.2.3 Multivariate logistic regression analysis:

The clinical and demographic characteristics combined, that showed significant association with seizures, were fitted in a multivariate logistic regression model to identify the variables that are associated independently. Results are shown in **Error! Reference source not found.**.

Table 5.10: Multivariate regression analysis of the variables showing significant association with seizures when compared with the RONC

Characteristics	p-value	OR	CI
Treatment risk group allocation			
Standard	0.03	1.3	1.01-1.8
Medium risk	0.006	1.5	1.1-2.0
High-risk group	-	1(ref)	
Phase of treatment			
Induction	0.0002	2.2	1.4-3.4
Consolidation	9.19e-05	2.4	1.5-3.9

Consolidation with HDMTX		1(ref)	
Maintenance	0.003679	2.1	1.2-3.5
Other	5.12e-06	2.5	1.7-3.9*

* Logistic regression analysis

Standard and medium risk and Induction, consolidation, maintenance and "Other phase of therapy" were found independently significant associated with seizures cases.

5.3.2.4 Comparison of demographic and clinical features of seizures cases with the UKALL 2003 published cohort

A major limitation of analysis using RONC is the lack of no neurotoxicity control group for comparative analysis and that the analysis is done between the different types of neurotoxicity. To investigate whether patients with seizures had distinct demographic or clinical variables compared to children presenting with ALL, the dataset was also compared with information obtained from the UKALL 2003 cohort. UKALL 2003 was a randomised trial for children with acute lymphoblastic leukaemia, where it included all the patients aged one year up to 25 years and recruitment levels were high (>90%) [47,136] therefore the patient characteristics can be considered "typical" of childhood ALL.

Table 5.11: Demographics of seizures cases comparison with UKALL 2003 cohort

Characteristic	**Seizures (n)**	**Seizures (%)**	**UKALL 2003 cohort (n)**	**UKALL 2003 cohort (%)**	**p-value**
Total	707		3113		
Age at diagnosis (years)					
Less than 10 years	407	57.5	2279	73	
10-15 years	185	26.1	501	16	.0001*

>15 years	115	16.2	333	11	
Gender					
Male	394	55.7	1767	57	0.61*
Female	313	44.2	1346	43	
Immunophenotype					
B-lineage	584	82.8	2727	88	0.007*
T-lineage	121	17.1	386	12	
WCC (x109/L) at diagnosis					
<50 ($x10^9$/L)	540	81	2428	78	.41*
>50 ($x10^9$/L)	165	19	685	22	
CNS status at diagnosis					
Total	680				
CNS 1 (no involvement)	551	81	2713	87	
CNS 2 (<5/μL)	85	12.5	366	11.6	< 0.00001*
CNS 3 (>5/μL) by cytology	44	6.4	34	1	
Treatment risk group allocation					
Standard risk	228	36.4	1537	49	
Medium risk	296	47.3	842	27	< 0.00001*
High risk	101	16.3	734	24	

* Logistic regression analysis

The comparison showed that there was a difference seen in age groups (<10 years, 10 -15 years and >15 years). Seizure cases show a higher proportion of cases in older age groups (10-15 years and >15 years) when compared with the age groups of ALL patients reported in the UKALL 2003 cohort, indicating that older age ALL was more likely to get seizures as shown in (**Error! Reference source not found.**).

There was no statistically significant difference seen in gender distribution in seizure cases from the PdL cohort when compared with ALL patients from UKALL 2003 cohort. (**Error! Reference source not found.**)

T- cell immunophenotype was over-represented in seizures cases from the PdL cohort when compared with the UKALL 2003 cohort but this may be due to the variation in inclusion and exclusion of the trials included in the PdL cohort. In this case where T cell immunophenotype is overrepresented in PdL, and is showing a significant association with seizures, this might be due to the cases included in the PdL cohort from a trial exclusively for T-cell ALL. (COG 0434)

White blood cell (WBC) counts at the time of diagnosis showed no statistically significant difference in seizures cases from the PdL cohort as compared to the UKALL 2003 study cohort.

CNS involvement (CNS 2 and CNS 3) were over-represented and showed significant association in seizures cases from the PdL cohort as compared with the CNS involvement (CNS 2 and CNS 3) group in UKALL 2003 cohort.

Medium risk group was commoner with a significant association in seizures cases from the PdL cohort as opposed to the standard risk and high-risk group allocation when compared with the treatment risk group allocations of the UKALL 2003 cohort as shown in **Error! Reference source not found.**.

5.3.2.5 Drugs administered as per protocol within 4 weeks of treatment

Information was collected regarding the drugs that were administered to the seizure cases four weeks prior to the event. Observations are shown in Table 5.12

Table 5.12: Comparison of the drugs administered before the event between the seizure cases and the RONC

Characteristic	Seizures		RONC		p-value	OR(CI)

	n=147		n=1660			
	n	%	n	%		
Methotrexate administration four weeks before the event						
Total	419		774		0.11*	1.6(0.9-2.9)
Yes	402	96	724	93.5		
No	17	4	50	6.4		
Routes of administration						
Total	375		634			
Intravenous <1g/m2	4	1.06	8	1.2		
Intravenous >1g/m2	0	0	10	1.5		
Intrathecal	299	79.7	465	73.3	-	
IT &IV<1 gm/mm2	18	4.8	49	7.7		
IT & IV >1 gm/mm2	36	9.6	84	13.2		
Oral	12	3.2	17	2.6		
IT & Oral	4	1.06	1	0.15		
Cytarabine was administered four weeks before the event						
Total	272		724			
Yes	129	47.4	285	39.3	0.02*	1.3(1.04-1.8)*
No	143	52.5	439	60.6		

* Logistic regression analysis

The use of methotrexate in the seizures cases was slightly over-represented, however, did not show significant association with administration of methotrexate within four weeks of the event, which might owe to the methotrexate induced SLS cases in the comparative RONC group. However, it was observed that seizures cases were predominantly administered methotrexate intrathecally, almost 95.2% (357/375) - (just intrathecal or with combination with intravenous) as compared with just intravenous route. As opposed to intravenous use of methotrexate which was reported in almost one-sixth (15.4%-58/375) of the cases (just intravenous or in

combination with intrathecal administration). However, this difference didn't show a statistically significant difference when compared with the RONC group.

Seizures cases also showed significant association with cytarabine, drugs that are administered during the consolidation, and delayed intensification (as per protocol), when compared with the RONC.

5.3.2.6 Administration of general anaesthesia four weeks before the event:

Another potential mechanism for methotrexate-induced neurotoxicity involves drug interactions due to simultaneous inhalation of nitrous oxide (N2O) and methotrexate administration, a practice that is common in paediatric haematology centres that perform lumbar punctures under general anaesthesia to deliver intrathecal methotrexate. Nitrous oxide may result in increased homocysteine levels and decreased methionine levels, indirectly by depleting the vitamin B12 levels, both of which can contribute to methotrexate's neurotoxic effects [66]. Although in the PdL database the information gathered was about general anaesthesia administered four weeks before the seizures event without specifying if Nitrous oxide was used, it was still worth exploring the effect of general anaesthesia administered on the occurrence of seizures, as methotrexate is one of the presumed drugs, based on assumption that Nitrous oxide might be commonly administered. Results are shown in Table 5.13.

Table 5.13: Comparison of the Seizures cases with the RONC for the administration of general anaesthesia four weeks before the event

Characteristic	Seizures		RONC		p-value	OR (CI)
General anaesthesia four weeks before the event	n	%	n	%		
Total	143		509			
Yes	112	78.3	374	73.4	0.2*	1.3(0.8-2.05) *
No	31	21.6	135	26.5		1(ref)

* Logistic regression analysis

It was observed that in seizures cases the use of general anaesthesia, was slightly more, in 78.3% (112/143) of cases, than the RONC, in 73.4% (374/509). Although the information gathered doesn't specify which agent was used and it is impossible to stratify the cases for which nitrous oxide was used. However, there was no statistically significant difference observed in the administration of general anaesthesia four weeks before the event between the Seizures and the RONC group.

5.3.2.7 Administration of cranial irradiation four weeks before the event

As per protocols, some of the trial groups were giving cranial irradiation to high-risk group/CNS involvement (CNS 3), or T-call immunophenotype, with variation in risk group stratified, lower age limit, the dose of the irradiation and phase of the therapy administered. On the PdL database information was gathered about the cranial irradiation administered to the neurotoxicity cases collected and to explore if there is an association of cranial irradiation with seizures, analysed. The results are shown in **Error! Reference source not found.**.

Table 5.14: Comparison of the Seizures cases with the RONC for the administration of cranial irradiation four weeks before the event

Characteristic	Seizures		RONC		p value	OR (CI)
Cranial irradiation four weeks before the event	**n**	**%**	**n**	**%**		
Total	607		912			
Yes	19	3.13	14	1.5	0.04*	2.07(1.03-4.2) *
No	588	96.8	898	98.4		1(ref)

* Logistic regression analysis

Seizures showed a significant association with cranial irradiation when compared with the RONC group. The odds of having a seizure with cranial irradiation four weeks before the event were 2 times higher than the RONC group.

In summary seizures showed significant association with older age group, CNS involvement, medium risk treatment allocation, "Other phase of therapy", cytarabine and cranial irradiation.

5.3.3 Aim 3: To identify any differences in the occurrence of Seizures and effects on neurological outcomes of Seizures between different treatment protocols:

The Ponte di Legno chemotherapy-associated central neurotoxicity study is retrospective, with cases reported from different study groups on different trials. There were variations in trial protocols for administering different drugs, dosage, and timing of administration (explained in detail in the introduction). For identifying the effect of these variations on incidence and outcomes of neurotoxicity across different protocols a database of Heterogeneity of protocols was built. Information regarding variations in protocols about 1) methotrexate CNS directed therapy, 2) leucovorin rescue timing, 3) leucovorin dose and 4) triple/single intrathecal therapy, that was relevant to seizures, was obtained from the heterogeneity of protocols database for the analysis below:

5.3.3.1 High Dose Methotrexate (HD MTX) vs Capizzi escalating methotrexate

One of the variations between the trial protocols included in the Ponte di Legno study was in the different approaches to administrating methotrexate during the CNS directed in the consolidation phase of therapy. Where in Capizzi-style escalating dose (starting at a dose of 100 mg/m2/day, and 50 gm added on every consequent MTX dose) of IV methotrexate is administered without leucovorin rescue and on the other hand high dose (3-5gm/m2/day over 24 hrs) of IV methotrexate is administered with leucovorin rescue. Results shown in **Error! Reference source not found.**.

Table 5.15: Comparison of the Seizures cases with the RONC group for association with HD MTX and Capizzi escalating methotrexate trials

Characteristic	Seizures		RONC		p value	OR (CI)
	n	%	n	%		
Total	400		715			
High dose methotrexate	266	66.5	538	75.2	5.89e-08*	1(ref)
Capizzi escalating methotrexate dose	134	33.5	177	24.7		1.7(1.4-2) *

* Logistic regression analysis

It was observed that seizures were statistically significantly associated with Capizzi escalating dose methotrexate when compared with the RONC. The odds of having seizures were 1.7 (1.4-2) times higher when Capizzi escalating dose methotrexate was administered as compared with the high dose methotrexate when compared with the ROCN.

5.3.3.2 Intrathecal triple vs single:

As part of intensifying the CNS-directed therapy protocols, a variety of intrathecal approaches were utilized during the maintenance phase of the treatment. One approach was to give a single drug intrathecally (methotrexate only) whereas the other was to administer triple-drug intrathecally (methotrexate, cytarabine and hydrocortisone). There were few protocols included in PdL that were either administrating Triple IT drugs to all risk groups (DCOG ALL10, LAL/SHOP-2005, LAL-SEHOP-PETHEMA 2013, DCOG ALL 11) or the high-risk group and/or T-cell immunophenotype (ALL-BFM 95, ANZCHOG ALL STUDY 8, ALL IC-BFM 2002, ALLIC, BFM 2009, CoALL 08-09, NOPHO 2008, ANZCHOG ALL STUDY 7) only, as per protocol. The effect of these different intrathecal CNS-directed approaches (Intrathecal methotrexate vs Triple intrathecal therapy) on seizures was explored and the results are shown in **Error! Reference source not found.**.

Table 5.16: Comparison of Seizures cases administered MTX with the RONC group for Intrathecal triple-drug therapy with single-drug therapy protocols

Characteristic	Seizures		RONC		p value	OR (CI)
	n	%	n	%		
Total	350		533			
Intrathecal triple drug	26	7.4	73	13.6	0.004*	1(ref)
Intrathecal single drug	324	92.5	460	86.3		1.9(1.2-3.2) *

* Logistic regression analysis

It was observed that seizures had a significant association with Intrathecal single drug when compared with the RONC group. The odds of having seizures were 1.9 times higher in the group being administered single drug intrathecally as compared with the triple drugs (**Error! Reference source not found.**).

5.3.3.3 Leucovorin rescue Early vs late:

The timing of the administration of leucovorin after methotrexate administration is critical because leucovorin is a folate analogue that effectively neutralizes the effects of methotrexate. As the leucovorin window cannot be too shortened as it would, on one hand, reduce the chances of toxicity but on the other hand can also affect the desired anticancer results. Cases were stratified into two groups depending on the time from start of methotrexate to first leucovorin dose (hours), as per protocols, 1) Early leucovorin rescue, where leucovorin is administered 24-36 hrs after post methotrexate administration 2) Late leucovorin rescue, where leucovorin is administered 42-60 hrs after methotrexate administration (**Error! Reference source not found.**).

Table 5.17: Comparison of the Seizures cases with the RONC cases for early leucovorin rescue early with late leucovorin rescue

Characteristic	Seizures		RONC		p value	OR (CI)
	n	%	n	%		
Total	276		541			
Early rescue 24-36 hrs	21	7.6	105	19.4	2.02e-05 *	1(ref)

Late rescue 42-60 hrs	255	92.3	436	80.5		2.9(1.8-4.9)

* Logistic regression analysis

The seizures group showed a statistically significant association with the late leucovorin rescue (42-60hrs) and the odds of having seizures were 2.9 times higher in the late leucovorin rescue group.

5.3.3.4 Leucovorin low dose vs high dose:

Leucovorin rescue, where it was administered, had a variation in the dose administered in different protocols. To see the impact of the difference in dose of leucovorin on the seizures with methotrexate cases with the RONC group, two groups of cases were identified in the database: a) low dose (5-10 mg/m2) b) high dose (15 mg/m2). Results of the analysis are shown in **Error! Reference source not found.**.

Table 5.18: Comparison of the Seizures cases with the RONC cases for leucovorin low dose with high dose

Characteristic	Seizures with MTX		Seizures without MTX		p- value	OR (CI)
	n	%	n	%		
Total	277		527			
Low dose leucovorin (5-10 mg/m2)	18	6.4	20	3.7	0.08*	1(ref)
High dose leucovorin (15 mg/m2)	259	93.5	507	96.2		0.5(0.2-1.1) *

* Logistic regression analysis

It was identified that there was no statistically significant difference observed between the seizures and the RONC group for the high dose (15 mg/m2) of leucovorin or the low dose (5-10 mg/m2) of leucovorin administered.

In summary Capizzi escalating MTX protocols, single intrathecal drug and late leucovorin rescue administration showed significant association with seizures.

5.3.4 Aim 4: To investigate the effects of neurotoxicity on the leukaemia outcomes in Seizures cases

5.3.4.1 Leukaemia relapse outcome

There were 78 (78/707 – 11.03%) seizures cases that had leukaemia relapse and 629 (629/707 – 88.9%) seizures cases that reported to have no leukaemia relapse for the duration that they were followed up (mean 5.4 years).

There was no statistically significant difference between the Seizures group and the RONC group for the leukaemia relapse post-event as compared with not having relapse post-event on chi-square analysis. (Seizures, leukaemia relapse 11.03% (78/707) **vs** RONC 9.9% (110/1105), p-value 0.4)

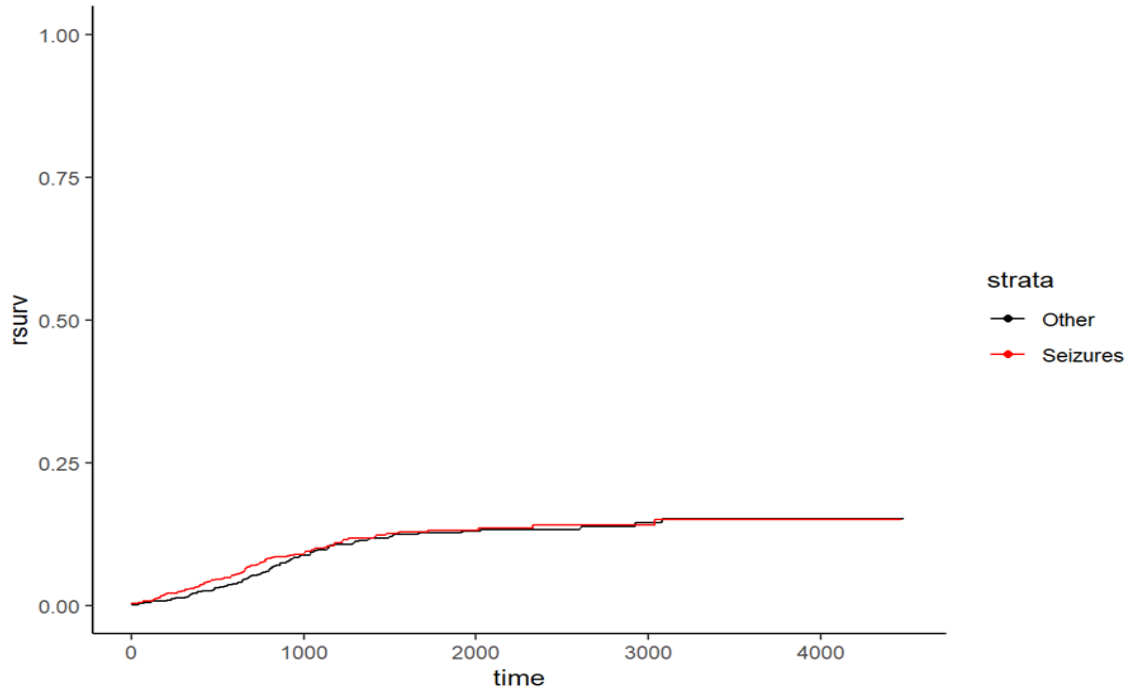


Figure 5.2: Kaplan Meier relapse probability plot for seizure cases

Kaplan Meier showing the calculated relapse probabilities of each patient of two groups, seizures cases and RONC cases, with censoring for relapse or last follow-up, whichever occurred first. There is no significant difference between the groups. (p-value 0.4)

On further examining the Seizures cases that were reported to have a leukaemia relapse, the most common site for the relapse was isolated bone marrow (51.3% - 39/76), followed by isolated CNS relapse reported in 23.6% (18/76) cases, 14.4% (11/76) reported to have combined bone marrow and CNS relapse, 6.5% (5/76), 2.6% (2/76) had a relapse in another extra medullary site (testis, ovary, skin, lymph node – details not given) and bone marrow and 1 case reported with CNS relapse with another extra medullary site.

5.3.4.2 Overall survival

There were 13.1% (92/702) seizures cases that are reported dead and 75% (528/702) seizures cases that were reported alive, whereas there were 82 (11.6%) cases that were lost to follow up, mean follow up was 5.4 years. (Table 5.19)

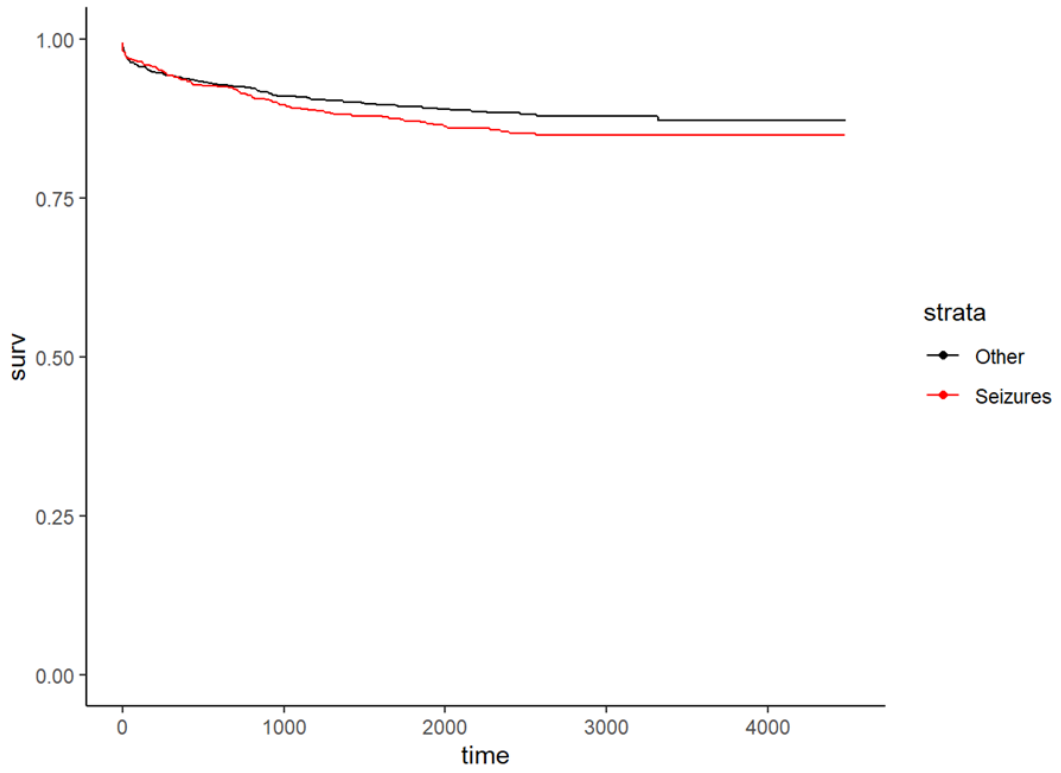


Figure 5.3: Kaplan Meier overall survival plot for seizures

Kaplan Meier calculated the survival probabilities of each patient of two different groups, seizures and RONC group. Seizures group showed lower probability of survival than the RONC group, with censoring for death or last follow-up, whichever occurred first. Log-regression test's showed significant association with seizures (p-value 0.02)

Table 5.19: Comparison of overall survival between seizures and RONC group

Characteristic	Seizures		RONC		p-value	
Overall survival	**n**	**%**	**n**	**%**		
Total	702		1090			
Alive	528	75.2	907	83.2	0.02	1(ref)
Dead	92	13.1	112	10.2		1.4(1.05-1.9)*
Lost to follow-up	82	11.6	71	6.5		

The seizures group was statistically significantly associated with cases reported as dead with the RONC group when compared to the overall survival post-event.

To summarize seizures showed no difference in relapse rate and showed reduced overall survival rates compared to the rest of the neurotoxicity cohort.

5.3.5 Aim 5: To explore the effects of neurotoxicity on neurological outcomes in the seizures group

5.3.5.1 ALL therapy modifications in response to neurotoxic events

Of the Seizures cases, there were 35.3% (46/130) where chemotherapy was modified after the event whereas the rest of 64.6% (84/130) cases reported continuing having chemotherapy as per protocol post neurotoxic event.

Table 5.20: Comparison of seizures and RONC group for modification of chemotherapy

Characteristic	Seizures		RONC		p value	
Change in chemotherapy	n	%	n	%		
Total	130		541			

Yes	46	35.3	278	51.3	0.001*	1(ref)
No	84	64.6	263	48.6		1.9(1.3-2.8)

In the comparison of Seizures cases with the RONC cases, there was a statistically significant association observed with the "no change in chemotherapy" after their seizure event. Seizures cases were 1.9 times more likely to continue their chemotherapy as per protocol as compared to the RONC group (Table 5.20).

5.3.5.2 Change in Intra-thecal Methotrexate (IT MTX) chemotherapy:

The seizures cases were predominantly administered intrathecal methotrexate four weeks before the event, so it was of interest to investigate any modifications (dose-reduced or dose with-held) in their IT MTX dose post-event of these cases.

Table 5.21: Comparison of modifications in chemotherapy between Seizures and the RONC

Characteristic	Seizures		RONC		p value	
IT MTX	**n**	**%**	**n**	**%**		
Total	64		258			
Continued as per protocol	33	51.5	109	42.2	0.14*	-
Dose reduced	5	7.8	44	17		-
With-held	26	40.6	105	40.6		-

* Logistic regression analysis

In the comparison of seizures group with the RONC group, it was identified that there was no statistically significant difference observed between intrathecal methotrexate modified and continued as per protocol (Table 5.21).

5.3.5.3 Change in High Dose Methotrexate (HD MTX) chemotherapy:

The information regarding if the chemotherapy was modified post-event was gathered for cases that had 1) continued as per protocol 2) if the dose was reduced/ one dose skipped 3) if the HD MTX was withheld. Results are shown in **Error! Reference source not found.**.

Table 5.22: Comparison of modifications in chemotherapy between seizures and the RONC

Characteristic	Seizures		RONC		p-value	
HD MTX	**n**	**%**	**n**	**%**		
Total	49		200			
Continued as per protocol	42	85.7	129	64.5	0.04*	4.5 (1.2-28) *
Dose reduced	2	4	28	14		1(ref)
With-held	5	10.2	43	21.5		-

* Logistic regression analysis

It was identified that seizures cases, in comparison with the RONC group, were 4.5 times more likely to get their HD MTX dose continued as per protocol.

5.3.5.4 Change in IT MTX administration post-event and its effect on leukaemia relapse:

The effect of change (dose reduced or dose with-held) in intrathecal methotrexate on the occurrence of leukaemia relapse was examined. Out of the responses obtained for the continuation as per protocol or modification in the intrathecal methotrexate for seizures, there was no statistically significant difference observed when compared with the RONC.

Table 5.23: Comparison of IT MTX dose modification in seizures for impact on leukaemia relapse

		Leukaemia relapse in Seizures					Leukaemia relapse in RONC				
Characteristic	**Total**	**Yes**		**No**		**Total**	**Yes**		**No**		**p-value**
		n	**%**	**n**	**%**		**n**	**%**			

IT MTX post-event											
Total	64					258					
Continued as per protocol	33	8	24.2	25	75.7	109	13	11.9	96	88.07	0.15*
Dose reduced/with-held	31	4	12.9	27	87	149	17	11.4	132	88.5	

There was no statistically significant difference observed between the seizures and RONC group for the modification of IT MTX and leukaemia relapse in these groups. (**Error! Reference source not found.**)

5.3.5.5 Change in HD MTX chemotherapy and its effect on leukaemia relapse:

The effect on leukaemia relapse in the seizures group, out of responses obtained, if there was a change in high dose methotrexate (dose reduced or dose with-held) of high dose methotrexate was explored. Results are shown in **Error! Reference source not found.**.

Table 5.24: Effect of change in HD MTX post-seizures on leukaemia relapse

		Leukaemia relapse in Seizures					Leukaemia relapse in RONC				
Characteristic	**Total**	**Yes**		**No**		**Total**	**Yes**		**No**		**p-value**
		n	**%**	**n**	**%**		**n**	**%**			
HD MTX post-event											
Total	49					200					
Continued as per protocol	42	10	23.8	32	76	129	17	13.1	112	86.2	

Dose reduced/with-held	7	0	0	7	100	71	9	12.6	62	87.3	-

* Logistic regression analysis

Looking at the responses obtained for the modification in the high dose methotrexate for seizures group, in comparison with the RONC group, “no cases” in one of the categories made it difficult to analyze.

5.3.5.6 Secondary neurotoxicity:

Recurrent episode or secondary neurotoxicity upon re-exposure to the presumed cause (mix of drugs in this case) was of interest in seizures cases. (**Error! Reference source not found.**)

Table 5.25: Comparison of secondary neurotoxicity event between seizures and RONC group

Characteristic	**Seizures**		**RONC**		**p-value**	
Secondary neurotoxicity	**n**	**%**	**n**	**%**		
Total	124		450			
Yes	63	50.8	91	20.2	0.0001	4.1(2.7-6.3)
No	61	49.1	359	79.7		1(ref)

Sixty-three patients, half of the seizures group, had a secondary neurotoxic event, out of the responses obtained, upon re-exposure to the presumed drug. The seizures group showed a statistically significant association with recurrent neurotoxicity, where the odds of having another neurotoxic episode were four times higher than the RONC group. (**Error! Reference source not found.**)

5.3.5.7 Type of Secondary events in Seizures

The recurrent events cases on re-exposure were explored to see the type of recurrence in this group, shown in **Error! Reference source not found.**.

Table 5.26: Recurrence in Seizures – types of the secondary event

Type of Secondary event (Total 63)	Number	Percentage
Seizures	50	79.3%
Encephalopathy	6	9.5%
SLS possible	3	4.7%
PRES	1	1.5%
Unspecified	3	4.7%

Seizures were the most common type of secondary neurotoxic event upon re-exposure to the presumed causative agent (**Error! Reference source not found.**), observed in 79.3% of cases, followed by Encephalopathy (9.5% of cases), followed by 4.7% of cases that had a SLS secondary neurotoxic event and there was 1 case with PRES and 3 cases with an unspecified recurrent episode.

5.3.5.8 Effect of Capizzi and High Dose methotrexate protocols on the recurrence of Seizures:

To explore if the Capizzi style and High Dose of methotrexate had an association with the recurrence of seizures in this cohort, the recurrent seizures cases were compared for the above-mentioned two different treatment protocols (**Error! Reference source not found.**).

Table 5.27: Comparison of seizures recurrence between HD MTX and Capizzi methotrexate protocols

Characteristic	Seizures Recurrence (Yes)	Seizures Recurrence (No)	p-value	OR (CI)

	n	%	n	%		
Total	63		60			
High dose methotrexate	26	41	51	85	2.45e-06*	1(ref)
Capizzi escalating methotrexate dose	37	58.7	9	15		8.06(3.5-20) *

It was observed the odds of having recurrent seizures were 8 times more likely in cases receiving Capizzi style of methotrexate administration protocol.

5.3.5.9 Effect of early and late leucovorin rescue on the recurrence of Seizures:

It was of interest to explore if early leucovorin rescue (24-36 hours after methotrexate exposure) and late leucovorin rescue (42-60 hours) had an association with recurrence of seizure on re-exposure to the presumed agent. (**Error! Reference source not found.**)

Table 5.28: Comparison of recurrence in seizures between the early and late leucovorin rescue

Characteristic	Seizures Recurrence (Yes)		Seizures Recurrence (No)		p-value	OR (CI)
	n	%	n	%		
Total	42		47			
Early rescue 24-36 hrs	2	4.7	18	38.2	0.01*	1(ref)
Late rescue 42-60 hrs	40	95.2	29	61.7		7.3(1.7-50)

Logistic regression analysis*

Recurrent seizures showed significant association with the late leucovorin rescue, and the odds were 7.3 times more likely to have a recurrent seizure with late leucovorin rescue when re-exposed to the presumed agent.

5.3.5.10 Effect of Intrathecal Single drug administration vs Intrathecal triple drug administration on the recurrence of Seizures:

To identify if there was an association of recurrent episodes of seizures with the administration of Intrathecal single drug administration (methotrexate only) vs the triple intrathecal drug administration (methotrexate, cytarabine and hydrocortisone) cases with seizure recurrence in this cohort were analysed (**Error! Reference source not found.**)

Table 5.29: Comparison of recurrence in seizures between the single and triple intrathecal drug

Characteristic	Seizures Recurrence (Yes)		Seizures Recurrence (No)		p value	OR (CI)
	n	%	n	%		
Total	56		33			
Intrathecal triple drug	2	3.5	7	21.2	0.017*	1(ref)
Intrathecal single drug	54	96.4	26	78.7		7.2(1.6-51) *

Fisher's test*

Cases that were on the protocol administering Intrathecal single drug were 7 times more likely to get a recurrent seizures episode as compared with the Intrathecal triple drug administration. (**Error! Reference source not found.**)

However, it is important to mention here that the number of cases in subgroups is small as there were few protocols included in the PdL database that were administering triple intrathecal drug therapy to high risk/T-cell cases only.

5.3.5.11 Admission to Intensive Care Unit (ITU):

Admission required to the Intensive Care Unit (ITU) is a way to assess the severity of the seizures event. It was reported that 45% (66/146) of seizures cases required admission to the intensive care unit for the management of their neurotoxic episode. (

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Table 5.30: Comparison of admission in ITU between the Seizures and the RONC group

Characteristic	Seizures		RONC		p-value	OR (CI)
Admission to ITU	**n**	**%**	**n**	**%**		
Total	146		584			
Yes	66	45.2	231	39.5	0.2*	-
No	80	54.7	353	60.4		

* Logistic regression analysis

In the comparison of the seizures group with the RONC cases, there was no statistically significant difference observed in the seizures cases that didn't require admission in the ITU and the cases that did require admission for the management of their event.

5.3.5.12 Requiring Ventilatory support in ITU:

Of the seizures cases that required ITU admission, out of the responses obtained, almost half of the cases, 44% (65/145) with severe enough conditions that required ventilatory support as part of the management of their neurotoxic episode.

Table 5.31: Comparison of requiring ventilatory support between seizures and the RONC group

Characteristic	Seizures		RONC		p value	
Required ventilation	**n**	**%**	**n**	**%**		
Total	145		585			
Yes	65	44.8	232	39.6	0.2*	-
No	80	55.1	353	60.3		

* Logistic regression analysis

However, Seizures cases compared with the RONC group requiring ventilatory support or not didn't show any statistically significant difference between the two groups. (

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5.3.5.13 Requiring intubation in the ITU:

One other way to measure the severity of seizures cases was to look at the cases that required intubation during their stay in the ITU. In the comparison of seizures cases with the RONC, there was no statistically significant difference observed in requiring intubation during the ITU stay. (**Error! Reference source not found.**)

Table 5.32: Comparison of requiring intubation between Seizures and the RONC group

Characteristic	Seizures		RONC		p value	
Required intubation	**n**	**%**	**n**	**%**		
Total	32		179			
Yes	22	68.7	93	51.9	0.2*	-
No	10	31.2	86	48		

* Logistic regression analysis

5.3.5.14 Duration of ventilatory support required:

One other measure to see how severe the Seizures event might be in these cases is to see for how long these cases required the ventilatory support. Data were collected for two timepoints 1) Less than 48 hours and 2) More than 48 hours of ventilatory support required for the Seizures cases post neurotoxicity episode.

Table 5.33: Comparison of the Seizures group with the RONC group for the duration of ventilatory support required

Characteristic	Seizures		RONC		p value	
Duration of ventilatory support required	**n**	**%**	**n**	**%**		
Total	13		77			
Less than 48 hors	7	53.8	31	40.2	0.5*	-
More than 48 hours	6	46.1	46	59.7		

* Logistic regression analysis

Of the Seizures cases, more than half the cases (53.8% - 7/13) required ventilatory support for less than 48 hours and (46% - 6/13) that ventilatory support for more than 48 hours. In comparison with the RONC group, there was no statistically significant difference observed between the two groups. (**Error! Reference source not found.**)

5.3.5.15 Use of parenteral anti-seizures:

Information regarding seizures group management during the event was gathered for the use of parenteral anti-seizures, out of the responses obtained, it was observed that the seizures were statistically significantly associated with the use of parenteral anti-seizures as compared with the RONC group.

Table 5.34: Comparison of seizures with the RONC group for the use of oral anti-seizures

Characteristic	Seizures		RONC		p-value	
Parenteral anti-seizures	**n**	**%**	**n**	**%**		
Total	113		480			
Yes	64	56.6	174	36.2	0.0001*	2.2(1.5-3.4) *
No	49	43.3	306	63.7		1(ref)

*Logistic regression

Seizures cases were 2.2 times more likely to get a parenteral anti-seizure administered when compared with the RONC group. (**Error! Reference source not found.**)

5.3.5.16 Use of long-term oral anti-seizure medication:

The association of seizures cases with the use of long-term oral anti-seizures to manage their neurotoxic events was explored, with the assumption that oral anti-seizures were administered for the long term.

Table 5.35: Comparison of seizures with the RONC group for the use long-term oral anticonvulsants

Characteristic	Seizures		RONC		p value	
Oral anti-seizures	**n**	**%**	**n**	**%**		
Total	153		548			
Yes	87	57.1	191	34.8	1.47e-06*	2.4(1.7-3.5) *
No	66	42.8	357	65.1		1(ref)

Logistic regression*

Seizures were 2.4 times more likely to start on long-term oral anticonvulsants as compared to the RONC group. (**Error! Reference source not found.**)

This association shown is despite the fact that seizures as a symptom of other types of neurotoxicity were probably receiving the anticonvulsants in the other types of neurotoxicity (PRES, vascular, metabolic, possible SLS) but most of the other types e.g., leukoencephalopathy didn't have seizures and will be in the comparative RONC group.

It was also observed that of the cases that were on the oral anticonvulsants, 87 cases, out of the responses obtained, for the recurrence of seizures on re-exposure 23% (9/38) had a recurrent episode and the rest 77% (29/38) did not experience a recurrent episode.

5.3.5.17 Persistence of neurological symptoms:

One of the neurological outcomes post seizures events of interest was to explore how many of the cases had persistent prolonged neurological symptoms at their last follow-up. As the PdL data collected was from both the clinical notes and SAE's trial databases whereas the latter lacks the details about the long term follow up. Out of the responses obtained, there were 47 (18.3% - 47/256) cases that were reported to have long term (more than 1 month to 1 year follow up) unresolved neurological symptoms whereas 209 (81.6% - 209/256) cases whose symptoms were fully resolved after the first event. Results of the analysis are shown in the (**Error! Reference source not found.**).

Table 5.36: Comparison of persistence of neurological symptoms in the seizures group and the RONC group

Characteristic	Seizures		RONC		p-value	
Persistence of neurological symptoms	**n**	**%**	**n**	**%**		
Total	256		757			
Yes	47	18.3	138	18.2	1.000	-
No	209	81.6	619	81.7		
Persistence of neurological symptoms with persistent radiological findings						
Total	16		58			
Yes	11	68.7	41	70	1.000	-
No	5	31	17	30		

* Logistic regression analysis

On comparison with the rest of the neurotoxicity cohort (RONC), seizures cases showed no statistically significant difference in association with having persistent neurological symptoms and with those that didn't.

The cases that had persistent neurological findings, out of the responses obtained, there was no statistically significant difference observed between the seizures and the RONC group for

the cases that had persistent neurological symptom and had persistent radiological findings. (**Error! Reference source not found.**)

5.3.5.18 Influence of change in chemotherapy on the persistence of neurological symptoms in seizures cases:

The impact of change in chemotherapy (dose reduced/one dose skipped/with-held), for both intrathecal methotrexate and high dose methotrexate dose, on recovery from the seizures event was explored. The (

) below shows changes in methotrexate (both intrathecal and high dose methotrexate) chemotherapy subset in seizures cases and having the long-term neurological persistent symptoms.

Table 5.37: The impact of change in methotrexate administration (IT MTX and HD MTX) post Seizures on the long-term persistence of neurological symptoms

	Long-term persistent neurological deficits (More than 1 month-1 year)			
Seizures	**Total responses obtained**	**Yes (%)**	**No (%)**	**p-value**
Changes in Intrathecal MTX				
Total	51			
Continued as per protocol	31	10 (32.2%)	21(67.7%)	0.5*
Dose reduced/ Skipped one dose/ withheld	20	5(25%)	15(75%)	
Changes in HD MTX				
Total	45			
Continued as per protocol	40	15(37.5%)	25(62.5%)	0.4*
Dose reduced/ Skipped one dose/	5	1(20%)	4(80%)	

withheld				

* Chi-square test

Out of the seizures cases, looking at the changes (dose reduced/with-held) in intrathecal methotrexate and continuation of intrathecal methotrexate as per protocol, there was no statistically significant difference observed between these for long term persistent neurological symptoms. (

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Similarly, observing the change (dose reduced/with-held) or the lack of it in the administration of high dose methotrexate post seizures event, there was no statistically significant difference observed between the two groups for the unresolved neurological symptoms post seizures episode.

5.3.5.19 Time to resolution of neurological symptoms:

In the responses obtained for the duration of resolution of symptoms, out of the Seizures cases it was observed that 90% of cases had their neurological symptoms resolved within a week, whereas 3.6% of cases with the event completely resolved in one week to one-month duration and the same 3.6% in one month to one-year duration. There were only 4 cases (2%) cases that were reported to have unresolved neurological symptoms for more than a year. (**Error! Reference source not found.**)

Table 5.38: Time to resolution of neurological symptoms post seizures event

Time to resolution of neurological symptoms post-event	n	%
Total responses obtained	192	
Less than one week	174	90.6
From one week to one month	7	3.6
From one month to one year	7	3.6
More than one-year duration	4	2

5.3.5.20 Death due to neurotoxicity:

Seven seizures cases were reported to die as a part of their neurotoxicity complications, out of the total (7/31) responses obtained, where the rest was missing data. While on the other hand, there have been 10 (10/72) deaths reported in the RONC group resulting due to complications in the neurotoxic event.

To summarise seizures showed significant association with recurrence, recurrence on Capizzi MTX protocol, Single IT MTX protocol, late leucovorin rescue protocols. Also, seizures showed significant association with use of oral anticonvulsants and parenteral anticonvulsants, no change in chemotherapy.

5.4 Discussion

To the best of our knowledge, the present study is the largest case series of seizures during ALL therapy reported. This allowed identification of variables associated with seizures such as medium risk regimen allocation, "Other treatment phase" including delayed intensification I &II, interim-maintenance I &II, and re-induction phase, secondary neurotoxic event, Downs syndrome, Capizzi methotrexate dose, Single intrathecal methotrexate, late leucovorin rescue administrating protocol. The Ponte di Legno database was a retrospective collection of neurotoxicity cases where there was heterogeneity of protocols, which allowed to do the comparison of variations in treatment protocols for seizures. However, there were a few limitations. There was no neurotoxicity comparative group, and the analysis was run with the rest of the neurotoxicity cohort (RONC). Also, the PdL database is a collaborative collection of retrospective cases from different sources a) clinical notes b) SAE's (Severe Adverse Events) trial databases c) from both clinical notes and SAE's trial databases. Almost 40% of data combined for the PdL neurotoxicity cases was just from the SAE's trial databases; on the one hand, this allowed us to obtain a larger sample size for these relatively rare cases and to be able to find novel findings, but on the other hand, there were a few limitations such a limited ability to carry out meaningful analysis on all cases, due to them not always being well-characterised and thus containing missing data. In addition, regarding the trial databases of Severe Adverse Events (SAE), it is well-reported that when SAE's were compared with clinical notes there were discrepancies [92,137].

Only three isolated seizures, a seizure episode with reports of no other neurological symptoms and normal radiological findings, out of 707 seizures cases, were observed. This finding of only three isolated seizures leads to the question of whether that is an isolated seizure an entity or are all the seizures potentially classifiable with the other related presentation (PRES, hypertensive, methotrexate-induced with some SLS-like features, metabolic imbalances, infection). In the PdL dataset, it might not be possible to show it with clarity because of the varying degree of missing data for each category; however, the cases that had information available were classifiable with other presentations and were not isolated seizures. This observation is contrasting to the earlier reports that showed 15 cases of isolated seizures without any other identifiable cause, although 1) Lacked the information regarding MRI changes, specifically stratified in types of seizures 2) Isolated seizures reported with other signs and symptoms of neurotoxicity (Supplementary table 1) [79]. Probably, there is a need to have a consensus on the definition of the chemotherapy-induced isolated seizures event.

Increased risk of seizure in Down syndrome (**Error! Reference source not found.**), is a novel finding, where previously only 2 isolated seizures were reported in UKALL 2003 Down syndrome outcomes sub-study [90]. Neurological deficits in ALL children on chemotherapy were shown to have increased risk of recurrence of chemotherapy-induced seizures previously [80], although this was not demonstrated in a later study [78].

The demographic and clinical features identified as significantly associated with seizures in comparison with UKALL 2003 cohort were older age group, CNS involvement at the time of ALL diagnosis, and Medium risk group, of which older age group and CNS involvement (**Error! Reference source not found.**) have already been shown in previous reports [79]. The reasons speculated can be the more treatment intensity, older age group is allocated more intense therapy and if there is CNS involvement (CNS 3) they are allocated more intense chemotherapy regimen, such as extra IT MTX doses, cranial therapy or allocated to HR regimen.

Clinical presentation of seizure cases included headache (27%), pyramidal focal weakness (20%), Altered mental status (17%), Waxing and waning pattern of symptoms (14%), Dysphasia (10%), Hypertension (6%), Sensory disturbances (6%), Constipation (4%), Ataxia (4%), Visual disturbances (2%), dyspraxia (1.1%) (**Error! Reference source not found.**). The accompanying symptoms reported in PdL seizure cases are consistent with the accompanying symptoms reported with isolated seizures in the literature (Supplementary Table 1) [79].

The aetiologies reported included unknown (8%), infection (4.8%) and metabolic imbalances with seizures reported in 3 cases and hypoglycaemia in 1 case. However, it was observed that in 18% of cases the cause was "Other" than the options provided and, in many cases, in 60%, the cause was missing information (Table 5.4). However, these reported aetiologies were not very easily fitted in the aetiologies described by Fisher at *al.* [77], except the seizures due to infectious and metabolic reasons. It is difficult to map the chemotherapy -induced seizures, especially where the presumed drug is methotrexate, under the aetiologies defined here since the underlying mechanism is excitotoxic analogues and is reversed by the excitotoxic analogue antagonist, based on this the metabolic aetiology might be the best fit. The seizures with the presumed cause of cerebral venous sinus thrombosis (CVST) may be multifactorial. As seizures due to CVST are induced by hypoxia they can be grouped under structural abnormalities. Hypertensive seizures are transient and occur due to direct and indirect effects of the drug (vincristine and steroids) induced auto-hypertension, which are reversed in most of the cases with the completion of treatment, can be classified as unknown aetiology.

The leukoencephalopathy (white matter changes) finding on CT scan and MRI reported in 38% and 68% respectively, (**Error! Reference source not found.**), is reduced than the earlier reports (abnormal MRI reported in 87% [79]) but this difference can be explained with difference in classification of seizures, where in PdL we included seizures not otherwise specified cases that were not classifiable as other types of neurotoxicity, but in the report by Stavroula *et al.* abnormal MRI findings are stratified into different aetiologies for seizures [79].

Persistence of radiological findings was observed in 23% of cases in this cohort, out of the responses obtained (**Error! Reference source not found.**). Additionally, persistent neurological symptoms with persistent radiological findings in 68% shown (**Error! Reference source not found.**), is consistent with Bhojwani et al. reported leukoencephalopathy in 77% of symptomatic cases until the end of the therapy in overall methotrexate-induced neurotoxicity patients [138]. Although this report is of overall methotrexate-induced neurotoxicity and thus includes both persistence of radiological findings of other types of neurotoxicity too, the most common presentation was seizures (7/14) [51].

Non-specific EEG abnormalities in most of the cases (80%) in **Error! Reference source not found.**, the reason speculated might be because of a mix of underlying aetiologies along the same lines of the previous reports where the 84% of abnormal EEG findings were reported [79].

The “Other phase of treatment”, entailing delayed intensification I &II and interim maintenance I&II, showed independent association with seizures (**Error! Reference source not found.**). This finding coupled with the significant association with cytarabine (Table 5.9

) implies that cytarabine is most likely to play the key role, as this compound is known to enhance neuronal cell sensitivity to glutamate-mediated damage, like that of methotrexate-induced toxicity [139,140]. In previous reports, cytarabine has been shown to cause a cerebellar type of neurotoxicity, of which ataxia is part of the clinical presentation. Ataxia has also been reported as part of the clinical presentation for seizures, so it might be caused by the concomitant effect of cytarabine [38].

Seizures cases start occurring from early induction phase and peaking around 10 weeks of therapy (Figure 5.**Error! Reference source not found.**). This suggests that seizures start appearing right after exposures to IT methotrexate and/or vincristine pulses and continues to be observed during the following phases, i.e., delayed intensification, interim maintenance phase, maintenance and consolidation; this is consistent with the findings in previous reports that seizures cases continued to occur throughout the consolidation, maintenance and delayed intensification phases of therapy [78,79].

Medium-risk treatment allocation showing significant independent association with seizures (**Error! Reference source not found.**), a finding also supported by comparison with UKALL 2003 (**Error! Reference source not found.**), can potentially be explained by the difference in intensity of drugs administered as compared to the standard risk groups. One other explanation can be the allocation of medium risk treatment to the older age group as opposed to the standard risk where older age has shown significant association with the chemotherapy-induced neurotoxicity [79]. However, this finding is in contrast with the earlier reports of association of chemotherapy-induced seizures with high-risk treatment group allocation [79].

The Capizzi escalating MTX (escalating by 50 mg/m2 every 10 days) protocols showed a significant association, where they were 1.7 times more likely to have seizures in contrast to protocols using high dose MTX (**Error! Reference source not found.**). One explanation for the significance of this association can be attributed to the differences in how methotrexate is administered among the treatment protocols, where intensive hydration for 24 hours after administration, frequent monitoring of serum MTX levels, and leucovorin rescue are the

contemporary practices used to reduce MTX-related toxicities in patients receiving HD MTX but not in patients receiving Capizzi escalating MTX protocols. As evidenced in our own previous work, in a Capizzi- MTX administrating protocol UKALL 2003, seizures were the most common type of neurotoxicity of the well-defined cases [141].

CNS-directed therapy intrathecally has different approaches: 1) Triple Intrathecal (TIT) and 2) Single IT MTX. The trials included in the PdL cohort were either randomising for ITT with SIT therapy (to only high-risk patients, or in one trial for all risk groups). Acknowledging this variation in CNS-directed therapy in different trials included in this cohort, it was interesting to explore if there was an association with seizures or an impact on the outcomes post-event in this cohort. It was interesting to observe that IT MTX showed a significant association with the occurrence of seizures (p-value 0.004, **Error! Reference source not found.**) and with the recurrence of seizures (p-value 0.01, Table 30). Reasons speculated can be that the triple intrathecal therapy has hydrocortisone included, an anti-inflammatory effect which might protect against the seizures and neurological outcomes [142]. Contrary to the previous reports, where (CCG 1952) study randomising for IT MTX and ITT, reported similar levels of CNS-toxicity on both study arms, 5.8% in IT MTX compared to an ITT report of 6.7% [143].

The most common route of methotrexate administration in the seizures group was intrathecal 95.2% (357/375), which is consistent with the previous findings where overall central neurotoxicity has been linked with intrathecal methotrexate [64,144]. Previously, intrathecal methotrexate-induced neurotoxicity has also been associated with elevated methotrexate CSF levels: in one study of intrathecal methotrexate neurotoxicity, CNS levels of the drug after 48 hours were 13 times higher in patients with neurotoxicity than those without (5/25) [119].

Exploring the other interactions with chemotherapy, cranial irradiation showed a significant association with seizures (**Error! Reference source not found.**Table 5.14: Comparison of the Seizures cases with the RONC for the administration of cranial irradiation four weeks before the event), where previously long-term intractable chemotherapy-induced epilepsy was hypothesized due to administration of cranial irradiation and IT MTX [145,50]. Historically, prophylactic CNS-directed cranial irradiation was administered [26], gradually the protocols limited the use of cranial irradiation to just high-risk, T-cell, CNS-relapse groups due to the reports of 1) undesired toxicities, including cognitive impairment [28,29] and 2) improved leukaemia outcomes with intensified chemotherapy without cranial irradiation [146]. Heterogeneity in the protocols

included in the PdL dataset, for the use of cranial irradiation for the risk group it's being administered to (high-risk/T-cell, CNS-3 disease), dosage (12, 18 or 24 Gy), and lower age limit, existed [147-149]. The mechanisms of chronic radiation damage include toxicity to oligodendrocytes and normal and progenitor neural cells, metabolism disturbances, and inflammatory responses [150-152].

Parenteral (rescue medication administered acutely) and long-term oral antiseizures administration, the interventions to manage the seizure episode was observed in almost half of the patients (Table 35 & 36). Use of antiseizures along the same lines has been reported previously 29/62 (46%) after the second seizure episode [78]. Enzyme-inducing antiseizures therapy is associated with increased systemic clearance of antileukemic drugs and worse leukaemia outcomes in patients [60]. In the PdL dataset, it was not possible to establish the type of antiseizures used; however, the finding of similar leukaemia relapse proportions in the seizures group, where half of the patients were on antiseizures, with the rest of the neurotoxicity comparative group makes it plausible that probably non-enzyme inducing antiseizures were administered.

The use of aminophylline, an adenosine antagonist, and dextromethorphan, non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor, to manage the methotrexate-induced seizures, reported in just 3 cases and 4 cases respectively, made it difficult to further explore the beneficial effects of these interventions in the PdL seizures cohort.

Interestingly, late rescues (**Error! Reference source not found.**) of leucovorin post-methotrexate administration were 6.8 times more likely to get a seizures episode. This finding is favoured by a report by Cohen I., a systematic review, showing that to prevent all forms of post-methotrexate neurotoxicity, the leucovorin administration should be started 24–36 hours after methotrexate dose administration [153].

Prolonged resolution of neurological symptoms (without stratifying which neurological symptom) was observed in only 18 % of cases (**Error! Reference source not found.**). Almost 94% of cases had their neurological symptoms completely resolved within less than one month duration (**Error! Reference source not found.**) and prolonged neurological symptoms were observed in only 6%, which is similar to the findings of a previous long-term follow-up study, the Childhood Cancer Survivor Study, which reported a 7% incidence of seizures in acute

leukaemia survivors [154]. More importantly, the finding of no statistically significant difference in prolonged neurological symptoms observed in the groups, seizures cases with IT MTX and HD MTX modified or not, post-event (Table 38), providing evidence that if cases are re-exposed, they are not more likely to have long-term neurological sequalae.

The observations regarding admission required to ITU in almost half (45%) of the seizure cases (

), and 46% cases required ventilatory support for more than 48% (**Error! Reference source not found.**), emphasises that in some of the cases, optimal supportive care is required to avoid the adverse outcomes. Almost half of the seizures cases requiring ITU admission is supported by the previous study reports where 42/81 cases required ITU admission [79].

Recurrence on re-exposure to the presumed drug after the initial event in seizures has been frequently speculated. In the PdL cohort, secondary neurotoxicity (50% of cases) showed significantly associated with recurrence of a neurotoxic event when re-exposed to the presumed agent (**Error! Reference source not found.**). Although, previous reports have shown recurrence in seizures when re-exposed in 35% cases, but the numbers are small [79].

The findings first time being reported here include seizures cases on the Capizzi-style MTX protocol, late leucovorin rescue protocol and single intrathecal therapy (IT MTX) protocol were at a higher risk for a recurrent episode when re-exposed to the presumed drug (**Error! Reference source not found.**, **Error! Reference source not found.**, **Error! Reference source not found.**).

Seizures recurrence upon re-exposure, despite the use of oral anticonvulsants was observed in 23% in PdL, however, it was difficult to establish if the oral anticonvulsants were administered before or after the recurrence. Information regarding recurrence of seizures on chemotherapy and oral anticonvulsants is not available in the literature, however, a previous study of long-term outcomes in chemotherapy-induced seizures study showed approximately one-quarter of patients had recurrent seizures after stopping antiseizure medication [78].

Reduced overall survival rates (p-value 0.02) in the seizures cases (**Error! Reference source not found.**) and seven neurotoxicity event-related deaths were reported. The reduced overall

survival along with the findings of 1) no statistically significant difference in leukaemia outcome overall p-value, 2) no effect on worsening of leukaemia outcomes after chemotherapy (IT MTX and HD MTX) modified and 3) coupled with the seizures cases reported to have an infection (24%), points towards the causes other than leukaemia relapse, probably infection, a most common cause of treatment-related mortality (TRM) [155]. However, from the available limited information, especially for the cases where data was obtained from the SAE's trial databases, it's difficult to determine with certainty. In contrast, in a recent study of overall methotrexate-induced neurotoxicity, Mateos et al. reported increased CNS relapses after modification in the methotrexate administration post event [57]. The reasons for not seeing the same effect of methotrexate therapy modification in seizures cases on leukaemia relapse can be explained by the small number of cases with relapse, and even fewer cases with CNS relapse, where out of the cases that had leukaemia relapse in our cohort, 23% had isolated CNS relapse in the seizures group irrespective of change in chemotherapy. The Mateos et al. study was a population-based study comparing MTX-induced neurotoxicity vs no neurotoxicity cases in paediatric ALL patients in a trial, while the PdL study compares seizures vs RONC group, therefore a direct comparison between these two studies is not always possible [57].

6 Characterization of re-classified PRES cases

6.1 Introduction

Posterior Reversible Encephalopathy Syndrome (PRES) was first described as a radio-oncological entity [71] with incidence variably reported between 3.8%-4.5% [49,59] making it one of the common types of chemotherapy associated neurotoxicity in paediatric acute lymphoblastic leukaemia (ALL). PRES is clinically characterized by seizures, hypertension, headache, altered mental status and visual impairment and on MRI classically presents as bilateral cortical or subcortical parieto-occipital white matter lesions [40,74,122,131,156].

The underlying pathology is speculated as an interplay between hypertension, leukaemia itself (either directly by infiltration or through the inflammatory response), and the cytotoxic effects of the presumed drugs that constitute the triggering factors that result in endothelial dysfunction and interstitial oedema in the brain [74,131,157]. PRES has been reported usually during the induction phase of ALL chemotherapy [2,72-74]. Older age, T- cell immunophenotype, CNS involvement, high risk treatment has been reported [49] as risk predictors but this assessment is based on limited numbers [49]. In therapy-related factors, more than 14 days of urinary alkalinization alongside methotrexate administration is reported as associated with PRES by Banerjee *at al* [59]. Inferior leukaemia outcomes, in terms of increased leukaemia relapse, have been shown by Banerjee *at el*. after a PRES event [59], but this was not observed in the other cohorts [3] making it imperative to test this possibility in comparison with other types of neurotoxicity in larger cohorts. Despite being reported as a reversible episode, there have been few reports of worse neurological outcomes post event and risk predictors of neurological outcomes are largely unknown [75].

6.2 Aims

1) To deep phenotype the PRES cases
2) To identify the differences and similarities in demographic and clinical features of PRES with the rest of the neurotoxicity cohort
3) To identify any differences in the occurrence of PRES and effects on neurological outcomes of PRES cases between different treatment protocols.
4) To explore the effects of neurotoxicity on neurological outcomes in the PRES group
5) To investigate the effects of neurotoxicity on the leukaemia outcomes in PRES cases

6.3 Results

Cases:

In the Ponte di Legno neurotoxicity clinical cohort, there were 158 cases of definite PRES classified according to the differential diagnostic scoring system (explained in detail in the chapter 2). There were 326 cases in this cohort including all the cases that were classified as definite PRES, probable radiological PRES, probable clinical PRES, and possible clinical PRES depending on the clinical and radiological information available for these cases.

Classified as	**n**	**Differential diagnostic scoring system (DDSS) scoring**
Definite PRES	158	3.5-5.5
Probable radiological PRES	69	2-3
Probable clinical PRES	31	3.5-5.5 with missing MRI
Possible clinical PRES	63	2-3 with missing MRI

Table 6.1: Showing number of definite, probable radiological, probable clinical and possible PRES cases re-classified based on DDSS

For the purpose of analysis, only the definite PRES cases were considered and are referred as PRES cases or PdL (Ponte di Legno) PRES cases.

Whereas, due to the lack of a "no neurotoxicity" control group the Rest of the Neurotoxicity Cohort (RONC, n= 1652) was taken as the comparative group.

6.3.1 Aim 1: To deep phenotype the PRES cases

6.3.1.1 Pre-existing neurological conditions:

In terms of having a pre-existing neurological condition there was one case with Down syndrome, one patient had epilepsy, one had autism and two had a history of febrile seizures.

Age:

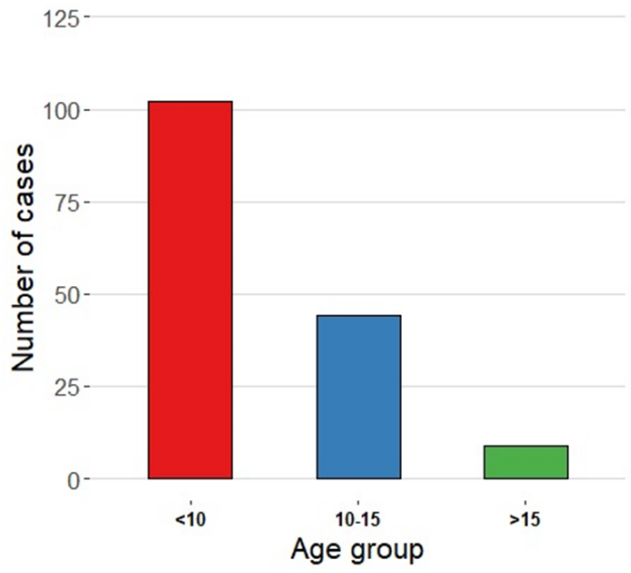

Figure 6.1: Bar graph showing PRES cases age distribution

Showing age distribution of PRES cases in age groups less than 10 years (<10 years), between 10-15 years (10-15 years) and more than 15 years (>15 years). (Figure 6.)

The 158 patients that were classified as definite PRES, at the time of ALL diagnosis, ranged in age from one to 19 years old with a median age of 8.62(IQR 5.45-11.12).

The majority of cases were in the younger (in <10 years) age group (102/155 - 65%) almost accounting for two thirds of the group, followed by age group 10-15 years (44/155 – 28%) and very few cases in the older age group >15 years (9/155 – 5.8%).

Gender:

Definite PRES was observed more in males (88/158 – 56%) than females (70/158 – 44 %).

ALL Immunophenotype:

Definite PRES patients were reported to mainly have a B-cell immunophenotype of leukaemia 70% (111/158) as compared to the T-cell 30% (47/158).

White blood cell counts:

At the time of their ALL diagnosis, more than two thirds of the cases, 69% (109/157) had a white blood cell count less than $50x10^9/L$, whereas the rest, 31% - (48/157), had white cell counts more than $50x10^9/L$.

Karyotype:

Out of the 120 patients for which the karyotype was reported, 23 had high hyperdiploidy (favourable prognosis) at the time of ALL diagnosis, followed by 15 with t (12;21) translocation (favourable prognosis) and 3 had MLL-rearranged (poor outcome) acute leukaemia with t (4;11) (q21; q23). There was a fairly large number of cases, 79, that reported to have “Other” type of karyotype, comprising of t (7;9), del 12p, t (9;18), iAMP21, t(1;19), hypodiploidy, as well as complex cytogenetics of uncertain origin.

CNS status:

The majority of the cases, 68% (103/154), had no leukaemic cells present in the CSF cytospin at the time of diagnosis (CNS1), followed by 22% (35/154) of cases that had CNS 2 type (<5 leukocytes/µl CSF with blasts) at presentation and 10% (16/154) had CNS 3 type (≥5 leukocytes/µl with blasts or signs of CNS involvement). There were no cases reported with traumatic lumbar puncture with or without blasts.

6.3.1.2 Timing of event:

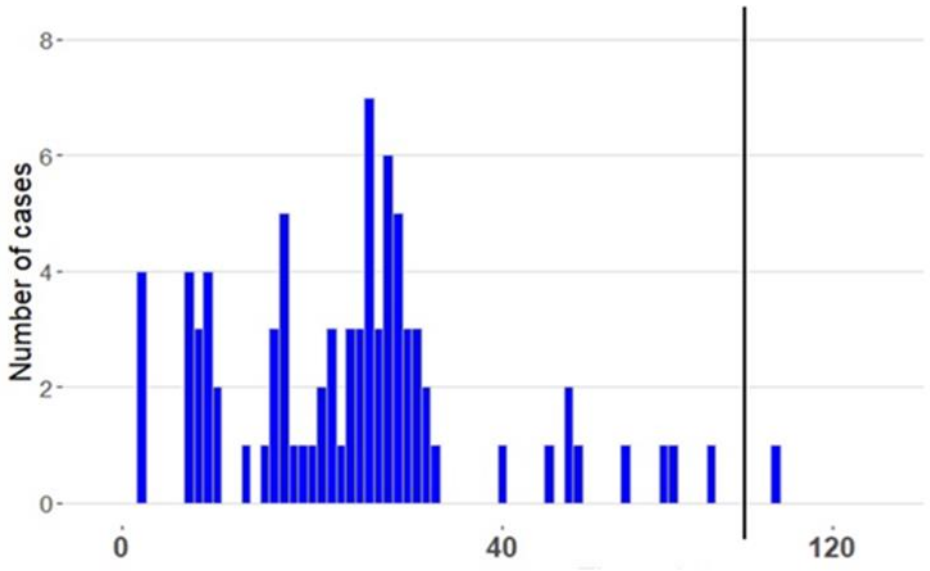

Figure 6.2: Bar graph showing distribution of PRES cases over treatment in weeks

(x-axis has been split to show the cases in later phase)

Most of the PRES events were concentrated during the initial phases of treatment. Most were observed up to first 30 weeks and there were a few sporadic cases spread across later phases of the ALL therapy going up to the 90 weeks. (Figure 6.)

6.3.1.3 Clinical characteristics of PRES cases:

To explore the clinical accompanying symptoms as part of the PRES event were of interest. Detailed data was collected for this purpose. However, due to variation of missing data in each category, the percentages were calculated out of the responses obtained for that category, hence the change in denominator for each category. The information is shown in **Error! Reference source not found.**.

Table 6.2: Clinical characteristics and lab findings of PRES cases

Clinical symptoms	Number of patients	Percentage (Out of the responses obtained)
Hypertension	158/158	100%
Seizures	140/157	89%
Altered mental status	113/150	75%
Headaches	46/122	38%
Visual disturbances	45/133	34%
Constipation	43/122	35%
Sensory disturbances	15/134	11%
Signs		
Infection	49/136	36%
Lab investigations		
Hyponatremia	9/155	5.8%
Hypoglycaemia	1/155	0.6%

Of the 158 cases that were picked up by the differential diagnostic scoring system as definite PRES, all the cases had experienced hypertension, due to the limitation of the differential diagnostic scoring system as to get diagnosed as definite PRES hypertension was a must. After hypertension seizures was the most common type of neurological symptoms observed in 89% (140/157). Altered mental status, as part of the PRES episode, was observed in three-quarters of the cases 75% (113/150) followed by headaches in 38% (46/122), visual disturbances in 34% (45/133), constipation in 35% (43/122) and sensory disturbances were observed only in 11% (15/134) of cases.

There were 49 cases (36% - 49/136) that were reported to have an infection along with the ongoing PRES event.

Looking at the lab investigations reported, there were 5.8% (9/155) with lower sodium blood levels at the time of the event. However, there was only one case with low blood sugar levels at the time of event. (**Error! Reference source not found.**)

6.3.2 Aim 2: To identify the differences and similarities in demographic and clinical features of PRES with the rest of the neurotoxicity cohort

In order to identify clinical and demographic features at the time of ALL diagnosis that are associated with PRES, these features were compared with the rest of the neurotoxicity group (RONC).

Table 6.3: Comparison of demographic and clinical features of definite PRES cases with the other neurotoxicity cases

Characteristic	**PRES n=158**		**RONC n=1655**		**p-value**	**OR (CI)**
	n	**%**	**n**	**%**		
Age						
Total	158		1652			
<10 years	102	65	941	56	0.032*	1.45(1.03-2) ф
>10 years	53	33	711	43		1(ref)
Age as continuous variable (Mean)	8.3		8.7		0.17*	
Gender						
Total	158		1653			
Male	88	55.6	884	53.4	0.6523*	-
Female	70	44.3	769	46.5		
Immunophenotype						
Total	158		1641			
B-cell immunophenotype	111	70	1358	82.7		1(ref)
T-cell immunophenotype	47	30	283	17.2	0.0002*	2.03(1.4-2.9) ф
White blood cell counts						
Total	157		1650			
<50x10^9/L	109	69.5	1291	78.2		1(ref)
>50x10^9/L	48	30.5	359	21.7	0.0152*	1.5(1.09-2.2) ф
White blood cell counts as continuous variable (Mean)	78099.8		53616.42		0.09*	-
CNS involvement						
Total	154		1595			
CNS 1	103	66	1307	81.9	9.87e-06*	1(ref)
CNS involvement (CNS 2 + CNS 3)	51	33	288	18		2.2(1.5-3.2) ф

Phase of treatment						
Total	158		1527			
Induction	65	40	401	26.2	0.00578*	2.1(1.26-3.7) ℏ
Consolidation	19	12	249	16.3		1(ref)
Consolidation HDMTX	16	10	162	10.6		-
Maintenance	14	8.8	140	9.1		-
Other	44	27	575	37		-

*Chi square testing

ℏLogistic regression analysis

Age:

For analysis the cohort was divided into two age categories, less than 10 years old and more than 10 years old. The younger age group (<10 years) was over-represented, 65% (102/158), and was statistically significantly (p value 0.032) associated with PRES when compared with the RONC group. The odds of having PRES were 1.45 (CI (1.03-2.06)) times higher in younger age group (<10 years) as compared with "RONC" when comparing with the older age (>10 years), age group.

However, age taken as a continuous variable did not show any statistically significant difference between PRES and the "RONC".

Gender:

There was no difference seen in the proportion of males and females between the PRES cases and the RONC groups. (**Error! Reference source not found.**)

ALL Immunophenotype:

Patients with T-cell immunophenotype had a higher risk of PRES when compared with the RONC group (p value 0.0002). The odds of having PRES were 2.03 times higher in T cell immunophenotype cases.

T cell immunophenotype remained a risk factor for PRES after adjusting for the inclusion of a treatment protocol that was exclusively for T cell ALL cases (COG 0434 trial) by restricting the analysis just to the rest of the protocols.

White blood cell counts:

Patients with higher White Blood Cell (WBC) counts (> 50 x 10^9/L) were at higher risk of having PRES as compared to the "RONC". There was a statistically significant (p value 0.0152) association of PRES with having higher WBC counts at the time of ALL diagnosis, 30.5% (48/157), as compared to the 21.7% (359/1650) cases of higher WBC counts in the "RONC". The higher WBC count (> 50 x 10^9/L) cases were 1.5 times more likely, OR (CI)1.5(1.09-2.25), to get PRES as an outcome compared to the RONC when compared with the group having lower WBC counts (<50 x 10^9/L) at the time of ALL diagnosis.

The WBC counts taken as a continuous variable did not show any statistically significant difference between the two groups compared, PRES and the "RONC".

CNS status:

The CNS status at the time of ALL diagnosis was categorized into two categories "No CNS involvement- CNS 1" and "CNS involvement- CNS 2 + CNS 3". The cases presenting with CNS involvement were at a higher risk of having PRES as compared to the "RONC". CNS involvement, seen in 33% (51/154) of cases, was statistically significantly (p value 9.87e-06) associated with having PRES as compared to the "Other type of neurotoxicity", where CNS involvement was reported in 18% (288/1595). The cases with CNS involvement were 2.24 times more likely to get PRES as compared to the RONC when compared with the cases having "No CNS involvement - CNS 1" at the time of ALL diagnosis.

6.3.2.1 Phase of therapy:

Almost 40% (65/158) of PRES episodes occurred during the induction phase of therapy followed by 12% (19/158) who had the event during consolidation, 10% (16/158) during their consolidation with High Dose methotrexate and 8.8% (14/158) during maintenance phase of therapy. There were 27% (44/158) in the "Other" treatment phase group including delayed intensification, interim-maintenance, and re-induction phase.

On comparison of PRES cases with the RONC group, Induction phase of therapy was found significantly associated with PRES, 40% (65/158) vs 26% (401/1527), p value 0.00578. The odds of having a PRES episode during the induction phase were 2.12 times higher than the RONC when compared with the group having an event during consolidation phase of therapy.

Multivariate logistic regression analysis:

The clinical and demographic characteristics combined, that showed significant association with seizures, were fitted in a multivariate logistic regression model to identify the variables that are associated independently. Results are shown in **Error! Reference source not found.**.

Table 6.4: Multivariate regression analysis of the variables showing significant association with PRES when compared with RONC

Characteristics	**p-value**	**OR**	**CI**
Age			
<10 years	0.019	1.53*	1.07-2.2
>10 years		1(ref)	
Lineage			
B cell immunophenotype		1(ref)	
T cell immunophenotype	0.028	1.60*	1.04-2.42
CNS involvement at the time of ALL diagnosis			
No CNS involvement - CNS 1		1(ref)	
CNS involvement (CNS 2 + CNS 3)	0.0003	1.98*	1.35-2.87
White blood cell counts at the time of ALL diagnosis			
<50 $x10^9$/L		1(ref)	
>50 $x10^9$/L	0.21	1.28*	0.85-1.9
Phase of treatment			
Induction	0.03	1.79*	1.1-3.1

Consolidation		1(ref)	

*Logistic regression analysis

The clinical and demographic characteristics combined, that showed significant association with PRES, were fitted in a multivariate logistic regression model.

Younger age group < 10 years, T cell immunophenotype of leukaemia, CNS involvement at the time of ALL diagnosis and Induction phase of treatment showed significant association with PRES and were identified as independent risk factors of PRES when compared with the "RONC".

6.3.2.2 Comparison of Demographic and clinical features of definite PRES cases with the UKALL 2003 published cohort:

A major limitation of analysis using neurotoxicity controls is the lack of no neurotoxicity control group for comparative analysis and the analysis is done between the different types of neurotoxicity. To investigate whether patients with seizures had distinct demographic or clinical variables compared to children presenting with ALL, the dataset was also compared with information obtained from the UKALL 2003 cohort. UKALL 2003 was a randomised trial for children with acute lymphoblastic leukaemia, where it included all the patients aged one year up to 25 years and recruitment levels were high (>90%) [47,13] therefore the patient characteristics can be considered "typical" of childhood ALL.

Table 6.5: Demographics of PRES cases comparison with UKALL 2003 cohort

Characteristic	PRES (n)	PRES (%)	UKALL 2003 cohort (n)	UKALL 2003 Cohort (%)	p value
Age at diagnosis (years)					
Total 158			3113		
<10 years	102	65	2279	73	
10-15 years	44	28	501	16	.000132*
>15 years	9	5.8	333	11	
Gender					
Male	88	55.6	1767	57	.7*
Female	70	44.3	1346	43	
Immunophenotype					
B-lineage	111	70	2727	88	.3*

T-lineage	47	30	1346	12	
White blood cell counts (x10^9/L) at diagnosis					
<50 (x10^9/L)	109	69.5	2428	78	.01*
>50 (x10^9/L)	48	30.5	685	22	
CNS status at diagnosis					
CNS 1 (no involvement)	103	66.8	2713	87	< 0.00001*
CNS 2 (<5/μL)	35	22.7	366	11.6	
CNS 3 (>5/μL) by cytology	16	10.3	34	1	
Treatment risk group allocation					
Standard risk	42	29.1	1537	49	< 0.00001*
Medium risk	66	45.8	842	27	
High risk	36	25	734	24	

*Chi square testing

The comparison showed that there was difference seen in age groups (<10 years, 10 -15 years and >15 years). PRES cases show higher proportion of cases in age group (10-15 years) and lower proportion of cases in the younger age group (<10 years) and older age (>15 years) as compared with the UKALL 2003 cohort, indicating that older age ALL was more likely to get PRES.

There was no difference seen in gender distribution in PRES cases from PdL cohort when compared with ALL patients from UKALL 2003 cohort.

T- cell immunophenotype was over-represented in PRES cases from the PdL cohort when compared with the UKALL 2003 cohort.

Higher white blood cell (WBC) counts (>50 x 10^9/L) at the time diagnosis showed higher occurrence in PRES cases from the PdL cohort as compared to the UKALL 2003 study cohort.

CNS involvement (CNS 2 and CNS 3) were over-represented in PRES cases as compared with the CNS involvement (CNS 2 and CNS 3) group in UKALL 2003 cohort.

Table 6.6: Comparison of treatment characteristics of PRES with the RONC

Characteristic	**PRES n=158**		**RONC n=1655**		**p-value**	**OR(CI)**
	n	%	n	%		
NCI risk group allocation						
Total	156		1647		0.9565*	-
Low risk	69	44.2	738	44.8		
Intermediate/high risk	87	55.7	909	55.1		
Treatment risk group allocation						
Total	144		1494			
Standard risk group	42	29.1	534	35.7	0.1528*	-
Medium risk group	66	45.8	671	44.9		
High risk group	36	25	289	19.3		

*Chi square testing

NCI risk group allocation:

Almost half of the cases, 44% (69/156), could be stratified in the standard risk NCI risk group i.e., they were less than 10 years old and had a white cell count less than 50,000 at the time of diagnosis. The rest of the 55% (87/156) fell into the Intermediate/high risk group.
There was no statistically significant difference observed between the two groups, PRES and the RONC.

6.3.2.3 Treatment risk group allocation:

As reported by the study groups there were 29% of cases stratified in standard risk group, whereas there were almost half of the patients, 46% (66/144), were put in medium risk group and only 25% (36/144) were assigned to high-risk group.

There was no statistically significant difference observed between the groups, PRES and the RONC.

6.3.2.4 Drugs administered as per protocol within four weeks of treatment

To explore the association of the drugs administered four weeks prior to the event information for the chemotherapy administered was collected. The results of the analysis are shown below in **Error! Reference source not found.**.

Table 6.7: Comparison of the drugs administered before the event between the PRES cases and the RONC

Characteristic	**PRES n=158**		**RONC n=1655**		**p-value**	**OR(CI)**
	n	%	n	%		
Vincristine administration four weeks before the event						
Total	130		820		<0.0001 1.27e-05*	1.12(0.06-3.2) ᵑ
Yes	111	85.3	536	65.3		
No	19x	14.6	284	34.6		
Steroids administered four weeks before the event						
Total	102		722			
Dexamethasone	38	37	271	37	0.08*	
Prednisolone	32	31	146	20		
Unspecified	15	14	83	11.4		
No	17	17	222	30		
Total steroid use Dexamethasone+ Prednisolone+ Unspecified						

Total steroid use - Yes	85	83.3	500	69.2	0.00408*	2.2(1.32-3.94) ⫛
Total steroid use -No	17	16.6	222	30.7		

*Chi square testing

⫛Logistic regression analysis

As it is reported that PRES occurs usually in the induction phase of the treatment so, a significant association was seen with drugs that are mostly administered during Induction phase such as Vincristine and use of Steroids (yes/no). Whereas steroids when stratified between types of steroids did not show significant association with dexamethasone, keeping the prednisolone as reference, when PRES was compared with the RONC.

6.3.2.5 Multivariate logistic regression analysis

As vincristine is mostly administered during the Induction of phase of treatment and both Vincristine and Induction phase of therapy showed significant association with PRES, it was interesting to fit them in a multivariate model to see if they are independently associated with PRES.

Table 6.8: Multivariate logistic regression analysis - Use of vincristine and phase of therapy

Characteristics	p-value	OR	CI
Use of vincristine within 4 weeks of event			
Yes	0.000175	2.6*	1.6-4.6
No		1(ref)	
Phase of treatment			
Induction	0.000485	1.98*	1.3-2.9
Rest of the phases of treatment		1(ref)	

*Logistic regression analysis

It was observed that the odds of having PRES with the use of vincristine within 4 weeks of the event were 2.6 times higher even after adjusting for the phase of the treatment. (**Error! Reference source not found.**)

6.3.2.6 Multivariate logistic regression analysis

As steroids are also mostly administered during the Induction of phase of treatment and both variables, use steroids and the Induction phase of therapy showed significant association with PRES, it was interesting to fit them in a multivariate model to see if they are independently associated with PRES.

Multivariate regression analysis of use of steroids within four weeks of administration and the phase of the treatment between PRES and the RONC group.

Table 6.9: Multivariate regression analysis - Use of steroids and phase of therapy

Characteristics	p-value	OR	CI
Use of steroids within 4 weeks of event			
Yes	0.0272	1.86*	1.09-3.35
No		1(ref)	
Phase of treatment			
Induction	2.03e-06	2.82*	1.84-4.34
Rest of the phases of treatment		1(ref)	

*Logistic regression analysis

It was observed that the odds of having PRES with the use of steroids within four weeks of the event were 1.86 times higher even after adjusting for the phase of the treatment. (**Error! Reference source not found.**)

6.3.2.7 Drugs administered as per protocol within four weeks of treatment compared for early and phase of therapy

To explore if there was any association of the drugs administered four weeks prior early (induction phase) and late (consolidation, maintenance, delayed intensification I & II and interim maintenance) phase of the therapy. The PRES cases occurring during the Induction phase of therapy (early PRES) were compared with the PRES cases that were occurring during the rest of the phases of therapy (late PRES).

Table 6.10: Comparison of drugs administered in early phase PRES and late phase PRES

Characteristic	**PRES in induction phase of therapy (Early phase)**		**PRES in the rest of the phases of therapy (Late phase)**		**p-value**
Use of vincristine within four weeks of event	**n**	**%**	**n**	**%**	
Total	61		69		
Yes	51	83	60	86	
No	10	17	9	13	0.7712*
Use of Steroid within four weeks of event	**n**	**%**	**n**	**%**	
Total	57		45		
Yes	48	84	37	82	
No	9	16	8	18	1.000*

*Logistic regression analysis

It was interesting to observe that there was no statistically significant difference seen for the administration of vincristine and steroids within the four weeks of event. Only 8 cases and 9 cases didn't have Vincristine and steroids in last four weeks, but this is probably because pulses

are given every four weeks in the maintenance phase (late phase). (**Error! Reference source not found.**)

6.3.2.8 Cranial irradiation before the event:

Only 1 case that received cranial irradiation before the event as compared with 151 cases that didn't receive the cranial irradiation treatment before the treatment. Whereas there were 32 did receive cranial irradiation and 1335 in the RONC did not receive cranial irradiation before the event.

6.3.3 Aim 3: To identify any differences in the occurrence of PRES and effects on neurological outcomes of PRES cases between different treatment protocols

As there was a variation in administering different dose and in total number doses of Vincristine being administered according to the different trial protocols so it was important to check if there is any difference in the occurrence of PRES between different protocols. The protocols were grouped in "higher dose of vincristine group" where Vincristine 2 mg/m2 dose was administered in induction and the "Lower vincristine group" where the 1.5 mg/m2 dose was administered.

Table 6.11: Comparison of different vincristine dosage administered as per protocols between PRES and RONC

Characteristic	Total	PRES		RONC		p-value	OR(CI)
		n	%	n	%		
Total	1728	158		1655			
Higher vincristine group	123	37	23.4	86	5.1	6.7e-15	5.4(3.53-8.3) *
Lower vincristine group	1659	121	76.5	1538	93		1(ref)

*Logistic regression analysis

The treatment protocols that administered higher vincristine dose comparatively were 5.4 times more likely to get PRES, however this finding is confounded by the fact that only one protocol was administering this higher dose of vincristine.

6.3.4 Aim 4: To explore the effects of neurotoxicity on neurological outcomes in the PRES group

6.3.4.1 Death due to neurotoxicity:

1 death out of total 10 responses, where the rest was missing data. This one case died of the complications of PRES and wasn't reported to have any other contributing factors such as infection, evidence of leukaemic infiltration or electrolyte imbalance.

6.3.4.2 Persistence of neurological symptoms:

There were 34 (22% - 34/148) cases that reported to have long term (more than 1 month to 1 year follow up) unresolved neurological symptoms whereas 114 (77% - 114/148) cases whose symptoms were fully resolved after the first event. When compared with the rest of the cohort there was no statistically significant difference seen it terms of having persistent neurological symptoms.

6.3.4.3 Comparison of persistence of neurological symptoms in early phase PRES and late phase PRES

It was of interest to explore the effect of different phases of therapy, early induction phase vs later phases including consolidation, maintenance, delayed intensification I & II, interim maintenance I & II, on the long-term persistence of neurological symptom in PRES cases.

Table 6.12: Comparison of persistence of neurological symptoms in early phase PRES and late phase PRES

Characteristic	PRES in induction phase of therapy		PRES in the rest of the phases of therapy		p-value
Persistence of neurological symptoms	n	%	n	%	
Total	62		86		
Yes	17	27	17	19	0.3714*
No	45	72	69	80	

*Logistic regression analysis

The PRES cases that had the neurotoxic event during the Induction phase of treatment were over-represented, 27% (17/62) in the group that had prolonged persistence of neurological symptoms (more than 1 month to 1 year follow up) when compared with the cases that had PRES event during the "rest of the phases of the therapy" – 19% (17/86). However, there was no statistically significant difference observed between the two groups of unresolved neurological symptoms of PRES in Induction phase and of PRES in the rest of phases of treatment.

6.3.4.4 Time to recovery

It was of interest to explore if there is any difference in the timing to recovery of the neurological symptoms in PRES cases when compared with the RONC group.

Most of the PRES cases, almost 90% (<1 week in 65%-73/112, 1 week to 1 month in 25%-29/112), had their neurological symptoms resolved within a month after the event however there were a few cases that had prolonged neurological symptoms. There were 7 patients with symptoms up to 1 year and there were 3 patients with persistent neurological symptoms up to more than a year (**Error! Reference source not found.**).

Table 6.13: Comparison of time to resolution of neurological symptoms between PRES and the RONC group

Characteristic	PRES n=158		RONC n=1655		p-value
	n	%	n	%	

Time to resolution of symptoms					
Total responses obtained	112		649		0.0032*
Less than 1 week	73	65	501	77	
1 week to 1 month	29	25	94	14	
1 month to 1 year	7	6	35	5	
More than 1 year	3	2.6	19	3	

*Logistic regression analysis

The PRES cases showed statistically significant association with time to recovery (1 week to 1 month) in comparison with the RONC group.

6.3.4.5 Secondary neurotoxicity

The PRES cases having recurrence of neurotoxicity upon re-exposure to the presumed drug were explored and it was observed that 25 patients (22% - 25/113) had a secondary neurotoxic event, out of the responses obtained, whereas 88 patients (77.8% - 88/113) did not have a recurrent neurotoxicity episode upon re-exposure to the presumed drug. However, there was no statistically significant difference observed between PRES group and the RONC in terms of having a secondary neurotoxicity episode on re-exposure to the presumed agents (PRES- (22%-25/113) VS Other neurotoxicity (28%-130/461), p value 0.2358)

6.3.4.6 Types of Secondary event:

The recurrent PRES cases on re-exposure were explored to see the occurrence of type of recurrence in this group, shown in Error! Reference source not found..

Table 6.14: Recurrence in PRES – types of secondary event

Type of Secondary event (Total 25)	n	%
Seizures	9	36%
Encephalopathy	7	28%
PRES	5	20%
AMS	1	4%

Unspecified	4	16%

The most common type of recurrence was seizures observed in 36% (9/25) followed by 28% (7/25), PRES observed in 20% (5/25), and there were 4 cases with no information regarding the type of recurrent neurotoxic episode.

6.3.4.7 Effect of early phase and late phase of treatment therapy on the recurrence of PRES

To explore if the early phase induction phase of therapy and late phase of had an association with the recurrence of PRES in this cohort, the recurrent PRES cases were compared for the above-mentioned two different treatment phases. The results are shown in (Error! Reference source not found.)

Table 6.15: Comparison of secondary event in early phase PRES and late phase PRES

Characteristic	**PRES in induction phase of therapy**		**PRES in the rest of the phases of therapy**		**p-value**
Secondary neurotoxic event	n	%	n	%	
Total	41		72		
Yes	14	34	11	15	0.0368*
No	27	66	61	85	

*Logistic regression analysis

PRES occurring during Induction phase was identified as significantly associated with secondary neurotoxic event when compared with the PRES occurring during the rest of the phases of treatment.

6.3.4.8 Use of oral anticonvulsants

The association of PRES cases with the use of long-term oral anticonvulsants to manage their neurotoxic events was explored, with the assumption that oral anticonvulsants were administered for the long term.

PRES patients were more likely to be put on the long-term oral anticonvulsant therapy 73% (90/122), out of the responses obtained. (

)

Table 6.16: Comparison of oral anticonvulsants administered between PRES and the RONC

Characteristic	PRES		RONC		p-value	OR (CI)
Use of oral anticonvulsants	n	%	n	%		
Total	122		580			
Yes	90	73	189	32.5	<0.0001	5.8(3.7-9.1) *
No	32	27	391	67.4		1 (ref)

*Logistic regression analysis

On comparison of PRES cases 73% (90/122) with the RONC group 32.5% (189/580) the long-term oral anticonvulsants therapy was significantly associated (p value <0.0001) with PRES and they were 5.8 CI (3.7-9.14) times more likely to be put on long term oral anticonvulsants.

6.3.4.9 Overall treatment strategies post-event

To explore the interventions given at the time of PRES event or the other drugs that were being administered at the time of event were of interest. Due to variation in missing data reported in each category, the denominator was different each category depending on the number of responses obtained. Data was collected and the information is shown in **Error! Reference source not found.**

Table 6.17: Treatment administered after PRES event

Group of the drug administered	Number of patients	Percentage
Oral antiepileptics	90/122	73%
Parenteral antiepileptics	65/100	65%
Antihypertensive	26/46	56.5%
Antibiotics	10/46	21%
Benzodiazepines	9/46	19.5%
Steroids	7/46	15%
Antivirals	6/46	13%

Diuretics	4/46	8.6%
Antifungal	3/46	6.5%
Anticoagulants	2/46	4.3%
Immunoglobulins	2/46	4.3%
Opioid painkiller	1/46	2.17%

PRES cases after the event, were more likely to be put on long-term oral antiepileptics 73% (90/122), and 65% (65/100) of PRES cases that were given parenteral antiepileptics as part of their event. There were 19.5% (9/46) of PRES cases that were administered benzodiazepines most likely to treat uncontrollable seizures.

There were 56% (26/46) of cases that were receiving antihypertensives as an intervention post PRES event and diuretics were administered in 8.6% (4/46) PRES cases post event.

Other noticeable drug administered is steroids given in 15% (7/46) which can be counterintuitive because steroids can also cause PRES, so might worsen the episode.

There were 6.5% (3/46) cases that were on antifungals post PRES event.

6.3.4.10 Admission to ITU:

There were 57% (72/126) PRES cases that got admitted to ITU for management of their neurotoxic episode.

Table 6.18: Comparison of admission in ITU between PRES and the RONC

Characteristic	PRES		RONC		p value	OR(CI)
Admission in ITU	n	%	n	%		
Total	126		604			
Yes	72	57	225	37	4.67e-05	1(ref)
No	54	43	379	63		2.2(1.5-3.3) *

*Logistic regression analysis

PRES group was 2.2 times more likely to be admitted to the ITU as compared with RONC group when compared with the "no admission required in the ITU". (**Error! Reference source not found.**)

6.3.4.11 Requiring Ventilatory support in ITU:

Of the PRES cases that required admission in ITU, it was observed that almost half of the cases condition was severe enough that required ventilatory support 48% (33/68) and the rest didn't require ventilatory support. Results are shown in the (**Error! Reference source not found.**)

Table 6.19: Comparison of requiring ventilatory support between PRES and the RONC

Characteristic	PRES		RONC		p value	OR(CI)
Requiring ventilatory support	n	%	N	%		
Total	68		143			
Yes	33	48	82	57	0.2921	-
No	35	52	61	43		-

On comparison of PRES cases requiring ventilatory support with the RONC, there was no statistically significant difference between the two groups.

6.3.4.12 ALL therapy modifications in response to neurotoxic events

6.3.4.13 Change in chemotherapy

Of the PRES cases there were 54% (63/116) whose chemotherapy was modified after the event whereas the rest of 46% (53/116) of cases reported to continue having their chemotherapy as per protocol post neurotoxic event.

On comparison of PRES cases with the RONC, there was no statistically significant difference seen between these two groups in chemotherapy being modified after the event.

6.3.4.14 Change in vincristine chemotherapy

It was interesting to explore if the chemotherapy, especially vincristine dose, of the PRES was modified after the event. The information collected was compared with the RONC group and is shown in the **Error! Reference source not found.**

Table 6.20: Comparison of modifications in chemotherapy between PRES and the RONC

Characteristic	PRES		RONC		p value	
Vincristine	n	%	n	%		
Total	35		212			
Continued as per protocol	16	45	181	85.3		1(ref)
Dose reduced	6	17	6	1.4	<0.0001	13.1(3.9-45.8)
With-held	13	37	25	11.7		5.8(2.5-13.7)

PRES cases were significantly more likely to get vincristine administration post neurotoxic event modified, either dose reduced or withheld, when compared with the rest of neurotoxicity. PRES cases were 13.1(3.9- 45.8) times more likely to get the dose of vincristine reduced post event and they were 5.8(2.5-13.7) time more likely to get their vincristine dose completely withheld when compared with the RONC group taking continuation of the vincristine dose as per protocol as a reference.

6.3.5 Aim 5: To investigate the effects of neurotoxicity on the leukaemia outcomes in PRES cases

6.3.5.1 Leukaemia relapse outcome:

There were 13 (13/158 – 8.2%) PRES cases that had leukaemia relapse and 145(145/158 – 91.7%) PRES cases that reported to have no leukaemia relapse for the duration that they were followed up (mean 5.3 years).

There was no statistically significant difference between the PRES group and the RONC group for the leukaemia relapse post event as compared with not having relapse post event on chi square analysis. (PRES, leukaemia relapse 8.2% (13/158) **vs** RONC 10.5% (175/1654), p-value 0.4137)

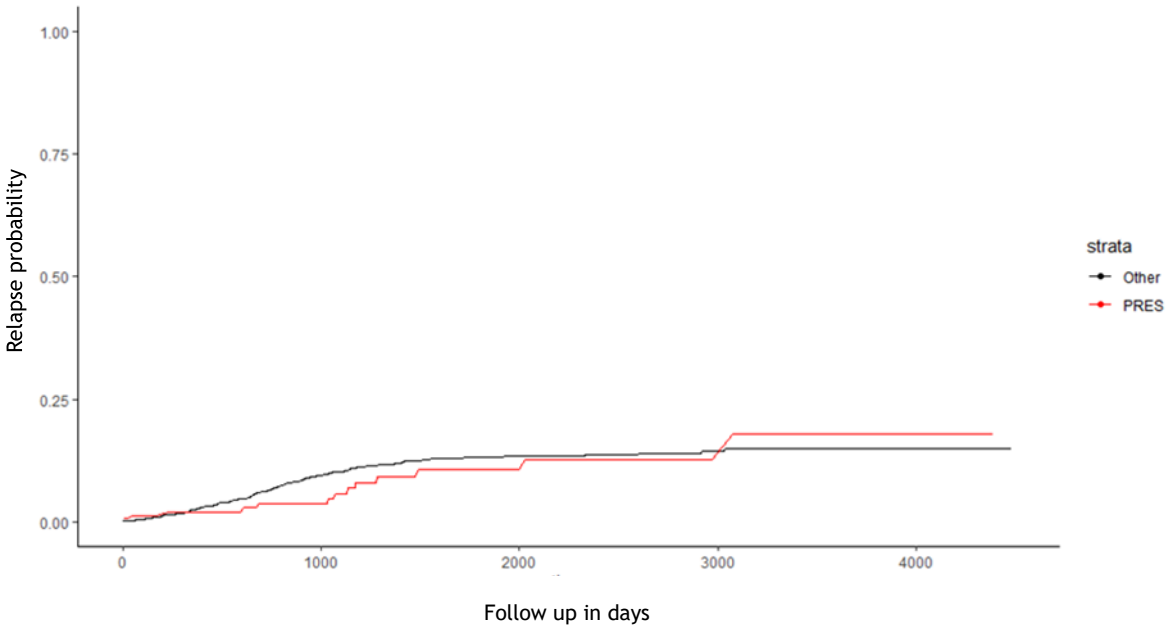

Figure 6.3: Kaplan Meier relapse probability plot

Kaplan Meier calculated the relapse probabilities, on y-axis, of each patient of two different groups, PRES and RONC group, with censoring for relapse or last follow-up, whichever occurred first. Log-regression tests showed no significant association with the two groups.

6.3.5.2 Overall survival:

There were 13 (13/157 – 8.2%) PRES cases that died and 142(142/157 – 90.4%) PRES cases that reported alive, whereas there were 2 cases that were lost to follow up, for the duration that they were followed up (mean 5.3 years).

There was no statistically significant difference between the PRES group and the RONC group when compared for the overall survival post event. (PRES, death events 8.2% (13/142) **vs** RONC 12.8% (191/1484), p value 0.1245)

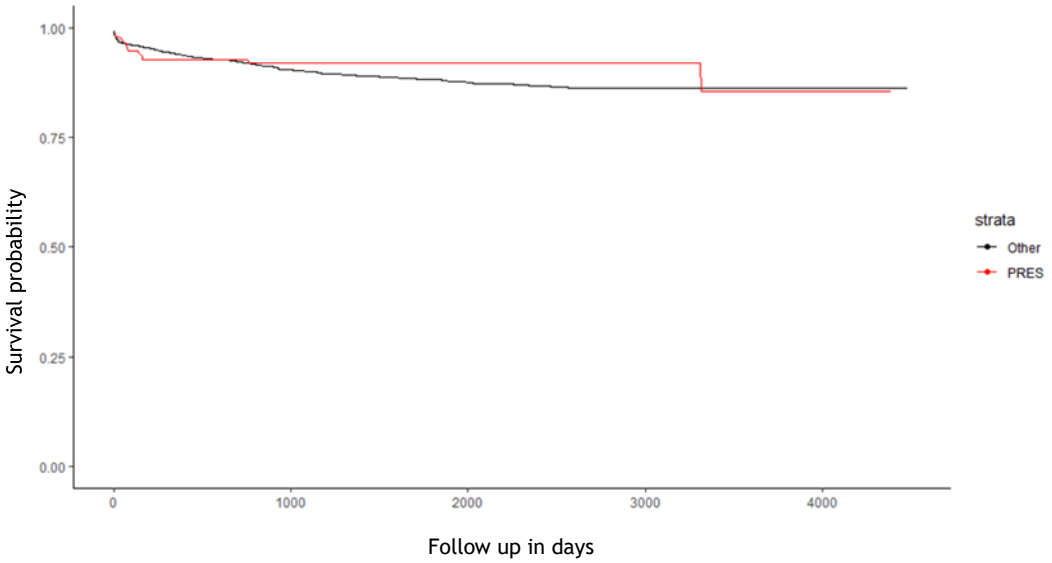

Figure 6.4: Kaplan Meier overall survival plot

Kaplan Meier calculated the survival probabilities, on y-axis, of each patient of two different groups, PRES and RONC group, with censoring for death or last follow-up, whichever occurred first. Log-regression tests showed no significant association with the two groups.

Showing comparison of Overall survival between PRES and the RONC group when followed up in days.

6.4 Discussion:

This is the largest case series of PRES cases that allowed us to make a few interesting findings. Deep phenotyping of PRES cases showed younger age group (< 10 years), T-cell and CNS involvement, high vincristine dose protocol and looking at the neurological outcomes-use of oral antiseizures were significantly associated with PRES.

A variety of symptoms that can be attributed to PRES was reported; in most cases, more than one symptom was experienced, as seen in **Error! Reference source not found.**. Observation that hypertension was the most common type of clinical feature in PdL PRES cases is because these cases were picked with differential diagnostic scoring system, which agrees with previous studies that found hypertension to be associated with PRES [59]. Increased blood pressure was also a major presenting feature of PRES reported in previous studies [49,158,159]. Seizures were reported as the second most common presenting symptom, which was also reported previously. [49,59,160] Headache and visual field disturbances were reported in 38% and 34% of PRES cases in our PdL study, similar to what has been reported by others [49], and are most likely linked with the high blood pressure.

Vincristine-induced toxicities can occur as a mix of polyneuropathies, affecting both sensory and motor systems, so it is not surprising that 35% of PdL PRES cases reported to have constipation. Constipation has been shown associated with PRES by Banerjee *et al.*.[59] Constipation is most likely caused by Vincristine's adverse effects involving sensory pathways and can take severe form as paralytic ileus.[161] Sensory disturbances, observed in 11% of PRES cases, are also likely due to vincristine's side effects on the sensory system and consistent with observations made in earlier studies [49].

Hyponatremia just seen in 6% of PdL PRES cases, is reported in previous case series [59,162,163] as one of the presenting features of PRES induced by vincristine. The hypothesized mechanism of

hyponatremia-induced encephalopathy in hospitalized patients is that, with hyponatremia and a hypotonic environment, the movement of water through water channel aquaporins (AQP4) is increased, causing swelling of the glial cells.[164]

Most of the events were reported in the initial phase of the therapy, due to the administration of vincristine and steroids in that phase, shown in Figure 6., which is consistent with the findings of other studies where PRES was reported mostly during the initial/induction phase of chemotherapy [49,165].

This study identified younger age group (< 10 years), T-cell and CNS involvement at the time of ALL diagnosis as independent variables significantly associated with PRES when compared with the RONC. (**Error! Reference source not found.**,**Error! Reference source not found.**)

The finding that the younger age group (<10 years) was found to be at increased risk of PRES compared with the older age group (>10 years), is in contradiction with the older age group reported as being at higher risk by Anastasopoulou *et al*. [49] and also the findings from our own comparison with the UKALL 2003(**Error! Reference source not found.**). The main explanation for the contradictory finding that the Anastasopoulou study comparator group was patients without neurotoxicity; possible reasons for this contradiction in detail are speculated below:
a) The NOPHO study was a population-based study, and the analysis was run between PRES and no PRES cases in paediatric ALL patients in a trial, but in the PdL study the analysis is between PRES group and the "RONC", therefore the finding might be driven by the cases in the "RONC". In this case, it might be because of the SLS group that is known to be associated with older age.
b) The NOPHO study under discussion had smaller number of cases while in the PdL study there was a larger PRES group; it is possible that in an analysis of a larger group of PRES, with almost 60% of the neurotoxicity occurrences in a younger (<10 years) age group, younger age (<10 years) shows association with PRES.
c) There was a difference in inclusion age criteria between the two studies. As the NOPHO study's inclusion age criteria was between 1-18 years, but in PdL study we had cases with age 0-29 years, it is plausible that the extended age range might affect the association.

T-cell immunophenotype showing significant association with PRES when compared with the RONC is consistent with previous findings [49]. T cell immunophenotype leukaemia are reported to have more inflammatory response compared with B cell immunophenotype, which can lead

to the increased inflammatory response resulting in endothelial injury and brain interstitial oedema [131]. There have also been reports of T-cell tumorigenic signalling pathways driving glucocorticoid resistance, which might lead to increased glucocorticoid toxicity, hypertension, and PRES [166,167].

Presence of CNS leukaemia at the time of diagnosis showed significant association with PRES as is also reported by others.[49] This might be because the blood-brain barrier is disrupted by the presence of leukaemic cells, making it susceptible to endothelial injury, directly or through cytokines (e.g., tumour necrosis factor-α, interleukin-1, and endothelin-1), which could result in PRES [131].

The findings of T-cell immunophenotype and CNS leukaemic involvement is consistent with previous findings that, compared to B-cell immunophenotype, the T-cell immunophenotype ALL cases have higher frequency of blast cells in cerebrospinal fluid, due to CNS disease status 2 (<5 leucocytes per μL with blasts) or CNS3 (≥5 leucocytes per μL with blasts) at diagnosis [120].

Previous research has shown association of PRES with high-risk treatment regimen allocation [49], but we were not able to see this association in the PdL study, which might be explained by the fact that there were study groups that contributed neurotoxicity cases just from high risk treatment protocols such as COG ALL 0434.

The observation made regarding the association of the induction phase of treatment with PRES (**Error! Reference source not found.**), owing to the administration vincristine and steroids in induction phase, is along the lines with what has been reported by previous studies, where most of the PRES events were during the induction phase of treatment [49,59,72,165].

PRES or central neurotoxicity has been linked with the administration of vincristine.[168,169] Accidental overdosage was demonstrated to cause vincristine toxicity by one case series.[170] Accidental intrathecal administration is almost always fatal and recommendations to avoid accidental intrathecal administration for the ALL protocol guidelines were provided by Fernandez et al. [171] Vincristine is mostly administered during the Induction phase but is also administered spread all over the later therapy phases such as interim maintenance I & II, delayed intensification I & II and maintenance, e.g., in UKALL 2003, BFM 2009 and COG 0331 protocols.

The use of Vincristine within four weeks of the event showed significant association with PRES in PdL cases, even after the correction for Induction phase of the therapy. (**Error! Reference source not found.**,**Error! Reference source not found.**)

Vincristine dosage can be variable in different protocols and the higher the dose of vincristine, the more incidence of PRES has been reported.[59] As the PdL database was a collection of neurotoxicity cases from different trial protocols, there was some heterogeneity in drug dosages. The association between PRES and higher dose of vincristine, shown in **Error! Reference source not found.**, is consistent with what has been reported [59].

The administration of steroids within four weeks was associated with PRES cases in our study, shown in **Error! Reference source not found.**. The reason for this can be because the use of steroid increased the occurrence of hypertension during the Induction phase, as reported by others [165]. The association of PRES with dexamethasone in comparison with prednisolone, reported by Anastasopoulou *et al.*,[49] was not observed in our PdL study. The lack of association of PRES PdL cases with dexamethasone can be explained by the Steroid psychosis cases (103/722 cases) included in the comparative RONC group, which showed association with dexamethasone (dexamethasone 83% **vs** prednisolone 7%).

Steroids are mostly administered during the Induction phase but are also used at all phases of therapy such as interim maintenance I & II, delayed intensification I & II and maintenance, e.g., in UKALL 2003, BFM 2009 and COG protocols.

The finding that vincristine and steroid associated with PRES shown previously (**Error! Reference source not found.**) was observed at the same rate, regardless of the timing (early phase vs late phase of the therapy), seen in **Error! Reference source not found.**, shows that PRES can occur outside of the induction phase, therefore clinicians should always be on the lookout for adverse effects whenever vincristine and steroids are administered, regardless of treatment phase.

More than 14 days of alkalinization along with high-dose methotrexate was found to be associated with PRES in one study [2], but in PdL we did not have that information about alkalinization with HDMTX.

We observed no difference in overall survival and leukaemia outcomes between the PRES group and the RONC, Figures 3 & 4, however, Banerjee et al had reported higher relapse rates post PRES event compared to patients with no neurotoxicity [59]. Possible reasons for this increased relapse rate might be:

a) Modification in treatment that might have resulted in higher relapse rate because of suboptimal therapy. In our PdL cohort, the dose of vincristine was withheld or modified in 54% of PRES cases, compared to 13% of RONC cases (**Error! Reference source not found.**); relapse rate and leukaemia outcome were similar in these two groups.

b) Use of liver enzyme-inducing antiepileptic drugs, especially phenytoin, known to be associated with faster antileukemic drug clearance and a higher risk of relapse in ALL [60]. Even though PdL PRES cases were 5.8 times more likely to be put on antiepileptic drugs (

), the information gathered doesn't allow to stratify which type/class of antiepileptics were used. Based on the similar leukaemia outcomes observed between the two groups, PRES and the RONC, it can probably be assumed that options other than phenytoin were administered to treat seizures in this group.

c) Caveats in our data reporting issues such as selected trials, older trials, variable follow-up.

Seizures are the second most common symptom, after hypertension, reported in these PRES cases it was not surprising to observe that the use of oral anticonvulsants was significantly associated with PRES (

), and is consistent with previous studies [59,159].

Even though 100% of cases had hypertension, only half of the cases 56% received treatment for it. It's possible that this was due to transient hypertension during the event, which might resolve on its own without treatment. Close monitoring of blood pressure would be beneficial to provide treatment if necessary.

Interestingly there were only 3 cases that were receiving antifungals at time of the PRES event, which is reduced from the report earlier [59]. One of the limitations was that the information regarding antifungal drug administered was not asked separately in the PdL questionnaire and the information gathered is from the free text box. However, but looking at the very few cases

reported on antifungals, it can be assumed that probably the use of prophylactic antifungals was reduced in protocols included in PdL remarkably after the likely drug interactions of antifungals and vincristine, resulting in increased vincristine toxicity, as reported by a previous study [172].

22% (25/113) of PRES cases experienced recurrence, seen in **Error! Reference source not found.**, which is more than the 10% reported in a cohort of paediatric patients with PRES undergoing a cycle of chemotherapy or HSCT for malignant and non-malignant diseases [159].

Seizures was the most common type of recurrent neurotoxicity which is consistent with what is also reported by previous studies, two-thirds of the patients experienced repeated seizures over the short term [1, 2, 28].

Given that seizures were the most common type of recurrent episode, prophylactic administration of antiepileptics could help reduce the risk of secondary events. Although the prophylactic role of oral anticonvulsants is controversial; where it is suggested by some but also has shown not much value by Zama *et al*. [159]

Use of antihypertensives post event might also have reduced the number of recurrent episodes for the PRES spectrum (including PRES, seizures, and encephalopathy).

The PRES cases in PdL were 2.2 times, 57 %, more likely to get admitted in the ITU as compared to the RONC is higher than the 28% reported by Zama *et al*. [159] which can be explained by the fact that later study is a cohort of paediatric patients with PRES for malignant and non-malignant diseases with high variability in drug dosages. Out of those PdL PRES cases admitted in ITU, 50% required ventilatory support (**Error! Reference source not found.**, **Error! Reference source not found.**).

There was one case reported to have died of complications due to PRES and there was no other evidence of other factors that might have contributed to the death. It is an adverse outcome that is rare but has been reported by other studies [159].

Conclusion:

PRES cases were more likely to occur during induction phase, in T-cell and CNS involvement, high vincristine dose protocol and require supportive care in the form of use of oral anticonvulsants.

7 Characterization of SLS

7.1 Background

SLS (Stroke-Like Syndrome), also described as subacute methotrexate encephalopathy, is a well-recognised methotrexate-associated condition. The reported incidence of SLS varies from <1%-3% [61,62] and tends to vary according to the scheduling and intensity of methotrexate and co-administration of other agents such as cytarabine and cyclophosphamide.[61]

SLS is characterised by focal pyramidal weaknesses or hemiparesis, often accompanied with disturbances in speech, with a classical waxing and waning pattern of these symptoms over the course of hours to days. CT scan is usually normal; however MRI shows periventricular white matter changes on DWI and FLAIR [63-65]. SLS occurs within three weeks of methotrexate administration and in most of the cases tends to resolve completely, although long-term deficits in some may occur [57]. One of the potentially intervenable mechanisms to reverse the neurotoxic effect of methotrexate is the accumulation of homocysteine and its metabolites, which has an excitatory effect on the N-methyl-D-aspartate receptor (NMDA). These changes can be managed and used for prevention of recurrence by dextromethorphan, a non-competitive antagonist to NMDA receptor [67-69], or aminophylline [70](an adenosine antagonist). Both drugs have shown promise in small case series, but larger clinical studies are needed. Older age group (more than 10 years) has been identified as a risk predictor, but only in small case series [61,62]. There is also lack of clarity regarding neurological outcomes for safety to re-expose to methotrexate post event, especially in the light of a recent study that reported higher CNS relapse rates in methotrexate-induced neurotoxicity after modification in the ALL treatment [57]. Effect of heterogeneity in methotrexate administration schedule and intensity in different protocols on

occurrence, severity, disease pattern and impact on neurological outcomes are largely unknown.

7.2 Aims:

1) To deep phenotype the re-classified SLS cases
2) To identify the differences and similarities in demographic and clinical features of SLS compared to the rest of the neurotoxicity cohort
3) To identify any differences in occurrence of SLS and effects on neurological outcomes of SLS between different treatment protocols.
4) To investigate the effects of neurotoxicity on the leukaemia outcomes in SLS cases
5) To explore the effects of neurotoxicity on neurological outcomes in the SLS group

7.3 Results:

Cases:

In the Ponte di Legno neurotoxicity clinical cohort, there were 45 cases that were identified as the re-classified definite SLS cases with the help of differential diagnostic scoring system (explained in detail in the chapter 2). And there were 286 cases in this cohort including all the cases that were classified as definite SLS, probable SLS, and possible SLS.

Table 7.1: Showing number of Definite, probable, and possible SLS cases re-classified based on DDSS

Classified as	n	Differential diagnostic scoring system (DDSS) scoring
Definite SLS	45	=>3
Probable SLS	102	=2

Possible SLS	139	=1

For the purpose of analysis, the group with definite and probable SLS cases was taken only. It will be referred as SLS cases or SLS PdL (Ponte di Legno) from here onwards. Whereas, due to the lack of "no neurotoxicity" control group the Rest Of the Neurotoxicity Cohort (RONC, n= 1666) was taken as the comparative group.

7.3.1 Aim 1: To deep phenotype the SLS cases

7.3.1.1 Demographic and clinical features:

7.3.1.2 Pre-existing neurological conditions:

In terms of having a pre-existing neurological condition there were four cases with ADHD, three cases with down syndrome and one patient had epilepsy.

7.3.1.3 Age:

The 147 SLS cases, at the time of ALL diagnosis, ranged in age from 2to 19.3 years old with a median age of 8.65 (IQR 6.35).

Most cases were in the middle age group 10-15 years (59/147 – 40.1%) followed by younger (in <10 years) age group (50/147 – 34%) almost accounting for one third of the group and then followed by age group age group >15 years (37/147– 25%).

7.3.1.4 Gender:

SLS cases were observed more in the females (79/147 – 54 %) than in the males (68/147 – 46%).

7.3.1.5 ALL Immunophenotype:

SLS cases were reported to predominantly have the B-cell immunophenotype of leukaemia 83% (122/147) as compared to the T-cell immunophenotype 17% (25/147) at the time of ALL diagnosis.

7.3.1.6 White blood cell counts:

At the time of their ALL diagnosis, most of the cases, 81% (119/147) had a white cell count less than 50x109/L, whereas the rest of the cases 19% (28/147) had white cell counts more than 50x109/L.

7.3.1.7 Karyotype:

Out of the 107 patients for which the karyotype was reported, 16 cases were reported with t(12;21) translocation (favourable prognosis), followed by 15 cases that had high hyper-diploidy (favourable prognosis) at the time of ALL diagnosis, and followed by, 3 cases with good prognosis without specifying the karyotype and 2 had MLL-rearranged (poor outcome) acute leukaemia with t(4;11)(q21;q23). However, there were 71 cases that were reported to have "Other karyotypes" entailing t(7;9), del 12p, t(9;18), iAMP21, t(1;19), hypodiploidy, as well as complex cytogenetics of uncertain origin.

7.3.1.8 CNS status

The majority of the cases, 82% (121/147), had no leukaemic cells present in the CNS at the time of diagnosis, followed by 10% (15/147) of cases that had CNS 2 status (<5 leukocytes/µl CSF with blasts) at presentation and 7.4% (11/147) had CNS 3 status (≥5 leukocytes/µl with blasts or signs of CNS involvement). Whereas there were 5% (7/147) cases reported with traumatic lumbar puncture (TLP) with blasts and 1 case of TLP without blasts.

7.3.1.9 Timing of event

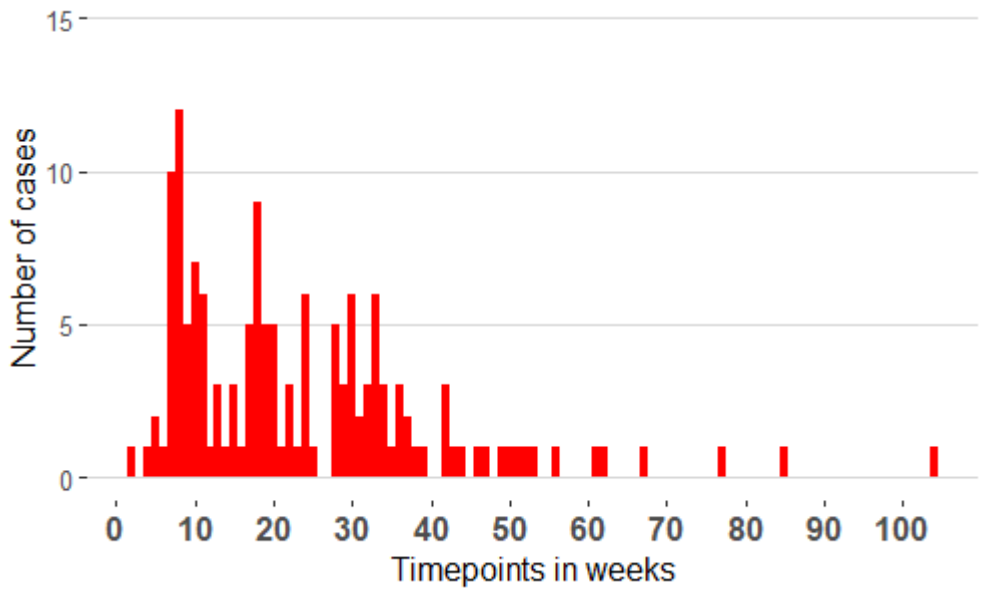

Figure 7 Error! Use the Home tab to apply 0 to the text that you want to appear here.**1: Bar-graph of timing of events of SLS.**

The bar graph shows the number of time points from the beginning of ALL treatment to the occurrence of neurotoxic event with number of SLS cases.

Figure 7**Error! Use the Home tab to apply 0 to the text that you want to appear here.** shows the timing of SLS events during ALL treatment from the start of therapy. The SLS events peak between weeks 8-10 weeks and occur throughout the mid-phase (consolidation, delayed intensification I&II and interim maintenance I&II), while there were few cases occurring late in the treatment phase, with the last case reported at week 104.

7.3.1.10 Clinical presentation of the SLS cases:

Table 7.2: Clinical characteristics and lab findings of SLS cases

Clinical symptoms	Number of patients	Percentage

		(Out of the total responses for that category)
Focal weakness	137/145	94.4%
Dysphasia	122/141	86.5%
Waxing and waning of symptoms	66/90	73.3%
Altered mental status	53/135	39.2%
Ataxia	39/112	34.8%
Dyspraxia	36/107	33.6%
Symptoms non-specific to SLS		
Sensory disturbances	38/109	34.8%
Headache	29/96	30.2%
Visual disturbances	9/109	8.2%
Hypertension	5/91	5.4%
Constipation	4/81	4.9%
Signs		
Infection	9/120	7.5%
Lab investigations		
Hypoglycaemia	2/13	15.3%
Raised CSF homocysteine	0/6	0

Of the 147 SLS cases, the most common symptoms were pyramidal focal weakness, waxing and waning of symptoms and dysphasia as these were part of the differential diagnostic scoring system, with which these cases were picked from the database. Out of the responses obtained, the most common symptom reported was focal pyramidal weakness in 94.4% (137/145) out of which 46.2% (50/108) lasted for less than twenty-four hours, and equally in 46.2% (50/108) duration of focal weakness lasted for less than seven days and only 7.4% (8/108) had symptom for longer duration and lasted for more than seven days.

After focal weakness, dysphasia was the most common type of neurological symptom observed in 86.5% (122/141). Out of which most of the cases ,48.9% (46/94), had their dysphasia resolved in less than twenty-four hours, then followed by in 42.5% (40/94) lasted for the duration of less than seven days, and in 8.5% (8/94) was more severe and lasted for more than seven days.

Waxing and waning pattern of the neurological symptoms, classical presentation of SLS, was observed in almost two-thirds of the cases, 73.3% (66/90).

Altered mental status, as part of the SLS episode, was observed in 39.2% (53/135) where the confused state most lasted for less than seven days in 62.8% (22/35), followed by symptoms lasting for less than twenty-four hours in 28.5% (10/35) and just in 8.5% (3/35) for more than seven hours.

Out of the responses obtained, 34.8% (39/112) of cases had ataxia as part of their SLS event, in 48.5% (17/35) of cases this lasted for less than seven days, followed by in 45.7% (16/35) the symptoms lasted for less than twenty-four hours and in only 5.7% (2/35) the ataxia lasted for more than seven days.

Dyspraxia was a presenting symptom in 33.8% (36/107) of cases. In most of the cases, the dyspraxia lasted in 53.3% (16/30) for less than twenty-four hours and in 46.6% (14/20) of cases lasted for more than seven days.

Additional symptoms that are not part of the classical presenting symptoms of SLS were also seen, sensory disturbances were reported in 34.8% (38/109), headache in 30.2% (29/96), and visual disturbances in just 8.2% (9/109), hypertension was reported in 5.4%(5/91) and constipation in 4.9% (4/81) of SLS cases.

There were 9 cases 7.5% (9/120) reported to have an infection along with the ongoing SLS event.

Looking at the lab investigations reported, there were 2 cases with low blood sugar levels at the time of the event. However, there was very limited information regarding homocysteine levels at the time of event.

7.3.1.11 Para-clinical findings in SLS cases:

Table 7.3: Radiological and EEG features of SLS cases

Para-clinical findings	Number of cases	Percentage

		(Out of the total responses for that category)
CT scan findings		
Normal	10/13	76.9%
MTX encephalopathy	3/13	23%
MRI findings		
Leukoencephalopathy	58/90	64.4%
Consistent with SLS	30/90	33.3%
Normal scan	2/90	2.2%
Diffusion Weighted Imaging (DWI)		
DWI hyperintense	11/21	52.3%
DWI hypointense	6/21	28.5%
DWI normal	4/21	19%
T2 FLAIR MRI findings		
T2 FLAIR hyperintense	15/25	60%
T2 FLAIR normal	9/25	36%
T2 FLAIR hypointense	1/25	4%
ADC MRI findings		
ADC hypointense	7/10	70%
ADC hyperintense	3/10	30%
EEG findings		
Generalised abnormality	12/21	57.1%
Focal abnormality	7/21	33.3%
Other abnormal finding	2/21	9.52%
Persistent radiological findings	15/65	23%

It was observed, out of the responses obtained, that in almost three-quarters of the SLS cases the CT scan findings were reported as normal (76%-10/13) whereas the rest of the cases had CT scan findings consistent with MTX encephalopathy.

Looking at the MRI findings, Leukoencephalopathy (with white matter changes) was the most common type of presentation, reported in 64.4% (54/90), followed by 33.3% (30/90) reports consistent with SLS (Ischemia/Infarct/Oedema/SLS) and 2 cases with normal MRI scan. Out of the responses obtained, frontal region (93.3%- 14/15) was the most common area with changes,

followed by parietal region (80% -16/20), then by basal ganglia (36.6%-4/11), then by temporal (18.1%-2/11), then in cerebellum region (18.8%-2/11) and then in the occipital region (10%-1/10). Hyper-intense diffusion weighted imaging (DWI) was reported in the majority of cases 52%, out of the responses obtained, followed by hypointense DWI reported in 28.5% of cases, whereas there were four cases reported to have normal DWI MRI reports. The cases reported to have Fluid-Attenuated Inversion Recovery Imaging (T2 FLAIR imaging) done, the most common abnormality reported was hyperintense (60%), while there were (36%) of cases with hypointense and one case with normal T2 FLAIR findings. Apparent diffusion coefficient (ADC) mapping hypointense, out of the responses obtained, was observed in 70% of cases.

Observing the Electroencephalogram (EEG), of the SLS cases, generalised abnormality (57%) was the most common presentation, whereas 33.3% had focal abnormalities.

Looking at the long-term persistent radiological findings of these SLS cases, 23% (15/65) cases had persistent radiological findings consistent with leukoencephalopathy at their last follow-up. (**Error! Reference source not found.**)

7.3.1.12 Overall treatment strategies post-event:

Table 7.4: Treatment administered after SLS event

Group of the drug administered	Number of patients	Percentage (Out of the total responses for that category)
Treatment for recovery		
Aminophylline	12/89	13.4%
Leucovorin/Folinic acid	6/53	11.3%
Dextromethorphan	6/86	6.9%
Oral antiepileptics	7/94	7.4%
Parenteral antiepileptics	8/93	8.6%
Aspirin	2/53	3.7%
Antibiotics	3/53	5.6%
Benzodiazepines	2/53	3.7%
Steroids	5/53	9.4%
Antivirals	6/53	11.3%

Diuretics	1/53	1.8%
Anticoagulants	10/53	18.8%
No other drugs specified	8/53	15%

Treatment for recovery:

Post-event the SLS cases, out of the responses obtained, 12 cases (13.4%- 12/89) were administered aminophylline, followed by leucovorin given to 6 cases (11.3%-6/53) and dextromethorphan was administered to 6 cases (6.9%- 6/86) to promote the recovery from the episode. (**Error! Reference source not found.**)

Use of oral anti-epileptics (7.4%-7/94) and parenteral anti-epileptics (8.6%-8/93) in SLS cases is surprising as none of these cases had seizures as part of their presentation.

7.3.1.13 Effect of administration of aminophylline on resolution on recovery

To explore the effects of administrating aminophylline post SLS event, a) neurological symptoms completely resolved b) time to recovery (one week vs more than one week) and c) persistent radiological findings at the last follow-up d) secondary neurotoxicity were examined.

Table 7.5: Comparison of SLS cases for administration of aminophylline to explore the impact on neurological outcomes.

Characteristic	**SLS cases administered aminophylline**		**SLS cases not administered aminophylline**		**p value**	**OR (CI)**
Neurological symptoms completely resolved	**n**	**%**	**n**	**%**		
Total	12		74			
Yes	11	91.6	69	93.2	0.8*	0.7(0.1-16) *
No	1	8.4	5	6.7		1(ref)
Time to recovery less than one week						
Total	11		65			

Yes	11	100	57	87.6		
No	0	0	8	12.3		
Persistent radiological anomalies at the last follow-up						
Total	8		48			
Yes	5	62.5	8	16.6	0.01*	8.3(1.7- 47.9) *
No	3	37.5	40	83.3		1(ref)
Secondary neurotoxicity						
Total	8		45			
Yes	1	12.5	8	17.7	0.7*	0.6(0.03-4.5) *
No	7	87.5	37	82.2		

*Logistic regression analysis

Out of the responses obtained, the cases that were administered aminophylline in the SLS group, most of the cases 91% of cases had their neurological symptoms completely resolved within a week's time without any residual symptoms.

There was no case that had prolonged resolution of neurological symptoms. However, almost 62% of cases had persistent radiological findings at the last follow up. In the SLS group, when the cases that were administered aminophylline post event and those who were not, compared for the neurological symptoms resolved, and the time to recovery less than one week, there was no statistically significant difference observed. Although, the cases administered aminophylline were 8.3 times more likely to have persistent radiological findings. However, the occurrence of secondary neurotoxicity in the SLS cases administered aminophylline to the SLS cases that didn't, there was no statistically significant difference observed between these two groups.

7.3.1.14 Effect of administration of dextromethorphan on recovery

Dextromethorphan, a non-competitive antagonist to NMDAR, is administered for its probable effect in reversing the neurotoxic effect and the prevention of secondary neurotoxicity. In order

to investigate whether dextromethorphan can aid in the recovery process following SLS in this cohort, the variables examined were a) neurological symptoms completely resolved, b) the recovery time (one week vs more than one week) and c) persistent radiological findings at the last follow-up d) Secondary neurotoxicity.

Table 7.6: Comparison of SLS cases for administration of dextromethorphan to explore the impact on neurological outcomes

Characteristic	SLS cases administered dextromethorphan		SLS cases not administered dextromethorphan		p value	OR (CI)
Neurological symptoms completely resolved	n	%	n	%		
Total	5		77			
Yes	4	80	71	92.2	0.3*	0.3(0.04-7.1) *
No	1	20	6	7.7		1(ref)
Time to recovery less than one week						
Total	5		67			
Yes	4	80	59	88	0.6*	1.8(0.08-14.5) *
No	1	20	8	11.9		1(ref)
Persistent radiological anomalies at the last follow-up						
Total	3		53			
Yes	3	100	11	20.7		
No	0	0	42	79.3		
Secondary neurotoxicity						
Total	4		48			
Yes	2	50	6	12.5	.07*	7.0(0.7-68.3) *

No	2	50	42	87.5		1(ref)

* Logistic regression analysis

The SLS cases that were administered dextromethorphan in the SLS group, 80% of the cases had their neurological symptoms completely resolved without any residual symptoms. Most of the cases, 80%, had their recovery from neurological symptoms in less than a week's time and there was only one case, 20%, with prolonged resolution of neurological symptoms. However, there were very few responses obtained for persistent radiological findings at the last follow up and all those cases administered had persistent radiological abnormalities at the last follow up. The SLS group did not administer dextromethorphan post-event to the cases that did, compared for the neurological symptoms resolved, time to recovery less than one week and persistence of radiological findings, there was no statistically significant difference observed. There was also no statistically significant difference observed between the SLS cases that were intervened with dextromethorphan compared with the cases that did not for secondary neurotoxic event.

7.3.2 Aim 2: To identify the differences and similarities in demographic and clinical features of SLS with the rest of the neurotoxicity

7.3.2.1 Comparison of demographic and clinical features of SLS cases with the RONC group

Table 7.7: Comparison of Demographic and clinical features of SLS cases with the rest of the neurotoxicity cases

Characteristic	SLS (n=147)		RONC (n=1660)			OR(CI)
	n	%	n	%	p-value	
Age						
Total	146		1664			
<10 years	50	34.2	995	60	7.31e-09*	1(ref)
>10 years	96	65.7	669	40		2.85(2-4.1) *
Age as continuous variable (Mean)	11.64		8.4		5.54e-15Ψ	
Gender						
Total	147		1664			
Male	68	46	904	54.3	0.0728*	1(ref)
Female	79	54	760	45.6		1.3(0.9-1.9) *
Immunophenotype						
Total	147		1652			
B-cell type	122	82	1347	81.5	0.744*	1(ref)
T-cell type	25	18	305	18.4		9.04(0.5-1.3) *
White cell counts						
Total	147		1660			
<50x10^9/L	119	81	1281	77.1	0.3423*	1(ref)
>50x10^9/L	28	19	379	22.8		0.7(0.5-1.2) *

White blood cell counts as continuous variable (Mean)	43647.91		56860.57		0.1596Ψ	-
CNS involvement						
Total	142		1607			
CNS 1	116	81	1294	80	0.8*	1(ref)
CNS involvement (CNS 2 + CNS 3)	26	19	313	20		0.9(0.5-1.4) *

Ψ Two-tailed t- test analysis

* Logistic regression analysis

Age:

For analysis the cohort was divided into two age categories, less than 10 years old and more than 10 years old.

The older age group (>10 years-65.7% (96/146) of SLS cases), was found statistically significantly (p value 7.31e-09) associated with SLS when compared with RONC 40% (669/1664). The odds of having SLS were 2.85(CI (2-4.1)) times higher in older age group (>10 years) as compared with the RONC

Age taken as a continuous variable was also found statistically significantly associated with SLS compared with age of the RONC.

Gender:

There was no statistically significant difference observed in gender distribution of males and females between the SLS group and the RONC.

Immunophenotype:

SLS group had predominantly B cell immunophenotype (82%- 122/147) and there was no statistically significant difference observed in the B-cell and T-cell immunophenotype distribution between SLS and the RONC.

CNS involvement at the time of ALL diagnosis:

The CNS status at the time of ALL diagnosis was categorized into two categories “No CNS involvement- CNS 1” and “CNS involvement- CNS 2 + CNS 3”. There was no statistically significant difference observed in the distribution of cases with CNS involvement and No CNS involvement at the time of ALL diagnosis between SLS group and the RONC.

7.3.2.2 Comparison of treatment characteristics of SLS cases with the RONC

It was of interest to see if the SLS cases had differences or similarities with the RONC group for the treatment characteristics at the time of ALL diagnosis or allocated later on.

Table 7.8: Comparison of treatment characteristics of SLS with the RONC group

Characteristic	SLS (n=153)		RONC (n=1660)		p value	OR(CI)
	n	%	n	%		
NCI risk group allocation						
Total	145		1658		0.0003*	
Low risk	44	29	763	46		1(ref)
Intermediate/high risk	101	71	895	54		1.95(1.3-2.8) *
Treatment risk group allocation						
Total	144		1494			
Standard risk group	31	22	545	36	6.44e-05*	1(ref)
Medium risk group	70	49	667	45		1.8(1.2-2.8) *
High risk group	43	30	282	19		2.6(1.6-4.3) *
Phase of treatment						
Total	147		1538			
Induction	11	7	455	29	-	1(ref)
Consolidation	33	22	235	15	8.48e-07	5.8(2.9-12.2) *

Consolidation with HDMTX	40	27	138	9	2.28e-12*	11(6.19-25.13) *
Maintenance	8	5.2	146	9.5	0.0845	2.2(0.8-5.7) *
Other	55	37	564	36	3.36e-05	4.03(2.1-8.2) *

*Logistic regression analysis

NCI risk allocation:

It was observed that in SLS cases, 71% of cases were initially allocated to Intermediate/high risk regimen at the time of ALL diagnosis and were found statistically significantly associated with SLS. The odds of having SLS were 1.95 times higher than RONC. (**Error! Reference source not found.**)

Risk group allocation:

The cases allocated high risk treatment regimens (30%-43/144) were identified as significantly associated (p value 0.0002) with having SLS. The odds of having SLS in high risk allocated group were 1.62(OR CI (1.62(1.2-2.06)), followed by odds of medium risk 1.8 (1.2-2.8), times higher, as an outcome when compared with the standard risk regimen allocated.

Phase of treatment:

Almost 27% (40/147) of SLS episodes occurred during the consolidation with HD-MTX phase of therapy followed by 22% (33/147) who had the event during consolidation, 7% (11/147) during their induction phase and 5.2% (8/147) during maintenance phase of therapy. There were 37% (55/147) in the "Other" treatment phase group including delayed intensification, interim-maintenance, and re-induction phase.

It was identified that SLS cases on comparison with the RONC group, that consolidation with HDMTX phase of therapy showed the most significant association with SLS, 27% (40/147) vs 9% (138/15238), p value 2.28e-12. The odds of having SLS episode during the consolidation with HD MTX phase were 11 times higher (11(6.19-25.13)). Followed by which, the consolidation

phase of treatment showed significant association with SLS cases, where cases were 5.8(2.9-12.2) times more likely to have an SLS during consolidation phase as compared with the RONC. The cases that were in the "Other" phase of therapy, which comprises of delayed intensification I &II and interim maintenance I&II phases of therapy depending on the protocol, the odds of having an SLS episode were 4.03(2.1-8.2) times more when compared with the RONC group. (**Error! Reference source not found.**)

Multivariate logistic regression analysis:

The clinical and demographic characteristics combined, that showed significant association with SLS, were fitted in a multivariate logistic regression model to identify the variables that are associated independently.

Table 7.9: Multivariate regression analysis of the variables showing significant association with SLS when compared with the RONC

Characteristics	p value	OR	CI
Age			
<10 years		1(ref)	
>10 years	0.000417*	4.6*	2.13- 12.01*
NCI risk			
Low risk group		1(ref)	
Intermediate/high risk	0.08*	0.45*	0.16- 1.04*
Treatment risk group allocation			
Standard/Medium risk		1(ref)	
High risk group	0.06*	1.5*	0.96- 2.6*
Phase of treatment			
Rest of the phases of treatment		1(ref)	
Consolidation with HD MTX	1.07e-08*	3.4*	2.2- 5.1*

* Logistic regression analysis

Older age group > 10 years and the consolidation with HD MTX phase of treatment showed significant association with SLS when compared with the RONC.

7.3.2.3 Comparison of Demographic and clinical features of SLS cases with the UKALL 2003 published cohort

A major limitation of analysis using neurotoxicity controls is the lack of no neurotoxicity control group for comparative analysis and the analysis is done between the different types of neurotoxicity. To investigate whether patients with SLS had distinct demographic or clinical variables compared to children presenting with ALL, the dataset was also compared with information obtained from the UKALL 2003 cohort. UKALL 2003 was a randomised trial for children with acute lymphoblastic leukaemia, where it included all the patients aged one year up to 25 years and recruitment levels were high (>90%) [47,136] therefore the patient characteristics can be considered "typical" of childhood ALL.

Table 7.10: Demographics of SLS cases comparison with UKALL 2003 cohort

Characteristic	SLS (n)	SLS (%)	UKALL 2003 cohort (n)	UKALL 2003 cohort (%)	p value
Total	147		3113		
Age at diagnosis (years)					
Less than 10 years	102	65	2279	73	
10-15 years	44	28	501	16	.0001*
>15 years	9	5.8	333	11	
Gender					
Male	88	55.6	1767	57	0.79*
Female	70	44.3	1346	43	
Immunophenotype					
B-lineage	111	70	2727	88	0.00001*
T-lineage	47	30	386	12	
WCC (x109/L) at diagnosis					
<50 ($x10^9$/L)	109	69.5	2428	78	.012*
>50 ($x10^9$/L)	48	30.5	685	22	
CNS status at diagnosis					
CNS 1 (no involvement)	103	66.8	2713	87	
CNS 2 (<5/μL)	35	22.7	366	11.6	< 0.00001*
CNS 3 (>5/μL) by cytology	16	10.3	34	1	

Treatment risk group allocation					
Standard risk	31	22	1537	49	
Medium risk	70	49	842	27	< 0.00001*
High risk	43	30	734	24	

* Logistic regression analysis

The comparison showed that there was difference seen in age groups (<10 years, 10 -15 years and >15 years). SLS cases show higher proportion of cases in older age groups (10-15 years) and lower in >15 years when compared with the age groups of ALL patients reported in the UKALL 2003 cohort, indicating that older age ALL was more likely to get SLS as shown in (**Error! Reference source not found.**).

There was no statistically significant difference seen in gender distribution in SLS cases from PdL cohort when compared with ALL patients from UKALL 2003 cohort. (**Error! Reference source not found.**)

T- cell immunophenotype was over-represented in SLS cases from the PdL cohort when compared with the UKALL 2003 cohort but this may be due to the variation in inclusion and exclusion of the trials included in the PdL cohort. In this case where T cell immunophenotype is overrepresented in PdL and is showing a significant association with SLS might be due to the cases that were included in PdL cohort from a trial exclusively for T cell ALL. (COG 0434)

Higher white blood cell (WBC) counts (>50 x 109/L) at the time diagnosis showed higher occurrence and significant association with SLS cases from the PdL cohort as compared to the UKALL 2003 study cohort.

CNS involvement (CNS 2 and CNS 3) were over-represented and showed significant association in SLS cases from the PdL cohort as compared with the CNS involvement (CNS 2 and CNS 3) group in UKALL 2003 cohort.

Medium and Higher treatment risk group were commoner with significant association in SLS cases from the PdL cohort as opposed to standard risk group allocation when compared with the treatment risk group allocations of the UKALL 2003 cohort as shown in Table 10.

7.3.2.4 Drugs administered as per protocol within 4 weeks of treatment:

Table 7.11: Comparison of the drugs administered before the event between the SLS cases and the RONC

Characteristic	SLS (n=147)		RONC (n=1660)		p value	OR(CI)
	n	%	n	%		
Methotrexate administration four weeks prior to the event						
Total	147		1046		0.0023*	6.3(9.3-1.53)
Yes	147	100	979	94		
No	0	0	67	6		
Routes of administration						
Total	134		875			
Intravenous <1g/m2	1	0.7	11	1.2		
Intravenous >1g/m2	4	2.9	6	0.6		
Intrathecal	98	73.1	666	76.1	-	
IT &IV<1 gm/mm2	3	2.2	64	7.3		
IT & IV >1 gm/mm2	27	20	95	10.8		
IT & oral	1	0.7	33	3.7		
Mercaptopurine administered four weeks prior the event						
Total	128		743			
Yes	113	88	362	49	<0.0001*	7.9(4.6-14.3)*
No	15	12	381	51		
Cyclophosphamide administered four weeks prior to the event						
Total	130		738			
Yes	90	69	247	33.4	<0.0001*	4.4(3-6.7)*
No	40	30.7	491	67.5		
Cytarabine administered four weeks prior to the event						
Total	134		862			
Yes	93	69.4	321	37.2	<0.0001*	3.8(2.5-5.7)*
No	41	30.5	541	62.7		

* Logistic regression analysis

SLS cases were picked by the differential diagnostic system and to enter the scoring system for SLS it was a must condition that the cases were had evidence of methotrexate administered four weeks prior to the event. However, it was observed that SLS cases were predominantly administered methotrexate intrathecally, almost 96.2% (129/134 - just intrathecal or with combination with intravenous) as compared with just intravenous route. As opposed to intravenous use of methotrexate which was reported in almost one-quarter (26%-35/134) of the cases (just intravenous or in combination with intrathecal administration). However, this difference didn't show a statistically significant difference when compared with the RONC group.

SLS cases also showed significant association with mercaptopurine, cyclophosphamide and cytarabine, drugs that are administered during the consolidation, interim maintenance, and delayed intensification, when compared with the RONC.

7.3.2.5 Administration of general anaesthesia four weeks before the event

Another possible drug interaction in methotrexate induced neurotoxicity involves the concomitant inhalation of nitrous oxide (N2O) and methotrexate, a practice that is common in paediatric haematology centres that perform lumbar punctures under general anaesthesia in order to deliver intrathecal methotrexate. Nitrous oxide may result in increased homocysteine levels and decreased methionine levels, indirectly by depleting the vitamin B12 levels, both of which can contribute to methotrexate's neurotoxic effects. Although in the PdL database the information gathered was about general anaesthesia administered four weeks prior to the SLS event without specifying if Nitrous oxide was used, it was still worth exploring the effect of general anaesthesia administered on the occurrence of SLS based on assumption that Nitrous oxide might be the most common drug administered.

Table 7.12: Comparison of the SLS cases with the RONC for the administration of general anaesthesia four weeks prior to the event

Characteristic	SLS	RONC	p value	OR (CI)

General anaesthesia four weeks prior to the event	n	%	n	%		
Total	96		556			
Yes	76	79.1	410	73.7	0.2*	1.3(0.8-2.3) *
No	20	20.8	146	26.2		1(ref)

* Logistic regression analysis

It was observed that in SLS cases the use of general anaesthesia, was slightly more, in 79.1% (76/96) of cases, than the RONC, in 73.7% (410/556). However, there was no statistically significant difference observed in the administration of general anaesthesia four weeks before the event between the SLS and the RONC group.

7.3.3 Aim 3: To identify any differences in occurrence of SLS and effects on neurological outcomes of SLS between different treatment protocols:

Ponte di Legno chemotherapy associated central neurotoxicity is a retrospective study which pooled cases reported from different study groups on different trials. There was variation in trial protocols for administering different drugs, dosage, and timing of administration (explained in detail in introduction). For identifying the effect of these variations on incidence and outcomes of the neurotoxicity across different protocols a database of Heterogeneity of protocols was built. Information regarding variations in protocols about 1) methotrexate CNS directed therapy, 2) leucovorin rescue timing, 3) leucovorin dose and 4) triple/single intrathecal therapy, that was relevant to SLS, was obtained from the heterogeneity of protocols database for the analysis below:

7.3.3.1 High Dose Methotrexate (HD MTX) vs Capizzi escalating methotrexate

One of the variations between the trial protocols included in Ponte di Legno study was in the different approaches of administrating methotrexate during the CNS directed in consolidation phase of therapy. Where in Capizzi-style escalating dose (starting at a dose of 100 mg/m2/day, and 50 gm added on every consequent MTX dose) of IV methotrexate is administered without

leucovorin rescue and on the other hand high dose (5gm/m2/day over 24 hrs) of IV methotrexate is administered with leucovorin rescue.

Table 7.13: Comparison of the SLS cases with the RONC group for association with High dose methotrexate and Capizzi escalating methotrexate trials

Characteristic	SLS		RONC		p value	OR (CI)
	n	%	n	%		
Total	146		1636			
High dose methotrexate	108	73.9	940	57.4	0.000137*	2.1(1.4-3.1) *
Capizzi escalating methotrexate dose	38	26	696	42.5		1(ref)

* Logistic regression analysis

It was observed that SLS was statistically significantly associated with high dose methotrexate when compared with the RONC. The odds of having SLS were 2.1 (1.4-3.1) times higher when high dose methotrexate was administered as compared with the Capizzi escalating methotrexate dose, when compared with the ROCN.

7.3.3.2 Intrathecal triple vs single

To intensify the CNS-directed therapy, protocols had varied approaches in administering intrathecal during the maintenance phase of the therapy. One approach was to give single drug intrathecally (methotrexate only) and whereas other was to administer triple drug intrathecally (methotrexate, cytarabine and hydrocortisone). There were few protocols included in PdL that were either administrating Triple IT drugs to all risk groups (DCOG ALL10, LAL/SHOP-2005, LAL-SEHOP-PETHEMA 2013, DCOG ALL 11) or to the high-risk group and/or T-cell immunophenotype (ALL-BFM 95, ANZCHOG ALL STUDY 8, ALL IC-BFM 2002, ALLIC, BFM 2009, CoALL 08-09, NOPHO 2008, ANZCHOG ALL STUDY 7) only, as per protocol.

Table 7.14: Comparison of SLS cases with the RONC for Intrathecal triple drug therapy with single drug therapy protocols

Characteristic	SLS		RONC		p value	OR (CI)
	n	%	n	%		
Total	117		1413			
Intrathecal triple drug	13	13.1	117	11.2	0.2*	1.38(0.7-2.4)*
Intrathecal single drug	104	86.9	1296	88.7		-

* Logistic regression analysis

It was observed that there was no statistically significant difference observed in the group being administered single drug intrathecally and triple drugs intrathecally when compared for the SLS group and the RONC group.

7.3.3.3 Leucovorin rescue early vs late

As leucovorin, a folate analogue, effectively neutralizes methotrexate's effects, the timing for administrating leucovorin after methotrexate administration is crucial. As the leucovorin window cannot be too shortened as it would, on one hand, reduce the chances of toxicity but on the other hand can also affect the desired anticancer results. Cases were stratified in two groups depending on the time from start of methotrexate to first leucovorin dose (hours), as per protocols, 1) Early leucovorin rescue, where leucovorin is administered 24-36 hrs after post methotrexate administration 2) Late leucovorin rescue, where leucovorin is administered 42-48 hrs after methotrexate administration.

Table 7.15: Comparison of the SLS cases with the RONC cases for early leucovorin rescue early with late leucovorin rescue

Characteristic	SLS		RONC		p value	OR (CI)
	n	%	n	%		
Total	107		1211			
Early rescue 24-36 hrs	14	13.1	136	11.2	0.563*	0.84(0.4-1.5) *
Late rescue 42-48 hrs	93	86.9	1075	88.7		-

* Logistic regression analysis

There was no statistically significant difference observed in the cases that were given early leucovorin rescue and late leucovorin rescue when compared for the SLS and RONC groups.

7.3.3.4 Leucovorin low dose vs high dose:

Leucovorin rescue, where it was administered, had a variation in the dose administered in different protocols. To explore the effect of difference in dose of leucovorin on the SLS cases with the RONC group, two groups of cases were identified on the database: a) low dose (5-10 mg/m2) b) high dose (15 mg/m2).

Table 7.16: Comparison of the SLS cases with the RONC cases for leucovorin low dose with high dose

Characteristic	SLS		RONC		p value	OR (CI)
	n	%	n	%		
Total	99		1203			
Low dose leucovorin (5-10 mg/m2)	5	5	293	24.3	0.0001*	1(ref)
High dose leucovorin (15 mg/m2)	94	94.9	910	75.6		6.05(2.6-17.2) *

* Logistic regression analysis

It was identified that high dose (15 mg/m2) of leucovorin rescue showed statistically significant association with SLS when compared with the low dose (5-10 mg/m2) of leucovorin administered. Likely to be a confounder i.e., that the low dose rescue was given in protocols that used low dose MTX. However at least it suggests that low dose rescue does not confer higher risks of MTX SLS.

In summary, High Dose MTX protocols and High dose leucovorin administration showed significant association with SLS.

7.3.4 Aim 4: To investigate the effects of neurotoxicity on the leukaemia outcomes in SLS cases

7.3.4.1 Leukaemia relapse outcome

There were 12 (12/153 – 7.8%) SLS cases that had leukaemia relapse and 141(141/153 – 92%) SLS cases that reported to have no leukaemia relapse for the duration that they were followed up (mean 5.3 years).

There was no statistically significant difference between the SLS group and the RONC group for the leukaemia relapse post event as compared with not having relapse post event on chi square analysis.

(SLS, leukaemia relapse 7.8% (12/153) **vs** RONC 10.6% (176/1659), p value 0.3330)

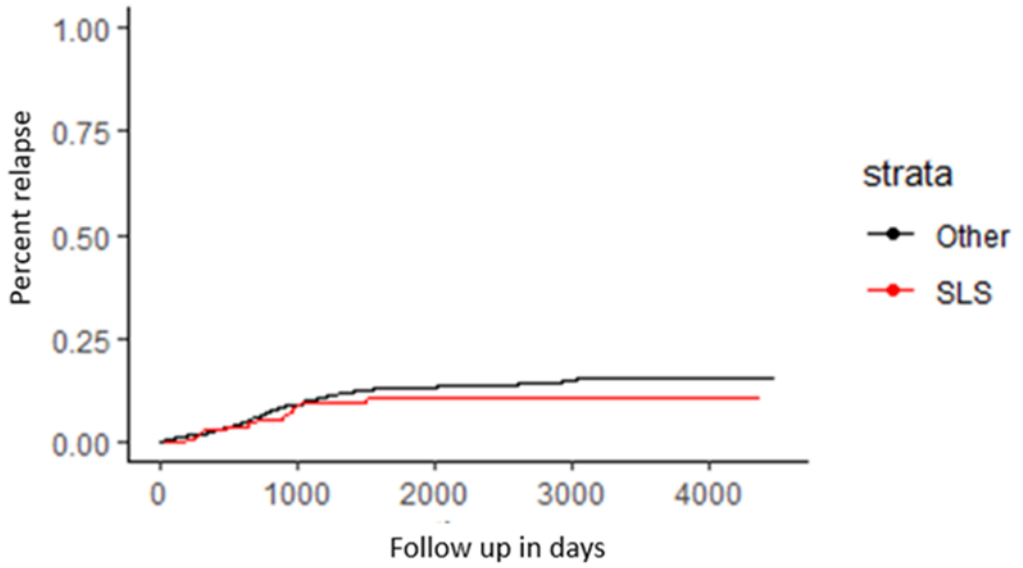

Figure 7.2: Kaplan Meier relapse probability plot, showing comparison of relapse rate between the SLS cases and the RONC group

On further examining the SLS cases that were reported to have a leukaemia relapse, the most common site for the relapse was bone marrow (58.3% - 7/12), followed by isolated CNS relapse reported in 16.6% (2/12) cases, 16.6% (2/12) reported to have combined bone marrow and CNS relapse, and 8.3% (1/12) had relapse in an extramedullary site and bone marrow.

7.3.4.2 Overall survival

There were 14 (14/148 – 9.4%) SLS cases that died and 134 (134/148 – 90.5%) SLS cases that reported alive, whereas there were 4 cases that were lost to follow up, for the duration that they were followed up (mean 5.3 years).

There was no statistically significant difference between the SLS group and the RONC group when compared for the overall survival (OS) post event. (SLS, death events 9.4% (14/148) **vs** RONC death events, 12.7% (190/1491), p value 0.2964)

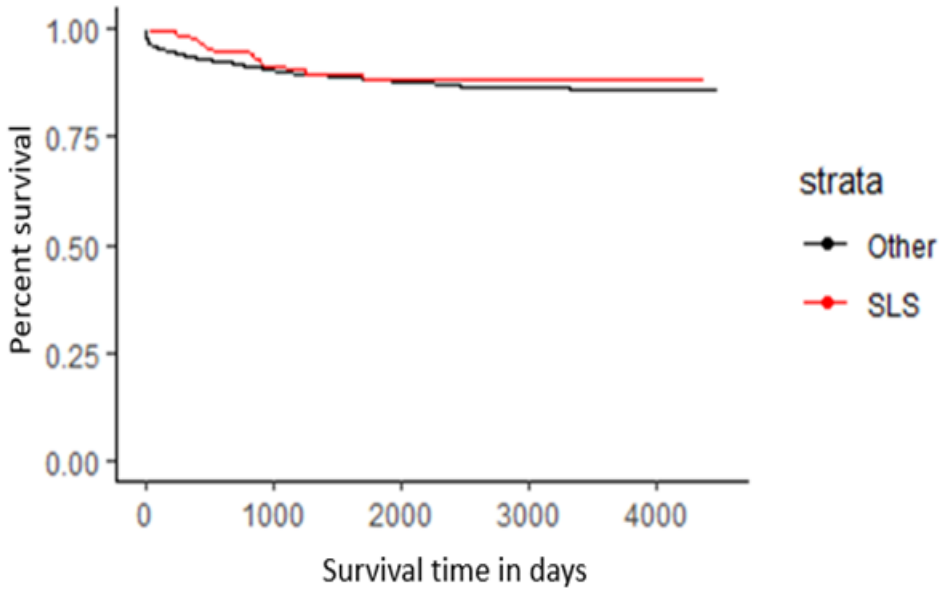

Figure 7.3: Kaplan Meier overall survival plot, showing comparison of Overall survival between SLS and the RONC when followed up in days.

This finding shows that OS is within the expected survival of paediatric ALL in general and suggests that an episode of neurotoxicity does not impact on OS, but limitations of the dataset make this impossible to conclude with any certainty.

7.3.5 Aim 5: To explore the effects of neurotoxicity on neurological outcomes in the SLS group

7.3.5.1 ALL therapy modifications in response to neurotoxic events:

Of the SLS cases there were 51% (53/103) whose chemotherapy was modified after the event whereas the rest of 49% (53/103) of cases reported to continue having their chemotherapy as per protocol post neurotoxic event.

On comparison of SLS cases with the RONC cases, there was no statistically significant difference seen between these two groups in chemotherapy being modified after the event. (SLS, Change in chemotherapy 51% (53/103) **vs** RONC 48% (271/565))

7.3.5.2 Change in Intrathecal Methotrexate (IT MTX) chemotherapy:

The SLS cases were predominantly administered intrathecal methotrexate four weeks prior to the event so it was of interest to see the modifications (dose-reduced or dose with-held) in their IT MTX dose post-event of these cases.

Table 7.17: Comparison of modifications in chemotherapy between SLS and the RONC

Characteristic	SLS		RONC		p value	
IT MTX	n	%	n	%		
Total	57		264			
Continued as per protocol	25	43	116	44	0.9801*	-
Dose reduced	8	15	41	15		-
With-held	24	41	107	40		-

* Logistic regression analysis

On comparison of SLS group with the RONC group, it was identified that there was no statistically significant difference observed between intrathecal methotrexate modified and continued as per protocol.

7.3.5.3 Change in High Dose Methotrexate (HD MTX) chemotherapy:

The information regarding if the chemotherapy was modified post-event was gathered for cases that had 1) continued as per protocol 2) if the dose was reduced/ one dose skipped 3) if the HD MTX was withheld.

Table 7.18: Comparison of modifications in chemotherapy between SLS and the RONC

Characteristic	SLS		RONC		p value	
HD MTX	n	%	n	%		

Total	43		206			
Continued as per protocol	22	43	149	44	0.0066*	1(ref)
Dose reduced	11	15	19	15		4.5(1.89-10.6) *
With-held	10	41	38	40		-

* Logistic regression analysis

It was identified that SLS cases, on comparison with the RONC group, were 4.5(1.89-10.6) times more likely to get their HD MTX dose reduced as compared to the continuation of the chemotherapy as per protocol.

7.3.5.4 Change in IT MTX administration post event and its effect on leukaemia relapse:

The effect of change (dose reduced or dose with-held) in intrathecal methotrexate on the occurrence of leukaemia relapse was examined. Out of the responses obtained for the continuation as per protocol or modification in the intrathecal methotrexate for SLS, the cases that had their intrathecal methotrexate dose modified leukaemia relapse was reported in 9.3% (3/32) which was slightly higher than the group that was administered intrathecal methotrexate as per protocol 3.8% (1/26), although the numbers are small.

On comparison with the RONC group, leukaemia relapse was reported in 12% (18/148) of the cases that had their intrathecal methotrexate dose modified. There was no statistically significant difference observed in leukaemia relapse if the intrathecal methotrexate was continued as per protocol and intrathecal methotrexate modified when compared between the SLS group and the RONC. (SLS, 9.3%-3/23 **vs** RONC,12%-18/148, p value 0.4, OR(CI) 1.3(0.6- 2.5)).

7.3.5.5 Change in HD MTX chemotherapy and its effect on leukaemia relapse:

Table 7.19: Effect of change in HD MTX post SLS on leukaemia relapse

		Leukaemia relapse in SLS					Leukaemia relapse in RONC				
Characteristic	**Total**	**Yes**		**No**		**Total**	**Yes**		**No**		**p value**
		n	**%**	**n**	**%**		**n**	**%**			
HD MTX post event											
Total	43					206					

Continued as per protocol	22	2	9	20	90.9	149	25	16.7	124	83	0.6*
Dose reduced/with-held	21	3	14	18	85.7	57	6	10.5	51	89	

* Logistic regression analysis

The effect on leukaemia relapse if there was a change in high dose methotrexate (dose reduced or dose with-held) of high dose methotrexate was explored. Looking at the responses obtained for the modification in the high dose methotrexate for SLS group, leukaemia relapse was reported in 14.2% (3/21) which was slightly higher than the group that was administered intrathecal methotrexate as per protocol 9% (2/22), albeit the numbers are small.

On comparison with the RONC group, leukaemia relapse was reported in 10.5% (6/57) of the cases that had their high dose methotrexate administration modified. There was no statistically significant difference observed in leukaemia relapse if the high dose methotrexate was continued as per protocol and high dose methotrexate modified when compared between the SLS group and the RONC. (SLS, 14.2%-3/21 vs RONC,10.5%-6/57, p value 0.42, OR(CI) 0.7(0.3 - 1.5))

7.3.5.6 Secondary neurotoxicity:

14 patients (18% - 14/75) had a secondary neurotoxic event, out of the responses obtained, whereas 64 patients (80% - 64/75) did not have a recurrent neurotoxicity episode upon re-exposure to the presumed drug. However, there was no statistically significant difference observed between SLS group and the RONC group in terms of having a secondary neurotoxicity episode on re-exposure to the presumed agents (SLS - (18%-14/75) **VS** RONC (28%-140/499), p value 0.1849)

7.3.5.7 Type of Secondary events in SLS:

Table 7.20: Recurrence in SLS – types of secondary event

Type of Secondary event (Total 14)	Number	Percentage
SLS	6	42%
Encephalopathy	5	35.7%
Unspecified	3	21.4%

The most common type of secondary neurotoxic event upon re-exposure to the presumed causative agent was SLS, observed in 42% of cases, followed by Encephalopathy (35.7% of cases) type of recurrent neurotoxic event and there were 21.4% of cases that had a secondary neurotoxic event, but it's not specified which type of neurotoxic event it was.

7.3.5.8 Admission to Intensive Care Unit (ITU):

Admission required in the Intensive Care Unit (ITU) is a way to assess the severity of the SLS event. It was reported that only 10.6% (11/103) of SLS cases required admission to intensive care unit for the management for their neurotoxic episode.

Table 7.21: Comparison of admission in ITU between the SLS and the RONC group

Characteristic	SLS		RONC		p value	OR (CI)
Admission to ITU	**n**	**%**	**n**	**%**		
Total	103		627			
Yes	11	10.6	286	45.6	3.18e-09*	0.14(.07-0.2)
No	92	89.3	341	54.3		1(ref)

* Logistic regression analysis

On comparison of SLS group with the RONC cases, there was a protective statistically significant difference seen in the SLS cases that didn't require admission in the ITU post event.

7.3.5.9 Requiring Ventilatory support in ITU:

Of the SLS cases that required ITU admission, out of the responses obtained, there were 3 cases (37.5% - 3/8) with severe enough condition that required ventilatory support as part of the management of their neurotoxic episode.

Table 7.22: Comparison of requiring ventilatory support between SLS and the RONC group

Characteristic	SLS		RONC		p value	
Required ventilation	**n**	**%**	**n**	**%**		
Total	8		203			
Yes	3	37.5	112	55.1	0.334*	0.4(0.09-2.03) *
No	5	62.5	91	44.8		1(ref)

* Logistic regression analysis

SLS cases compared with the RONC group requiring ventilatory support or not didn't show any statistically significant difference between the two groups.

7.3.5.10 Duration of ventilatory support required:

One other measure to see how severe the SLS event might be in these cases is to see for how long these cases required the ventilatory support. Of the SLS cases, where the numbers are small, that required ventilatory support only 2 cases required ventilatory support for more than 48 hrs and there was no case that required ventilatory support for less than 48 hrs. Comparison with the RONC group was not possible due to limitation of small numbers in the subgroup.

7.3.5.11 Persistence of neurological symptoms:

There were 16 (11.6% - 16/137) cases that were reported to have long term (more than 1 month to 1 year follow up) unresolved neurological symptoms whereas 121 (88.3% - 121/137) cases whose symptoms were fully resolved after the first event.

Table 7.23: Comparison of persistence of neurological symptoms in the SLS group and the RONC group

Characteristic	SLS		RONC		p value	
Persistence of neurological symptoms	**n**	**%**	**n**	**%**		
Total	137		876			
Yes	16	11.6	169	19.2	0.034*	0.5(0.3-0.9) *
No	121	88.3	707	80.7		1(ref)

* Logistic regression analysis

When compared with the rest of the neurotoxicity cohort (RONC), SLS cases showed a protective association with having persistent neurological symptoms and they were less likely to have unresolved neurological symptoms.

7.3.5.12 Prolonged resolution of neurological symptoms in different protocol groups

Of the SLS cases that had persistent neurological symptoms, the effect of variation in the treatment protocols, the High dose methotrexate or Capizzi escalating dose, on the unresolved neurological symptoms was examined.

Table 7.24: Comparison of persistence of neurological symptoms in the SLS group for Capizzi escalating and HD MTX protocols

Characteristic	Persistence of neurological symptoms in SLS						
	Total	**Yes**		**No**		**p value**	**OR CI**
Protocol		**n**	**%**	**n**	**%**		
Capizzi escalating dose	36	8	22	28	8	0.0289*	3.28(1.1-9.7) *
High dose methotrexate	100	8	78	92	92		1(ref)

* Logistic regression analysis

It was observed that the odds of persisting neurological symptoms in SLS cases on Capizzi escalating methotrexate protocol were 3.28 times higher when compared with those on the high dose methotrexate treatment protocol.

7.3.5.13 Effect of single intrathecal drug vs triple intrathecal on resolution of neurological symptoms from the SLS event

It was of interest to see if there is any difference in the resolution of neurological symptoms between the SLS cases that were administered single intrathecal methotrexate only and the SLS cases that were administered triple drug intrathecal (methotrexate, cytarabine and hydrocortisone) as per protocol.

Of the SLS cases, there were 12.5 % (12/96) cases that were administered single intrathecal and had persistent neurological symptoms post event. On the other hand, there were 15.3% (2/13) of cases administered triple intrathecal with remnant neurological symptoms and there was no statistically significant difference observed in the single and triple intrathecal administered for the unresolved neurological symptoms. (Single intrathecal, 12.5% (12/96) vs triple intrathecal, 15.3% (2/13), p value 0.6727)

7.3.5.14 Impact of early vs late and high dose vs low dose of leucovorin rescue on the persistence of neurological symptoms post event

Leucovorin rescue as per protocol after administering the methotrexate dose could be divided in early leucovorin rescue (24-42 hrs) and late leucovorin rescue (48-52 hrs). Other explorable variation was in dose of leucovorin administered: two groups of cases were identified on the database: a) low dose (5-10 mg/m2) b) high dose (15 mg/m2). The effect of early and late and high dose vs low dose of leucovorin rescue was examined on the persistent of neurological symptoms in SLS cases post event.

Table 7.25: Impact of early vs late & High dose vs low dose of leucovorin rescue on the persistence of neurological symptoms in SLS cases

Persistence of neurological symptoms in SLS	Total	Yes		No		p value	OR CI
Leucovorin rescue window	**97**	**n**	**%**	**n**	**%**		
Early leucovorin rescue	13	1	7.6	12	92.3	1.0000*	-
Late leucovorin rescue	84	7	8.3	77	91.6		-
Leucovorin dose							
High dose leucovorin rescue	86	8	9.3	78	90.6	0.9*	-
Low dose leucovorin rescue	3	0	0	3	100		-

*Logistic regression analysis

Of the SLS cases, the comparison between early vs late and for high dose vs low dose of leucovorin rescue for persistence of neurological symptoms post event, there was no statistically significant difference observed between these two groups.

7.3.5.15 Influence of change in chemotherapy on persistence of neurological symptoms in SLS cases:

The impact of change in chemotherapy (dose reduced/one dose skipped/with-held), for both intrathecal methotrexate and high dose methotrexate dose, on recovery from the SLS event was explored. The table below shows changes in methotrexate (both intrathecal and high dose methotrexate) chemotherapy subset in SLS cases and having the long-term neurological persistent symptoms.

Table 7.26: The impact of change in methotrexate administration (IT MTX and HD MTX) post SLS on long-term persistence of neurological symptoms

	Long-term persistent neurological deficits (more than 1 month-1 year)			
SLS	**Total responses obtained**	**Yes (%)**	**No (%)**	**p value**
Changes in Intrathecal MTX				
Total	54			
Continued as per protocol	24	4 (16.6%)	20(83.3%)	1.000*
Dose reduced/ Skipped one dose/ withheld	30	5(16.6%)	25(83.3%)	
Changes in HD MTX				
Total	40			

Continued as per protocol	21	3(14.2%)	18(85.7%)	1.000*
Dose reduced/ Skipped one dose/ withheld	19	2(10.5%)	17(89.4%)	

* Chi-square test

Out of the SLS cases, looking at the changes (dose reduced/with-held) in intrathecal methotrexate and continuation of intrathecal methotrexate as per protocol, there was no statistically significant difference observed between these for long term persistent neurological symptoms.

Similarly, observing the change (dose reduced/with-held) or the lack of it in administration of high dose methotrexate post SLS event, there was no statistically significant difference observed between the two groups for the unresolved neurological symptoms post SLS episode.

7.3.5.16 Time to resolution of neurological symptoms:

It was of interest to see the time taken in the resolution of neurological symptoms of these SLS cases. Observations are shown in Table 7.27

Table 7.27: Time to resolution of neurological symptoms post SLS event

Time to resolution of neurological symptoms post-event	n	%
Total responses obtained	120	
Less than one week	108	90
From one week to one month	9	7.5
From one month to one year	1	0.8
More than one year duration	2	1.6

The responses obtained for the duration of resolution of symptoms, out of the SLS cases it was observed that 90% of cases had their neurological symptoms resolved within a week's time and 7.5% of cases with the event completely resolved in one week to one month duration. There were only 2 cases (1.6%) of cases that were reported to have unresolved neurological symptoms for more than a year

7.3.5.17 Death due to neurotoxicity

There was no death reported due to SLS neurotoxicity complications, out of the total 10 responses obtained, where the rest was missing data. While on the other hand, there have been 17 deaths reported in the RONC group resulting due to complications in the neurotoxic event.

7.4 Discussion

To the best of our knowledge, the present study is the largest case series of neurotoxicity cases with the largest number of SLS cases reported. This allowed us to identify variables associated with SLS such as older age, consolidation with high dose methotrexate phase of treatment (first time in a multivariate analysis), High risk treatment regimen allocation, High dose methotrexate administrating protocol. The Ponte di Legno database is a retrospective collection of neurotoxicity cases where there was heterogeneity of protocols that allowed to do comparison for variations in treatment protocols for SLS. However, there were some limitations. There wasn't a no-neurotoxicity comparative group available for analysis, and therefore the analysis was run comparing SLS cases with the rest of the neurotoxicity cohort

(RONC). Also, the PdL database is a collaborative collection of retrospective cases from different sources a) clinical notes b) SAE's (Severe Adverse Events) trial databases c) from both clinical notes and SAE's trial databases. Almost 40% of data combined for the PdL neurotoxicity cases was just from the SAE's trial databases; on the one hand, this allowed us to obtain a larger sample size for these relatively rare cases and to be able to find novel findings, but on the other hand there was a limited ability to carry out meaningful analysis on all cases, due to them not always being well-characterised and thus containing missing data. In addition, regarding trial databases of Severe Adverse Events (SAE), it is well-reported that when SAE's were compared with clinical notes there were discrepancies [92,137].

As the cases were picked up by the Differential Diagnostic Scoring System (DDSS), most of the clinical presentations, focal weakness-94.4%, dysphasia-86.5%, Waxing and waning pattern of symptoms-73.3%, altered mental status-39.2%, ataxia-34.8%, and dyspraxia-33.6% reported were expected and are consistent with the literature. [61,62] According to the DDSS, there was no overlap of these SLS (Definite and probable) cases with definite PRES cases, but there was still some overlap with probable and possible PRES; for this reason, these overlapping cases had some clinical features that are associated with PRES and/or vincristine, such as sensory disturbances, headache, visual disturbances, hypertension, constipation reported. (**Error! Reference source not found.**)

As expected, CT scan was reported as normal in most (56%) of the SLS cases and characteristic MRI findings, leukoencephalopathy was observed in most (64%) of the cases, where subcortical white matter changes in areas classically often involving the frontal and parietal areas. The anomalies were generally hyperintense on DWI, hyperintense on T2 FLAIR and hypointense on ADC (**Error! Reference source not found.**), consistent with the literature. [56,64] However, it was observed in a few SLS cases in this cohort that they had enough/strong evidence clinically to be classified as SLS but radiologically they presented with normal MRI presentation in 2 cases, normal T2 FLAIR findings normal in 19% and normal DWI in 36% of cases. The normal MRI presentation was supported by previous studies where according to Rubnitz *et al.*, 8/259 patients developed transient encephalopathy caused by methotrexate (less than 3 days post exposure) with normal MRI in 7/8 cases and leukoencephalopathy in 1/8 cases. In a study reported by Bond et al. 7/28 for the cases MRI available were normal was also observed in SLS cases for which the MRI reports were available [61]. MRI imaging studies have reported abnormal

findings observed in 12/17 but there were also 4/17 cases that were reported as having normal CT and MRI reports [56].

Persistence of radiological findings was observed in 23% of cases in this cohort, out of the responses obtained. Rubnitz et al. reported persistent leukoencephalopathy just in one case (1/8,12.5%) [62]. In contrast, Bhojwani et al. reported leukoencephalopathy in 77% of symptomatic cases until the end of the therapy in overall methotrexate-induced neurotoxicity patients. [138] Although this report is of overall methotrexate induced neurotoxicity and thus includes both persistence of radiological findings and other presentations. The difference in the two previous reports referenced here and the results from PdL cohort regarding persistent MRI findings, coupled with the normal MRI, T2 FLAIR and DWI findings in SLS, makes it plausible that may be persistent radiology is more frequently observed in methotrexate associated neurotoxicity (which includes seizures, stroke like syndrome and encephalopathy presentations) than in SLS. One explanation can be very careful centralised radiology review in SJCRH, also SJCRH protocols are very MTX intense so may have been more problems. And MRI findings likely to be underreported here because they would only be recorded if case notes reviewed not from trial databases.

Older age (age more than 10 years) has shown significant association as an independent variable with odds of having SLS 4.6 times higher as compared with the younger age (**Error! Reference source not found.**) in a multivariate analysis. While previous studies have reported a similar association, to the best of our knowledge this is the first multivariate analysis to report this.[56,61,62] This finding of SLS being associated with older age is also supported by the comparison carried out with UKALL 2003 (Table 10). This association with older age may be explained by adolescents tendency to have reduced methotrexate clearance reported in a previous osteosarcoma study, however they also reported no difference in methotrexate induced toxicities. [173]

Consolidation with high dose methotrexate phase of treatment showed significant association with SLS as an independent variable (**Error! Reference source not found.**), where the doses that were reported to be administered intravenously less than 1 gm/mm2 and more than 1 gm/mm2 concomitantly administered with intrathecal methotrexate, is consistent with the observations made by the previous reports. [62]

Although consolidation phase with high dose MTX showed the highest association with SLS, consolidation phase of treatment and the "Other phase of treatment", entailing delayed intensification I &II and interim maintenance I&II, also showed association with SLS. Association with SLS with concomitant administration of the cytarabine and cyclophosphamide (**Error! Reference source not found.**) was shown by Bond et al. [114] where Ara-C is most likely to play the key role, as this compound is known to enhance neuronal cell sensitivity to glutamate-mediated damage, in a manner similar to that of methotrexate-induced toxicity.[139,140]

In previous reports cytarabine has been shown to cause cerebellar type of neurotoxicity, of which ataxia is part of the clinical presentation. Ataxia also constitutes the clinical presentation for SLS, so it might be caused by concomitant effect of cytarabine. [174] Mercaptopurine, on the other hand showing significant association with SLS, has limited CNS penetration and its toxicities are mostly Immuno-/myelosuppression, hepatotoxicity rather than neurotoxicity it might be a by chance finding in PdL due to the phase in which it is administered showing an association with SLS. [174]

SLS cases peak at approximately weeks 8-10 of therapy (Figure 7.1). This suggests that SLS appears after multiple exposures to methotrexate, including IT administration during Induction (4 or 5 weeks) and intravenous administration subsequently, making it plausible that methotrexate might have a cumulative effect. SLS cases in the PdL cohort continue to be observed during the following phases, i.e., consolidation, delayed intensification and interim maintenance phase, consistent with the findings by Bond et al. that SLS cases were clustered in consolidation and delayed intensification phases of therapy [61].

NCI Intermediate/high-risk cases and high-risk treatment allocation showing significant association with SLS can potentially be explained by the difference in intensity of drugs administered in standard risk groups, for both NCI and risk group treatment allocations, and the high-risk groups. In case of high-risk groups (NCI and risk group allocation) there are more drugs during different phases of therapy such as an additional drug (daunorubicin) during induction, duration of induction is longer, and an additional delayed intensification or interim maintenance (depending on the protocol) is administered. So, High-risk treatment allocation means more intense therapy which explains the higher rates of SLS because of the either direct combined cytotoxic effects of the drugs or through the other mechanisms. Higher risk treatment groups association is also supported by the comparison with UKALL 2003. (Table 8).

The high dose MTX protocols showing significant association, where they were 2.1 times more likely to have SLS in contrast to protocols using Capizzi-escalating MTX (Table 13). The finding of High dose MTX associated with SLS is especially interesting because with High dose methotrexate Leucovorin rescue is administered but it is not part of the Capizzi escalating methotrexate. This could be attributed to the use of high dose of methotrexate in a single dose in HD MTX protocol as opposed to slowly increasing the dose of methotrexate (escalating by 50 mg/m2 every 10 days) in Capizzi-escalating dose, wherein later all the signs of methotrexate induced toxicities are considered before increasing the next dose of methotrexate. Therefore, it might be that the Capizzi escalating methotrexate protocols are better in avoiding any type of methotrexate-induced toxicities in general and methotrexate induced SLS specifically. One other plausible explanation can be that escalating Capizzi also contains multiple chemo agents whereas HD MTX is usually given in isolation, though it's unlikely that other agents provide a protective effect.

High dose methotrexate was a significant risk of encephalopathy (n=20/1343), a subgroup in an acute neurotoxicity report, with HD MTX administration (p=0.03) [56].

More intense triple intrathecal therapy (ITT) has been suggested as a potential method for decreasing the risk of CNS relapse and few of the trials included in PdL cohort were either randomising for ITT vs IT MTX therapy to only high-risk patients or in one trial for all risk groups. Acknowledging this variation in CNS-directed therapy in different trials included in this cohort, if there was an association with SLS, or an impact in the outcomes post event but in this cohort was explored. Observation was made that there was no statistically significant difference seen in this cohort, between the two groups administering single IT or triple IT, for association with SLS (p value 0.2, Table 14) or for the persistence of neurological outcomes in SLS (p value 0.6). Reasons speculated can be that the number of cases receiving TIT were small and the analysis lacked the power to show significant results. In a study (CCG 1952) randomising for IT MTX and ITT, there were similar levels of CNS toxicity (grades 3 and 4) on both study arms, with 5.8% CNS toxicity when assigned to IT MTX versus 6.7% with ITT. [143]

Exploring the other drug interactions with methotrexate, Nitrous oxide may contribute to the neurotoxic effects of methotrexate [66], but administration of general anaesthesia four weeks prior to SLS event did not show a significant association (Table 12), perhaps because it was only

possible to look at general anaesthesia without stratification with respect to which agent was used.

In the SLS group the most common route of methotrexate administration was intrathecal (Table 11), which is consistent with the previous findings where SLS has been linked with intrathecal methotrexate .[64,144] IT methotrexate induced neurotoxicity has also been linked previously with increased methotrexate CSF levels where according to one study of intrathecal methotrexate neurotoxicity, the central nervous system concentrations of the drug after 48 hours were on average 13 times more in patients with neurotoxicity (5/25) than those who did not experience toxic effects.[119]

Interventions that can be administered in order to reverse neurotoxic effects of SLS are dextromethorphan and aminophylline. One potential mechanism of neurotoxicity is elevated homocysteine that results in neuroexcitatory metabolites that act on NMDA receptor. Dextromethorphan is a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor. Previous case series have shown improved resolution of symptoms, but the time to resolution is variable and in few cases extending beyond the usual course of resolution of SLS (within 7 days).[69] We don't see a beneficial effect of dextromethorphan administration in SLS when checked for parameters; complete resolution of neurological symptoms, time to recovery of neurological symptoms, secondary neurotoxicity and for the persistent radiological findings at the last follow-up (

). This may be due to the small number of cases and the fact that the time to recovery variable wasn't reported precisely enough to detect a difference (values for time to recovery would need to be in hours, and case numbers very large).

Aminophylline, an adenosine antagonist, which can counter the effects of the increased adenosine levels in the CNS of paediatric ALL patients with toxicity, has also been favoured as an intervention post SLS event. Bernini *et al.* tested whether infusions of aminophylline 2.5 mg/kg for 1 hour could displace adenosine from central receptors and showed improved resolution of neurotoxicity symptoms. However, in PdL cohort no statistically significant difference was observed for the variables; complete resolution of neurological symptoms, time to recovery after event, secondary neurotoxicity, and persistent radiological findings at the last follow-up, with aminophylline administration in the SLS cases with the cases without

(**Error! Reference source not found.**). The reason of which can be speculated by the findings from the Bernini *et al*. study patients received very high doses of IT methotrexate, compared to modern protocols, and the clinical symptoms tabulated are of acute methotrexate neurotoxicity (nausea, emesis, headaches and lethargy) and not of SLS.[70] So, it is possible that aminophylline might be beneficial for acute methotrexate induced neurotoxicity.

Interestingly, patients rescued with higher dose (15mg/m2) of leucovorin were 6.8 times more likely to get an SLS episode. This is in contrast with previous reporting that high dose of leucovorin causes improved recovery in overall methotrexate-induced toxicity. [175] However, high dose of leucovorin is administered with high dose of methotrexate, while low dose is administered in the protocols administering low dose of methotrexate or after a spinal administration of methotrexate (again relatively lower than high dose MTX). Thus, it is plausible that this association is not due to leucovorin toxicity, but rather to confounding. All this information is based on the protocol rather than the individual case i.e., and it's not known that the patient actually received the leucovorin just that the protocol mandated it.

However, there was no statistically significant association with SLS observed with difference of length in leucovorin window (early or late, Table 15) post MTX administration. Additionally, in SLS cases, there was no effect observed on persistence of neurological symptoms post-event with either difference in dose of leucovorin (low dose vs high dose, **Error! Reference source not found.**), or with the difference in leucovorin administration window (early vs late) (**Error! Reference source not found.**). In contrast with the Cohen I. findings from a systematic review showing that neurotoxicity is due to inadequate rescue post MTX [153], our results provide reassurance that SLS is not due to inadequate rescue.

Observing the impact of SLS neurological event on the leukaemia relapse rates outcomes of these SLS cases seems to be unaffected when compared with the rest the RONC group (p value 0.3). There was also no rise in leukaemia relapse incidence overall or CNS relapse reported in the SLS group, after change in IT methotrexate and High dose methotrexate treatment modification post SLS event compared with the SLS cases who continued as per protocol therapy (**Error! Reference source not found.**), despite the finding that the SLS cases were 4.5 times more likely to have their HD MTX modified post event (**Error! Reference source not found.**). In contrast, Mateos *et al*. reported increased CNS relapses in a recent study of overall methotrexate induced neurotoxicity after modification in the methotrexate administration post

event. [57] The reasons for not seeing the same effect of methotrexate therapy modification in SLS cases on leukaemia relapse can be explained with few cases with relapse, and even fewer cases with CNS relapse, where out of the cases that had leukaemia relapse on our cohort only 2 cases (16%) had isolated CNS relapse in SLS group irrespective of change in chemotherapy. The Mateos *et al*. study was a population-based study comparing MTX-induced neurotoxicity vs no neurotoxicity cases in paediatric ALL patients in a trial, while the PdL study compares SLS vs RONC group, therefore direct comparison between these two studies is not possible.

Prolonged resolution of neurological symptoms showed protective association with the SLS cases (**Error! Reference source not found.**). Around 97% of cases had their neurological symptoms completely resolved within less than one month duration (Observations are shown in Table 7.27

) and prolonged neurological symptoms were observed in only 11%, which is along the same lines with the findings previously reported where either most of the cases [61] or all of the cases [62] had no neurological sequalae. More importantly the finding of no statistically significant difference in prolonged neurological symptoms observed in both the groups with or without the SLS cases IT MTX and HD MTX modified post event (**Error! Reference source not found.**), providing favourable evidence for the safety of re-exposing to methotrexate after initial SLS event.

The finding of persistence of long-term neurological symptoms at the last follow up showed significant association with Capizzi MTX. The reason for this significant association can be speculated on the variation in the way methotrexate is administered in the treatment protocols, where intensive hydration for 24 hours after administration, monitoring serum MTX levels, and leucovorin rescue are standard practices for reduce MTX-related toxicities in patients receiving HD MTX but not in Capizzi escalating MTX protocols.

The findings regarding admission required to ITU reported in 11 SLS cases only (**Error! Reference source not found.**), out of which only 3 cases required ventilatory support (**Error! Reference source not found.**) and no neurotoxicity related death in SLS group are observations that shows that in most of the cases that symptoms were not severe enough to require intensive care intervention and there were rarely severe adverse outcomes and neurological sequelae, also supported by the previous studies reports. [61,62]

Safety to re-expose to methotrexate after the initial event in SLS has been frequently speculated. In the PdL cohort secondary neurotoxicity was observed in 13 cases only when re-exposed which is lower than the RONC group. This finding regarding secondary SLS or SLS-like (Encephalopathy, **Error! Reference source not found.**) episode post re-exposure to methotrexate is consistent with the findings shown by Bond *et al.* in which majority of cases 82.1% cases had no recurrence of symptoms.[61] MTX resumption also does not appear to be contraindicated with transient acute encephalopathy.[62] The findings from PdL study and the literature favours the guidelines in some protocols to re-expose to IT MTX in SLS patients who had a full recovery without any complications e.g., UKALL 2003.

7.4.1 Conclusion:

The dosage, the frequency, duration of methotrexate exposure and concomitant drugs administered to the central nervous system are all important factors. The beneficial effects of interventional drugs remained unproven.

8 Deep phenotyping of Chemotherapy-associated neurotoxic episodes in Down syndrome ALL from the Ponte di Legno Neurotoxicity study

8.1 Background

Down syndrome is a condition in which a child is born with an extra copy of chromosome 21 [82]. This not only causes physical and mental developmental delays and disabilities but also predisposes them to have higher incidence of acute lymphoblastic leukaemia with distinct biological characteristics [83,84]. Furthermore, research has shown inferior outcomes of Down syndrome acute lymphoblastic leukemia (DS-ALL) compared to non Down syndrome ALL (non DS-ALL) because of increased relapse rate and treatment related mortality (TRM) [85]. DS-ALL patients also have increased vulnerability to chemotherapy-associated toxicities, particularly MTX and anthracyclines, including a high risk of toxic death [86-88]. The reason for this increased susceptibility to methotrexate-induced toxicity in Down syndrome ALL patients is most likely due to altered MTX pharmacokinetics [89].

Although neurotoxicity in Down syndrome with ALL has been reported, only 2 isolated chemotherapy-induced seizures cases were reported from, a randomized controlled trial based on the MRD-based risk stratified adapted chemotherapy in UKALL 2003, also reporting DS-ALL outcomes as a sub study [90]. There is lack of detailed clinical description and there is no information about the severity of these events, extent of recovery and their risk of recurrence. Moreover, the risk predictors and their implications on neurological and leukaemic outcomes are unknown, as MTX-dose modifications are frequent and their impact on leukemic outcomes are uncertain. Also, there is a lack of clarity about clinical patterns of neurotoxicity and their effect on clinical outcomes in the presence of an existing neurological condition such as Down syndrome. This highlights the reason to investigate Down syndrome ALL neurotoxicity in a larger pooled cases dataset.

8.2 Aims

1) To deep phenotype neurotoxic episodes in patients with DS-ALL
2) To identify risk predictors of neurotoxicity in this group compared to non DS-ALL
3) To explore the effects of neurotoxicity on clinical outcomes in DS-ALL

Cases:

For analysis, the neurotoxicity cases reported as DS-ALL, n=42 were the test group, and the non DS-ALL (non DS-ALL, n=1741) was taken as the comparative group. Cases with missing DS information, 23 patients, were not included in the analysis.

8.3 Results

8.3.1 Aim1: To deep phenotype neurotoxic episodes in patients with DS-ALL

8.3.1.1 Timing of event:

It was of interest to explore the timing of different of types of neurotoxicity events in DS-ALL cases to see which type of neurotoxicity occurred during the different phases of therapy.Figure 8.1

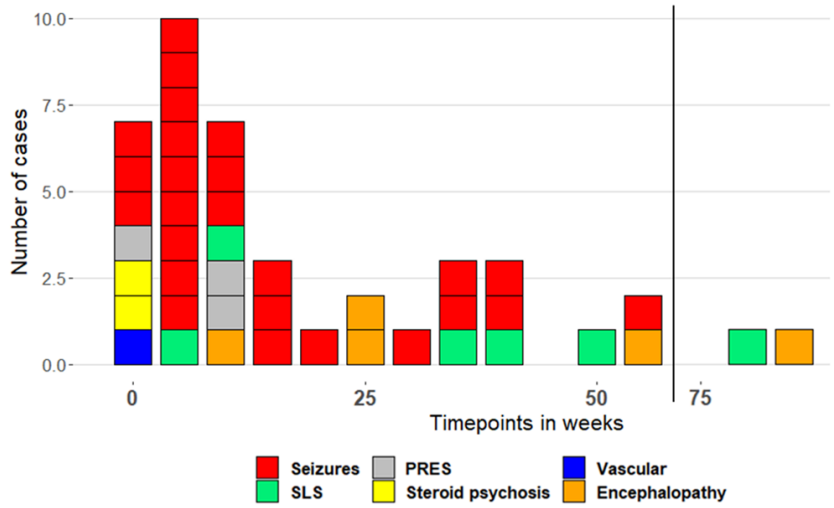

Figure 8.1. Stacked bar graph time points of different types of neurotoxicity in DS-ALL

The number of time points from the beginning of ALL treatment to neurotoxic event with number of cases of DS-ALL stacked as different types of neurotoxicity.

Figure 8.1 shows the timing of neurotoxic events according to type in DS-ALL. The events peak in the first 5-10 weeks. PRES and steroid psychosis are seen in the initial phase of therapy, SLS was occurring in the mid-phase, while seizures, with a peak in the initial phase, were occurring throughout the treatment period.

8.3.1.2 Types of neurotoxicity in DS-ALL

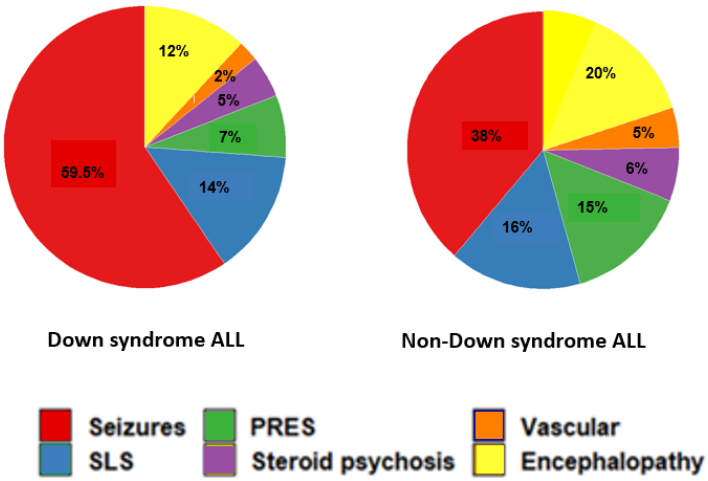

Figure 8.2: Pie chart showing comparison of types of neurotoxicity in DS-ALL and non DS-ALL

Out of these 42 cases of neurotoxicity in Down syndrome ALL, there were 25 cases of seizures, 6 of Stroke-like Syndrome, 3 of Posterior Reversible Encephalopathy Syndrome, 2 steroid psychosis, 1 neurotoxicity due to vascular disorder and 5 cases with unspecified encephalopathy.

Looking at the types of neurotoxicity, it was identified that isolated seizures were reported in almost 60% (25/42) of patients and were more common in DS-ALL than in Non-DS-ALL, 38% (723/1741) ($p<0.05$).

8.3.2 Aim 2: To identify risk predictors of neurotoxicity in this group compared to non DS-ALL

8.3.2.1 Demographic comparison of DS-ALL neurotoxicity with non DS-ALL with neurotoxicity

Table 8.1: Demographics of DS-ALL neurotoxicity cases compared with non DS-ALL neurotoxicity cases

Variable	DS-ALL neurotoxicity group		Non DS-ALL neurotoxicity group		
	n	%	n	%	p-value*
Age at diagnosis (years)					
Total	42		1749		
<10 years	22	52.3%	807	46%	
10-15 years	12	28.5%	940	54%	.06*
>15 years	8	19%	267	15.3%	
Median years(range)	8.1 (2-26)		8.10 (0-29)		-
Sex					
Male	21	50%	941	54%	.61*
Female	21	50%	806	46%	
Immunophenotype					
Total	41		1736		
B-cell type	40	97.5%	1408	81%	<0.05*
T-cell type	1	2.4%	328	18.8%	

*Chi square analysis

Looking at the demographics of neurotoxicity in the Down syndrome ALL group, there was no statistically significant difference in the proportion of males and females, and age groups (less than 10 years, 10-15 years, and more than 15 years) except that, as expected, Down syndrome was associated almost exclusively with the B-cell lineage ALL compared with Non-DS ALL cases.

8.3.2.2 Risk predictors

In order to investigate any demographic or clinical variables associated with neurotoxicity in Down syndrome ALL, due to the lack of "no neurotoxicity" control group, the dataset was compared with information obtained from Ponte di Legno Down syndrome ALL [85], UKALL 2003 Down syndrome ALL [90] and BFM Down syndrome ALL MTX toxicity published data [176].

Table 8.2: Demographics of DS-ALL neurotoxicity cases compared with DS-ALL publications

Variable	**DS-ALL neurotoxicity cases**		**PdL DS-ALL publication**			**UKALL DS-ALL publication**			**BFM DS-ALL MTX toxicity publication**		
	n	%	n	%	p-value	n	%	p-value	n	%	p-value
Age at diagnosis (years)											
Total	42		653			86			103		
Median years (range)	8.62(2-26)		5(1.2-17.9) *			4.5(1–23)			4.8(1.2-17.7) *		
Gender											
Male	21	50	343	52.5	.63[ṁ]	59	69	.01[ṁ]	64	62	.08[ṁ]
Female	21	50	310	47.4		27	31		39	38	

PdL Down syndrome ALL publication & BFM Down syndrome ALL MTX toxicity age inclusion <18 years*, therefore not included in age comparison.

ᵑChi-square analysis

The comparison showed that there was difference seen in median age, Down syndrome ALL neurotoxicity cases show higher median age 8.62 years as compared with the 4.5 years median age of Down syndrome ALL patients reported in the UKALL 2003 cohort, indicating that older age Down syndrome ALL were more likely to have a neurotoxic event. (Statistics on the continuous variables (age) could not be run due to inaccessibility to raw datasets of the studies included for comparison)

Gender distribution comparison showed that although DS-ALL with neurotoxicity cases on this dataset showed equal distribution between the two genders. However, on comparison with PdL Down syndrome ALL, and BFM Down syndrome ALL MTX toxicity cohorts showed no statistically significant difference for the two groups but with UKALL 2003 Down syndrome showed significant association (p-value 0.01) (**Error! Reference source not found.**)

8.3.2.3 Treatment allocation:

Table 8.3: Comparison of DS-ALL and non DS-ALL neurotoxicity cases for treatment allocation

Variable	DS-ALL neurotoxicity		Non DS-ALL neurotoxicity		p-value
	n	%	n	%	
Treatment allocation					
Total	41		1577		
Standard risk	17	42	556	36	

Medium risk	11	31	707	44	.04*
High risk	13	27	314	20	

*Chi square analysis

It was noted that most of the cases 42% (17/41) of DS ALL neurotoxicity cases were allocated Standard risk treatment regimen followed by High-risk treatment regimen-31.7% (13/41) and 26% (11/41) were allocated medium risk group. DS-ALL group was more likely to get standard risk treatment allocation (p-value .04). (**Error! Reference source not found.**)

8.3.2.4 Treatment phase

Most of the neurotoxicity events in DS ALL were occurring during the induction phase 40% (17/42) followed by delayed intensification phase (I&II) 30% (13/42), consolidation phase 11.9% (5/42), consolidation with HD MTX 7.4% and maintenance phase of treatment 4.7% (2/42).

When comparing DS-ALL and non DS-ALL neurotoxicity was more likely to occur during the induction phase of the treatment in DS-ALL (42% (17/40) of cases vs 27.6% (448/1620), p value .03)

8.3.2.5 Treatment on High Dose Methotrexate (HD MTX) or Capizzi methotrexate protocols

DS-ALL neurotoxicity cases (47.6% - 20/42) were treated on HD MTX type of treatment protocol and (52.3% - 22/42) Capizzi MTX treatment protocol. There was no statistically significant difference in treatment protocol observed on comparison with the non DS-ALL neurotoxicity cases.

Table 8.4: Comparison of DS-ALL and non DS-ALL neurotoxicity for HD MTX and Capizzi protocols

Variable	DS-ALL neurotoxicity HD MTX protocol		DS-ALL Neurotoxicity Capizzi protocol		p-value
	n	%	n	%	
Treatment protocol					
Total	42		1719		
HD MTX	20	48	1020	60	.12*
Capizzi escalating	22	52	699	40	

*Chi square analysis

8.3.2.6 Methotrexate administration within four weeks of the neurotoxicity event

There were 96% (26/27) of neurotoxicity DS ALL cases who were administered methotrexate within four weeks before the neurotoxic event. There was no statistically significant difference seen between DS ALL and Non-DS ALL (96.2% (26/27) vs 94% (1089/1154), p-value .66).

8.3.2.7 Route of methotrexate (MTX) administration

DS-ALL neurotoxicity cases were more likely to get MTX through intrathecal route, within four weeks of the event (92% (23/25) as compared to the Non-DS ALL 76.1% (735/965). However, there was no statistically significant difference observed between the two groups (p-value .06).

There was only one case that received Intravenous and IT (4% (1/25) and one case that had oral MTX (4% (1/25) within 4 weeks of the neurotoxic event in DS ALL neurotoxicity patients.

8.3.3 Aim 3: To explore the effects of neurotoxicity on clinical outcomes in DS-ALL

8.3.3.1 Severe adverse neurotoxicity outcomes

It was of interest to explore the severe adverse neurotoxicity outcomes of DS ALL cases post a neurotoxic event and compare them with the Non-DS ALL neurotoxicity cases.

8.3.3.2 Use of oral antiseizures post-event

Out of the responses obtained, 50% (5/10) of the Down syndrome ALL patients were put on long-term antiseizures as compared to 40% (232/578) cases in the Non-DS ALL neurotoxicity cases. There was no statistically significant difference observed between the two groups (p-value .52)

8.3.3.3 Requiring admission in ITU

Almost half of the DS-ALL neurotoxicity cases, 50% (6/12) required admission in ITU as part of their management of the event as compared with 40% (289/709) in Non-DS ALL neurotoxicity cases. There was no statistically significant difference between the two groups. (p-value .51)

8.3.3.4 Complete resolution of neurotoxicity symptoms

The neurological events were reported to have completely resolved in almost all, for the responses obtained, the DS ALL neurotoxicity cases (20/21–95.2%) without any neurological sequelae as compared to the 85% (819/955) cases in Non-DS ALL cases. However, there was no statistically significant difference observed between the two groups for complete resolution of neurological symptoms (p-value .12)

8.3.3.5 Death due to neurotoxicity

It was observed that there were no neurotoxicity-related deaths reported in DS-ALL as opposed to 17 deaths that were reported in Non-DS ALL neurotoxicity group although the numbers are small.

8.3.3.6 Recurrence in DS-ALL neurotoxicity on re-exposure

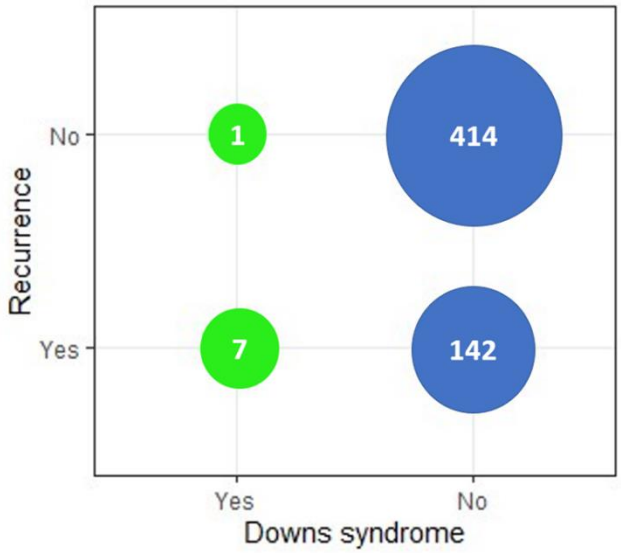

Figure 8.3. Bubble plot of DS-ALL with recurrence of neurotoxicity

DS-ALL cases with previous neurotoxicity were found to be at very high risk for a second neurotoxic event on re-exposure to the presumed causative agent. This was observed in 7/8 (87%) out of responses obtained, as compared to 25% (142/414) of non DS-ALL cases having a recurrent episode on re-exposure, ($p<0.001$). An odds ratio of 20.4, means that patients with DS-ALL were approximately 20 times more likely to get recurrent neurotoxicity when re-exposed to the presumed agent as compared to non DS-ALL patients. DS-ALL remained a risk factor for recurrence after adjusting for gender and age.

8.3.3.7 Demographic features of the Down syndrome ALL neurotoxicity recurrent cases:

Table 8.5: Demographic features of the Down syndrome ALL neurotoxicity recurrent cases

Variable	Recurrent neurotoxicity in DS-ALL subgroup	
	n	%
Age at diagnosis (years)		
Total	7	
Less than 10 years	3	42.8%
10-15 years	4	57.1%
More than 15 years	-	-
Sex		
Male	6	85.7%
Female	1	14.2%

It was observed that the Down syndrome ALL recurrence was more frequent in males 85.7% (6/7) and age groups below 15 years, but the small number of cases available makes it difficult to draw any conclusions.

8.3.3.8 Clinical presentations, change in chemotherapy and its impact on recurrence in DS ALL neurotoxicity cases

Table 8.6: Showing details of clinical events in recurrent DS-ALL

Initial event	Intervention given post initial event	Change in chemotherapy	Secondary event
Seizures	Dextromethorphan & Leucovorin	NA	Seizures
Seizures	Parenteral antiseizures	NA	Seizures

Seizures	NA	NA	Seizures
SLS	NA	No	Encephalopathy
SLS	Parenteral antiseizures & anticoagulants	No	SLS
PRES	Not given	Yes	Encephalopathy
Steroid psychosis	Not given	Yes	NA

In the seven DS-ALL cases with recurrent neurotoxic events, the information is available on the primary and secondary events. There were 3 cases of Seizures, 2 cases of SLS with recurrent events as SLS and encephalopathy, 1 case of PRES with recurrence as encephalopathy and 1 case with steroid psychosis as an initial event and no information about their secondary event.

There were only 3 cases out of these 7 recurrent DS-ALL neurotoxicity cases that were given an intervention to manage the neurotoxicity event. There was 1 case administered dextromethorphan and with leucovorin rescue, 1 case was just given parenteral anticonvulsants and there was 1 case given anticoagulants with parenteral anticonvulsants. There doesn't seem to be any effect of administering leucovorin rescue with dextromethorphan (although it's just one case) for recurrence.

Chemotherapy modification post initial neurotoxic event in DS-ALL was observed in only half of the cases 50% (2/4) although, there is very limited information available, on just 4 cases.

8.3.3.9 Impact on survival outcomes:

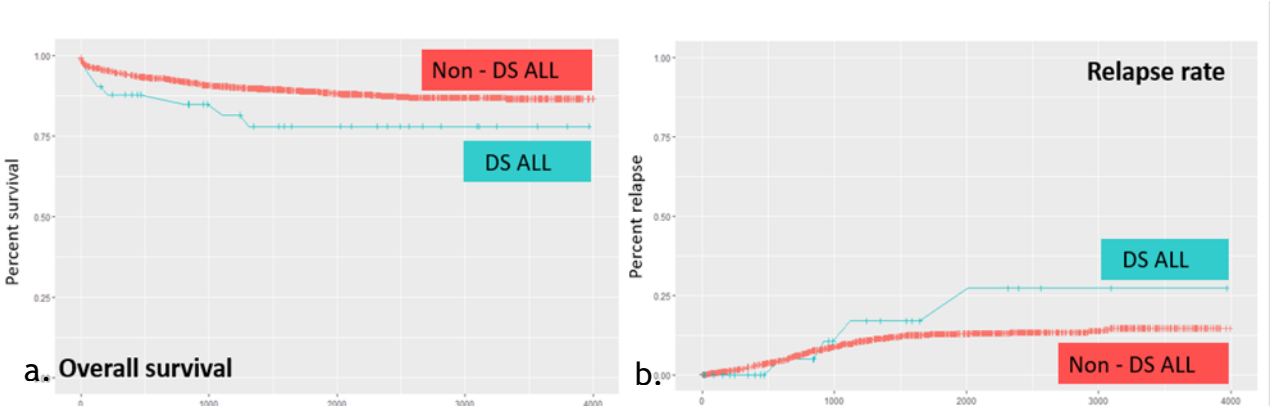

Figure 8.4. Kaplan Meier survival analysis of Down syndrome ALL and Non-Down syndrome ALL.

a) Showing comparison of overall survival and b) showing comparison of relapse rate.

Down syndrome is reported to have inferior leukaemia outcomes, so survival analysis was a point of interest. Despite seeing an increase in leukaemia relapse in DS-ALL neurotoxicity (4 cases), there was no statistically significant difference in overall survival (OS) and relapse rate (RR) between DS-ALL and non DS-ALL post neurotoxic event when followed up (mean 4.85 years).

8.4 Discussion:

To the best of our knowledge, this is the largest case series of Down syndrome ALL chemotherapy-induced neurotoxicity reported. This allowed identification of variables associated with DS-ALL such as seizures, standard risk, induction phase, recurrence upon re-exposure. The Ponte di Legno database was a retrospective collection of neurotoxicity cases where strength of DS-ALL neurotoxicity cases analysis was the "non DS-ALL" neurotoxicity cases.

The finding that B-cell type lineage was almost exclusively overrepresented in DS ALL neurotoxicity cases when compared with Non-DS ALL is consistent with other reports DS-ALL [177]. The comparison with Ponte di Legno Down syndrome ALL [85], UKALL 2003 Down syndrome ALL [178] and BFM Down syndrome ALL MTX toxicity [176] published data to identify risk predictors for DS ALL neurotoxicity showed that older age and female sex are more likely to get a neurotoxic event.

Although there have been isolated reports of Seizures, as chemotherapy induced SAE reports in Down syndrome ALL [178], our study identified, seizures being significantly associated with DS-ALL neurotoxicity as compared with Non-DS ALL neurotoxicity. Patients with Down syndrome are reported to have higher prevalence of seizures as compared with the rest of the population [179]. DS-ALL being at high risk of recurrence upon re-exposure is a novel finding by our study and seizures were the common recurrent episode 3/6-50% (**Error! Reference source not found.**). There is no information available in the literature regarding chemotherapy-induced recurrence in DS-ALL; however, neurological deficits (2 patients-unspecified) in ALL children on chemotherapy were previously shown to have increased risk of recurrence of chemotherapy-induced seizures [80].

It is known that treatment protocols have modifications (reduced intensity of treatment) for children with Down syndrome ALL, [90] which is consistent with the findings of our study showing significant association with standard risk allocation (**Error! Reference source not found.**). This finding of significant association with lower intensity treatment regimen allocation makes it plausible that the occurrence of neurotoxicity is probably not due to the intensity of the treatment but due to the underlying condition that predisposes to neurotoxicity. By contrast,

other methotrexate-induced toxicities (mucositis, myelosuppression, liver toxicity) are reported more with more intense treatment in DS-ALL [176].

Down syndrome ALL has inferior leukaemia outcomes as compared to the non DS-ALL that has been attributed to higher relapse rates [180] as well as higher levels of chemotherapy [89], especially methotrexate-associated toxicities [176] and treatment-related mortality [90]. However, in this limited cohort, there was no significant difference for the leukaemia outcomes was observed between DS-ALL and non DS-ALL post neurotoxic event. This finding is unexpected as DS-ALL are more likely to get their treatment modified, as previously reported in 43% of DS-ALL therapy-induced morbidity most probably due to HD MTX [181]. However, no difference in DS-ALL and non DS-ALL in OS, RR and EFS can be explained with small numbers, only 4 DS-ALL cases, for this comparative survival analysis.

The unexpected findings regarding the neurotoxicity outcomes of no statistically significant difference seen in DS ALL and Non-DS ALL for the use of oral anticonvulsants (despite of seizures being the more frequent type of neurotoxicity), requiring admission to ITU, the resolution of initial neurotoxic symptoms and the long-term persistence of neurological symptoms might owe to the small numbers and missing data. In Down syndrome, the premorbid mentally debilitating condition it would be expected to have more pronounced and long-term neurological outcomes after a neurotoxic event as compared to the rest.

8.4.1 Limitations

It is important to acknowledge that there was a fair amount of missing data since it was collected from different sources, including trial databases, and it was difficult to find more and robust detailed clinical information about the neurotoxic event. The lack of a “no neurotoxicity” control group limits the types of analysis that is possible to carry out on this dataset, restricting all the questions that could have been asked.

8.4.1.1 Conclusion

This study identifies, for the first time, DS as a risk factor for recurrent neurotoxicity upon re-exposure. Therefore, careful consideration should be given to optimising supportive care and potentially modifying therapy for children with DS who experience a first chemotherapy-induced neurotoxic event.

9 Ponte di legno Genotyping (GWAS meta-analysis)

9.1 Introduction

Although neurotoxicity is reported in 8-12% of children undergoing treatment for ALL, the individual numbers of patients with PRES/SLS and other types of neurotoxicity in each trial group are too small to allow genotype-phenotype correlations. Even though there have been some SNP array-based studies that have reported genetic polymorphisms associated with Methotrexate-induced neurotoxicity, both acute type of neurotoxicity and chronic long-term type, related to neuronal development, MTX clearance or folate metabolism by-products namely homocysteine [104] however, none reached GWAS significance level. GWAS studies for MTX associated neurotoxicity have shown an association with polymorphisms potential role in neurodevelopment, developmental delays phenotypes such as attention deficit hyperactivity disorder and autism [51,57] but they were looking at the polymorphism associated with overall methotrexate associated neurotoxicity without stratifying participants into different types of neurotoxicity and didn't reach GWAS significance level. Smaller studies have identified vincristine-related neurotoxicity [105] but none of these were able to show significance at GWAS level. Genetic variations depicting the severity of the neurotoxic event as measured by whether there was a full recovery or persistent neurological deficits, and the occurrence of a recurrent event are unknown.

9.2 Aims

The objectives of this analysis were as follows:

1) To identify genetic polymorphisms (SNPs) that are associated with the risk of acute chemotherapy-induced neurotoxicity in children and adolescents treated on childhood ALL protocols.

2) To investigate the association of SNPs of interest with different types of neurotoxicity (SLS, PRES, Seizures and Encephalopathy).

3) To explore the association of SNPs with recurrence of neurotoxicity and with persistent neurological deficits

9.3 Results

9.3.1 Aim 1: To identify genetic polymorphisms (SNPs) that are associated with the risk of acute chemotherapy-induced neurotoxicity in childhood ALL protocols

9.3.1.1 Phenotype 1: Neurotoxicity

All the cases reported as having a chemotherapy-induced neurotoxicity episode while being treated on the frontline protocol were analysed using the METAL software [182], the top nominally statistically significant results (p-value <0.05) are shown below:

Table 9.1: Top SNPs showing nominally statistically significant association with neurotoxicity at a significance level of p <0.05

SNP ID	Chr	Location	Allele1	Allele2	P-value	Weight	Z score	Direction	Gene
rs1801725	3	123486447	t	g	0.009337	3047	2.599	+?+	CaSR
rs2520464	7	87201086	t	c	0.01684	3148	2.39	+++	ABCB1
rs1953783	14	82903381	a	g	0.02103	3168	-2.307	---	
rs10888798	1	39958080	c	g	0.02374	3172	-2.261	---	BMP8A
rs199883435	2	199181697	g	g	0.02514	41	-2.239	?-?	
rs875740	16	16123048	a	c	0.02928	3170	2.18	+++	ABCC1
rs1198402	14	82898979	t	g	0.03038	3168	2.165	+++	
rs1809697	1	39959085	t	c	0.0313	3172	2.153	+++	BMP8A
rs864600	7	71151340	a	c	0.03193	3047	2.145	+?+	CALN1
rs12138051	1	39948224	a	g	0.03268	3172	-2.136	---	MACF1
rs7555699	1	39957301	a	g	0.03269	3172	2.136	+++	BMP8A
rs722357	1	39944768	t	c	0.03274	3172	2.135	+++	MACF1
rs6691194	1	39948741	a	g	0.03277	3172	2.135	+++	MACF1
rs6665948	1	39944180	a	g	0.03345	3172	-2.127	---	MACF1

rs246221	16	16138322	t	c	0.04078	3125	-2.046	---	ABCC1
rs246240	16	16138322	a	g	0.0431	3095	-2.023	---	ABCC1

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 1 i.e., overall neurotoxicity, showing an association of $p < 0.05$.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z- score; "Direction" refers to the direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

There were 16 top SNPs from the meta-analysis results that showed nominally significant association (<0.05) with the phenotype neurotoxicity. These SNPs were mapped to CaSR, ABCB1, BMP8A, ABCC1, AC005235.1, CALN1, and MACF1 genes where multiple SNPs showed nominally significant association.

However, none of the neurotoxicity associated SNPs on Bonferroni correction retained their significance. As they were corrected against 254 tests, the number of SNPs on the neurotoxicity candidate SNPs list, the Bonferroni adjusted p-value will be 0.0002.

9.3.2 Aim 2: To investigate the association of SNPs of interest with different types of neurotoxicity (SLS, PRES, Seizures and Encephalopathy)

9.3.2.1 Phenotype 2: Definite SLS

Definite SLS, based on their DDSS scoring, is causally linked to methotrexate use. The neurotoxicity candidate SNPs list had SNPs associated with different types of chemotherapy agents so for definite SLS the list was restricted to just the methotrexate-associated SNPs reducing the number of SNPs/tests from 254 to 154.

There were no SNPs that showed significant association (<0.05) with the definite SLS phenotype.

9.3.2.2 Phenotype 3: Definite, Probable and Possible SLS

This phenotype included within 21 days of methotrexate administration 1) SLS definite, where cases that were true to the differential diagnostic scoring system 2) probable SLS cases that had some clinical features 3) possible SLS had few clinical features like SLS. As SLS is associated with methotrexate, a restricted list of candidate SNPs (n=154) published just associated with methotrexate associated neurotoxicity (without stratifying into types of neurotoxicity) were included for the analysis. The results are shown in **Error! Reference source not found.**.

Table 9.2: Top SNPs showing nominally significant association with Methotrexate-induced definite, probable, and possible SLS at a significance level of p <0.05

SNP ID	Chr	Location	Allele1	Allele2	Weight	Z score	P-value	Direction	Gene
rs6971012	7	97315198	t	c	2705	-3.456	0.000549	-?-	ASNS
rs10886214	10	85117719	t	c	2748	2.427	0.01524	+0+	-
rs1106480	3	195925458	a	c	2705	-2.368	0.0179	-?-	ZDHHC19
rs486907	1	180821180	t	c	2757	2.297	0.0216	+++	RNASEL
rs17835197	17	2847632	a	g	2705	-2.234	0.02549	-?-	GARNL4
rs1106479	3	195925355	t	c	2731	2.23	0.02575	+++	ZDHHC19
rs6993813	8	120121419	t	c	2775	-2.196	0.0281	-0-	-
rs1465614	2	16516774	t	c	2790	2.141	0.03228	+++	-

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 3 i.e., definite, probable and possible SLS, showing association of $p < 0.05$.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z- score; "Direction" refers to direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

There were total 8 SNPs that showed nominally significant (p-value <0.05) association with definite, probable, and possible SLS. The top SNP rs6971012 showed 0.000549 p-value. The mapping of these SNPs to the ASNS, ZDHHC19, RNASEL, and GARNL4 genes.

However, none of the Definite, probable, possible SLS associated SNPs upon Bonferroni correction none remained significant. As they were corrected against 154 tests, with the restricted MTX only candidate SNPs list, the number of SNPs on the neurotoxicity candidate SNPs list, the Bonferroni adjusted p-value will be 0.00032.

9.3.2.3 Phenotype 4: Definite PRES

Definite PRES included the cases that were true to the DDSS scoring system, with the MRI findings. Full candidate SNPs list, n=254, was used because there are very few published SNPs associated with PRES.

Table 9.3: Top SNPs showing nominally significant association with definite PRES at a significance level p <0.05

SNP ID	Chr	Location	Allele1	Allele2	Weight	Z score	P-value	Direction	Gene
rs3758730	11	34431675	a	t	2711	-2.847	0.004415	--	CAT
rs2065920	1	164869647	a	g	2712	2.731	0.006319	++	FMO9P†
rs4149056	12	21178615	t	c	2711	-2.517	0.01182	--	SLCO1B1
rs1013940	2	108608648	a	g	2711	-2.463	0.01378	--	SLC5A7
rs6961419	7	87172136	t	c	2712	-2.29	0.02202	--	ABCB1
rs17626001	14	42139233	a	g	2711	-2.276	0.02286	--	-
rs7238784	18	70078832	t	g	2164	1.98	0.04773	+?	CYB5A

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 4 i.e., Definite PRES, showing an association of p < 0.05.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z- score; "Direction" refers to the direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" and "Gene function" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

There were total 7 SNPs that showed nominally significant association (<0.05) with Definite PRES. The genes that were mapping to these SNPs showing significant association were CAT, FMO9P†, SLCO1B1, SLC5A7, ABCB1, and CYB5A genes.

Although, after Bonferroni correction none of the above-mentioned SNPs remained significant as the adjusted p-value was 0.00020, as there were 254 candidate SNPs for this comparison.

9.3.2.4 Phenotype 5: Definite, Probable and Possible PRES

This phenotype includes 1) PRES definite cases that fulfilled the score with the radiological findings, 2) probable PRES cases that had radiological findings with some PRES-like clinical presentation, and 3) possible PRES where possible cases had some clinical features like PRES. The analysis was run against full list of neurotoxicity candidate SNP's list, n=254.

Table 9.4: Top SNPs showing nominally significant association with Definite, probable, and possible PRES at a significance level p <0.05

SNP ID	Chr	Location	Allele1	Allele2	Weight	Zscore	P-value	Direction	Gene
rs3865466	19	6510003	a	g	2752	-2.844	0.004449	-0-	TUBB4
rs4149056	12	21178615	t	c	2737	-2.641	0.008276	-?-	SLCO1B1
rs5986510	X	24428606	c	g	2174	2.538	0.01114	+??	PDK3
rs2074614	X	24431810	t	c	2174	-2.537	0.01119	-??	PDK3

rs2710057	X	86078659	t	g	2174	2.509	0.01212	+??	-
rs3758730	11	34431675	a	t	2737	-2.129	0.03322	-?-	CAT
rs6696880	1	66574083	a	g	2738	-2.103	0.03549	-?-	PDE4B

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 5 i.e., Definite, probable and possible PRES, showing an association of $p < 0.05$.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z- score; "Direction" refers to the direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

There were 7 SNPs that showed nominally significant association (<0.05 p-value) with the definite, probable, and possible PRES cases when compared with the controls. These SNPs mapped to the genes TUBB4, SLCO1B, PDK3, CAT, and PDE4B genes. (**Error! Reference source not found.**)

After Bonferroni correction none of the SNPs remained significant as the adjusted p-value of 0.00020, when corrected for 254 tests (number of neurotoxicity candidate SNPs).

9.3.2.5 Phenotype 6: Seizures NOS

This group includes the seizures that were not picked by DDSS for PRES and excludes seizures with an underlying vascular or infection cause as it is different pathology. Seizures with other underlying aetiologies (hyponatremia, hypoglycaemia, febrile seizures) were grouped under this category. On the basis that all the children experiencing hyponatremia, hypoglycaemia or febrile aetiology do not experience seizures pointing to the underlying probable genetic cause that makes some of them more susceptible to seizures in this case. The GWAS results from the collaborating study groups were analysed against the complete list of neurotoxicity candidate

SNP's list (n=254), as the causative agent for the phenotype Seizures NOS is likely to be a mix of chemotherapeutic agents.

Table 9.5: Top SNPs showing nominally significant association with Seizures NOS at a significance level p <0.05

SNP ID	Chr	Location	Allele1	Allele2	Weight	Z score	P-value	Direction	Gene
rs1801725	3	123486447	t	g	2899	3.34	0.000837	+?+	CASR
rs9545873	13	81288492	a	c	2930	2.709	0.006758	+0+	-
rs1953783	14	82903381	a	g	2925	-2.656	0.007901	---	-
rs4558416	16	90002767	a	g	2928	-2.539	0.01112	-0-	TUBB3
rs429358	19	45411941	t	c	2899	-2.45	0.0143	-?-	APOE
rs1198402	14	82898979	t	g	2925	2.424	0.01536	+++	-
rs2503057	10	36898986	c	g	2930	-2.236	0.02535	---	-
rs3006957	10	36898852	t	g	2930	-2.236	0.02535	---	-
rs2503056	10	36899573	a	g	2930	-2.197	0.02805	---	-
rs1211307	14	82906017	a	g	2369	2.181	0.02916	+??	-
rs4667729	2	164755339	t	c	2899	2.152	0.03139	+?+	-
rs10490626	2	118552311	a	g	2899	2.149	0.0316	+?+	-

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 6 i.e., Seizures NOS, showing an association of $p < 0.05$.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z- score; "Direction" refers

to the direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

There were in total 12 SNPs that showed association with Seizures NOS when compared with the controls. The top nominally significant SNP was rs1801725 with a p-value 0.000837, mapped to the CaSR gene. (**Error! Reference source not found.**)

However, after Bonferroni correction there were no SNPs that retained their significant association with the Seizures NOS. (Adjusted p-value 0.00020 in this case as there were 254 candidate SNPs on the list for the meta-analysis).

9.3.2.6 Phenotype 7: Encephalopathy NOS

The neurotoxicity cases that were not clearly classifiable as Seizures NOS, definite SLS, SLS-DPP, definite PRES, PRES-DPP. And other identifiable causes of encephalopathy had been ruled out e.g., encephalopathy due to SIADH or other metabolic disturbances. These mainly are the cases reported to have chemotherapy-induced altered mental status, with or without other accompanying neurological symptoms, and MRI findings either reported as leukoencephalopathy or missing MRI findings. The complete list of neurotoxicity candidate SNP's list was used for testing encephalopathy NOS

Table 9.6: Top SNPs showing nominally significant association with encephalopathy NOS at a significance level p <0.05

SNP ID	Chr	Location	Allele1	Allele2	Weight	Zscore	P-value	Direction	Gene
rs1923492	1	150651457	t	c	2753	-2.509	0.01211	-0-	CRNN
rs919463	17	44065741	a	g	2740	2.239	0.02514	+?+	MAPT
rs2034256	2	164734476	t	c	2740	2.213	0.02691	+?+	-
rs12026	7	94878952	c	g	2740	2.164	0.03045	+?+	PON2
rs7493	7	94872711	c	g	2740	2.155	0.03115	+?+	PON2
rs2529657	2	199197483	a	t	2740	2.123	0.03371	+?+	AC005235.1
rs8079215	17	44064851	t	c	2740	2.114	0.03455	+?-	MAPT

rs9466410	6	22722709	t	c	2218	2.053	0.04008	+??	-
rs13267761	8	138930469	a	g	2231	2.047	0.04065	+0?	FLJ45872
rs35307996	17	747700	g	gc	522	2.037	0.04162	??+	NXN
rs10490082	2	199204986	a	t	2740	-2.02	0.0434	-?-	AC005235.1
rs1799945	6	26091179	c	g	2740	-1.983	0.04732	-?-	HFE
rs2429090	2	199200332	a	t	2740	-1.98	0.04771	-?-	AC005235.1

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 7 i.e., encephalopathy NOS, showing an association of $p < 0.05$.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z- score; "Direction" refers to the direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

There were 13 SNPs that showed nominally significant association with Encephalopathy NOS when compared with the controls. These SNPs were mapped to the genes: CRNN, MAPT, PON2, AC005235.1, FLJ45872, NXN, and HFE genes.

Although none of the SNPs remained significant after the Bonferroni correction. The adjusted p-value was 0.00020 after correcting for 254 tests.

9.3.3 Aim 3: To explore the association of SNPs with recurrence of neurotoxicity and with persistent neurological deficits

9.3.3.1 Phenotype 8: Recurrence on re-exposure

Recurrence of same type (or similar type with the same presumed drug e.g., where the initial event was SLS and the recurrent episode was encephalopathy, presumed drug for both is MTX) of neurotoxicity for the phenotypes included before, definite PRES, PRES-DPP, definite SLS, SLS-DPP, Seizures NOS and MTX-associated encephalopathy. The complete neurotoxicity candidate SNPs list (SNPs= 254) was used for the cases that had a recurrent episode on re-exposure.

Table 9.7: Top SNPs showing association with recurrent neurotoxicity at a significance level p <0.05

SNP ID	Chr	Location	Allele 1	Allele 2	Weight	Z score	P-value	Direction	Gene
rs7320755	13	109829093	c	g	2179	2.611	0.009017	+??	COL4A2
rs11100491	4	164478878	t	c	2191	2.279	0.02268	+0?	NPY5R
rs1131199	3	113542458	c	g	2186	-2.233	0.02558	-0?	SMARCA4
rs3733242	4	77894529	t	c	2707	2.203	0.02757	+?+	SHROOM3
rs2073337	10	101567426	a	g	2707	-2.107	0.03508	-?-	ABCC2
rs11867549	17	44013235	a	g	2707	-2.091	0.03649	-?-	MAPT
rs2756109	10	101558746	t	g	2179	2.044	0.04093	+??	ABCC2
rs7887242	X	20931587	a	c	2179	2.014	0.04405	+??	-
rs7945554	11		a	g	2179	2.004	0.04502	+??	STIM1

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 8 i.e., recurrent neurotoxicity upon re-exposure to the presumed causative drug, showing an association of $p < 0.05$.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL

meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z- score; "Direction" refers to the direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

There were 9 SNPs that showed nominally significant association with cases having a recurrent episode on re-exposure to the presumed agent compared with the controls. The genes mapping to these significant association SNPs were COL4A2, NPY5R, SMARCA4, SHROOM3, ABCC2, MAPT, ABCC2, and STIM1 genes. (**Error! Reference source not found.**)

However, with Bonferroni correction none of the 9 SNPs that showed nominally significant association earlier remained significant, as the adjusted p-value was 0.00020 after correcting for 254 tests.

9.3.3.2 Phenotype 9: Long-term persistence of neurological symptoms

Persistence of neuro-disabilities after the first event for the phenotypes included in the phenotypes defined above, e, g. definite PRES, PRES-DPP, definite SLS, SLS-DPP, Seizures NOS and MTX-associated encephalopathy. As persistence of neurological outcomes is being analyzed in all the above defined phenotypes; the complete list of neurotoxicity candidate gene list was tested in the meta-analysis (n=254 SNPs).

Table 9.8: Top SNPs showing nominally significant association with persistence of neurological symptoms at a significance level p <0.05

SNP ID	Chr	Location	Allele1	Allele2	Weight	Z score	P-value	Direction	Gene
rs7801662	7	18921973	t	g	2740	2.397	0.01652	+++	HDAC9
rs3758730	11	34431675	a	t	2719	-2.109	0.03495	-?-	CAT

rs6971012	7	97315198	t	c	2719	-2.082	0.03737	-?-	ASNS
rs10244266	7	87188467	t	g	2719	-1.998	0.04575	-?-	ABCB1

Single-nucleotide polymorphisms (SNPs) in neurotoxicity meta-analysis for the phenotype 9 i.e., persistence of neurotoxicity, showing an association of $p < 0.05$.; "SNP ID", rs identification number for single-nucleotide polymorphism; "Chr", chromosome; "Location" Position on the chromosome; "Allele 1" and "Allele 2" refer to nomenclature from METAL meta-analysis software, where Allele 1 is the Reference allele and Allele 2 is the alternative allele; "P-value" meta-analysis p-value; "Weight" the sum of the individual study weights (typically, N) for this marker; "Z- score", combined weighted Z-score; "Direction" refers to the direction of effect of the SNP in each cohort, thus "+++" refers to concordant positively-associated effect with neurotoxicity risk. SNPs were excluded from this table if the SNP was only able to be analysed in one cohort within the meta-analysis. "Gene" refer to consensus gene and either function of that gene or association of that gene reported earlier determined through cross-referencing of UCSC, Refseq and Ensembl databases.

The SNPs, four in this case, that showed nominally significant association with the long-term persistence of neurological deficits post event when compared with the controls. These SNPs mapped to these genes HDAC9, CAT, ASNS, and ABCB1. (**Error! Reference source not found.**)

However, the SNPs did not hold their significant association with persistence of neurological symptoms after Bonferroni correction where the adjusted p-value was 0.00020, when corrected to 254 tests.

9.4 Discussion:

This is the first GWAS meta-analysis conducted for chemotherapy-induced symptomatic neurotoxicity in childhood paediatric ALL/LBL. A list of polymorphisms of interest, obtained from published research, was investigated for its association with overall neurotoxicity, as well as association with specific types of the neurotoxicity including SLS, PRES, Seizures NOS, Encephalopathy NOS, recurrent neurotoxicity, and long-term persistent neurological symptoms post event. The analysis was against a neurotoxicity candidate SNPs list from literature, which focuses on already known associations involving pathophysiologic pathways of toxicity based on what is known about the drug's transport, metabolism, or mechanism of action. Using such a hypothesis driven focused list increases the chances of finding a significant association. On the other hand, this also limits the possibility of finding novel associations. Three large international cohorts were combined yielding 3207 samples, including 422 cases and 2785 controls. To minimise confounding caused by population stratification, principal component analysis for ancestry on the individuals with non-Caucasian ancestry was conducted by the study groups. There were SNPs identified that showed nominally significant association (p-value <0.05) with overall or specific types of neurotoxicity phenotypes but, despite the relatively large sample size of the study, there were no genome-wide significant candidates after Bonferroni correction. Where nominally significant association is defined as …. Another point worth mentioning is that the SNPs/genes that showed association with SLS-DPP and with definite PRES and PRES-DPP are different, that possibly provides an internal validation for the differential diagnostic scoring system.

In this study, even though there was a considerable amount of heterogeneity between the treatment protocols, 16 SNPs were identified as nominally significantly associated with chemotherapy-induced overall neurotoxicity (**Error! Reference source not found.**). The top SNP rs1801725 with a p-value of 0.009 maps to the gene CaSR, which is abundantly expressed in neural tissue and has already shown association with Alzheimer's and epilepsy [183,184]. The second top result, rs864600, maps to the CALN1 gene, which possibly plays a role in neurophysiology, as well as being potentially important for memory and learning. This gene has also been associated with schizophrenia in a GWAS study [185, 186]. Moreover, the above-mentioned SNPs have also shown association with methotrexate-induced neurotoxicity previously [51], thus showing nominally significant association in the PdL study further strengthens the link of neurotoxicity with rs1801725 (CaSR gene) and rs864600 (CALN1).

The SNPs (rs2520464, rs875740, rs246221, rs246240) that show nominally significant association with neurotoxicity map to ABCB1 and ABCC1, which encode for efflux transporter genes that mediate the development of resistance to anticancer drugs. These transporter genes have shown nominally significant association with vincristine-induced neurotoxicity during induction phase, [105] making the association with neurotoxicity plausible, as overall neurotoxicity includes all the other types of neurotoxicity.

BMP8A (rs10888798, rs1809697, and rs7555699), MACF1 (rs12138051, rs722357, rs6691194, rs6665948) and AC005235.1 (rs199883435 - non-intronic region), shown in **Error! Reference source not found.**, showing nominally significant association with neurotoxicity are expressed abundantly in the frontal regions and its role in neuronal axon migration, make their role plausible in the occurrence of neurotoxicity and were reported in an Australian cohort by Mateos showing significant association with methotrexate induced neurotoxicity [57].

We did not find any SNP with a nominally significant association (p-value <0.05) with Definite SLS. One possible reason could be the very limited size of the definite SLS phenotype group (N=5) which limits the power of the analysis.

There were 8 SNPs that we found nominally significantly associated (p-value <0.05) with definite, probable, and possible SLS phenotype located near the genes ASNS, RNASEL, ZDHHC19, GARNL4.

The ASNS gene (rs6971012, p-value 0.000549) encodes for the asparagine synthetase enzyme and it is required for synthesising neurotransmitters. It is noteworthy that brain cells rely solely on asparagine synthetase to produce asparagine due to the inability of this amino acid to pass the blood-brain barrier. During normal neurodevelopment, asparagine is essential, and lack of it in the developing brain cells will result in a poor brain development and severe neurological problems [187]. ASNS gene is a key therapeutic vulnerability in leukaemia and asparaginase is used in these patients and is very significant, and asparaginase can potentiate the action of MTX and causing MTX-induced toxicity making this finding note-worthy [188].

The RNASEL gene encodes a component of the interferon-regulated 2'-5'oligoadenylate (2'-5'A) system, which carries out many different functions related to the exclusion of viruses as well as the inhibition of proliferation after viral infection to protect humans against virus-induced demyelination [187]. GARNL4 (also known as RAP1GAP2 gene) encodes an activating GTPase which

acts on the small guanine nucleotide-binding protein Rap1, which is found in platelets. Located in the brain tissue according to the one study [189], it is involved in cell signalling and communication, interaction with synaptotagmin-like protein 1 and Rab27, and regulating the secretion of dense granules from platelets at sites of endothelial damage [190]. Our finding of a nominally significant association between the three genes above (ASNS, RNASEL and GARNL4) and definite, probable, and possible SLS in methotrexate-treated patients from the PdL study is consistent with previous research which also found an association with methotrexate-induced neurotoxicity [51].

Another SNP (rs1106479) that showed nominally significant association (p-value 0.02575) with definite, probable, and possible SLS phenotype, was mapping onto the ZDHHC19 gene (Zinc finger DHHC- type containing 19). This gene represents a likely palmitoyl transferase that is thought to modulate the trafficking, activity, and localization of lipidated proteins via modifications made post-translationally. In particular, this gene contributes to the palmitoylation of R-RAS, which is involved in cell viability [191]. By reorganizing F-actin in the cortical neuron axons, R-Ras plays a critical role in the growth and development of cortical neurons [192]. The association shown here in the PdL study further strengthens the link of ZDHHC19 with methotrexate-induced neurotoxicity that has been previously reported in a study for the association with methotrexate-induced neurotoxicity [57].

Seven SNPs showed nominally significant association with Definite PRES phenotype, mapping on to the genes CAT, FMO9P†, SLCO1B1, SLC5A7, ABCB1 and CYB5A. (**Error! Reference source not found.**)

The top SNP, rs3758730 with a p-value 0.004, maps onto the CAT gene, which codes for the catalase enzyme and protects cells from the toxic effects of hydrogen peroxide and other reactive oxygen species (ROS). ROS can damage biomolecules such as DNA, proteins, and cell membranes [193]. An in-vivo study in which catalase was either administered alone or co-administered with superoxide dismutase mostly prevented the neurotoxic effects of homocysteine [194]. Another SNP (rs2065920) with nominally significant association (p-value 0.006319) with definite PRES maps onto the FMO9P† gene, which is of interest because this gene is overexpressed in parietal and frontal cortex regions (areas affected in PRES) compared to other brain areas, according to the Allen Brain Atlas Developing Human Brain Tissue Gene Expression Profiles by Microarray dataset [195]. Other consolidating evidence of linking this gene

with PRES is the association of FMO9P in GWAS with heritability of diastolic blood pressure, which is particularly interesting because High blood pressure is a salient feature of PRES [196].

SLCO1B1 and SLC5A7 encode for organic anion transporters that are expressed in hepatocytes and transport compounds from the blood into the liver to be detoxified and eliminated from the body. Although previously associated with MTX-induced neurotoxicity, especially SLCO1B1, these genes might also play a role in vincristine toxicity (PRES), given that this drug is also metabolised and eliminated through the liver. Interestingly, vincristine has been identified as a specific inhibitor of SLCO1B1, which might be a possible mechanism of toxicity for SNPs in this gene [197]. This gene showing association with other non-neoplastic and neoplastic drugs is also a link that it might be associated with both MTX and vincristine but has been possibly overlooked because of 1) previously reported with MTX and 2) lack of GWAS level exploration of polymorphisms associated vincristine-induced neurotoxicity.

CYB5A encodes for a membrane-bound cytochrome, which when activated by stearyl-CoA-desaturase reduces ferric haemoglobin (methaemoglobin) into ferrous haemoglobin. Variations in CYB5A gene have been connected with psychiatric disorders such as schizophrenia [198,199].

The fact that ABCB1 and ABCC1, belonging to the same ABC transporter efflux family, have already been reported by meta-analysis for the association with the peripheral neuropathy induced by vincristine [200] strengthens the link with vincristine-induced toxicity, although the association is with peripheral form of neurotoxicity.

There were 7 SNPs that showed nominally significant association with definite, probable, and possible PRES phenotypes. The genes that were identified in proximity to these SNPs were TUBB4, SLCO1B1, PDK3, PDE4B, and CAT. (**Error! Reference source not found.**)

The top SNP (rs3865466) with the lowest p-value (0.00444) maps to TUBB4, a gene involved in neurogenesis and neuronal migration [201].The association shown in our study with definite, probable and possible PRES (vincristine-induced) provides further evidence of a possible link between TUBB4 and neurotoxicity during induction phase of the therapy in childhood acute lymphoblastic leukaemia that has been reported previously [202].

SNP (rs4149056), maps to SLCO1B1, which encodes for an organic anion transporter, a member of the Solute carrier (SLC) transporter proteins. The synthesized protein is found in liver cells and transports compounds from the bloodstream into the liver tissues to be excreted.

PDK3 and PDE4B gene: The genes that are essential for regulating the glucose metabolism and in signal transduction respectively [203,204]. These genes have been mechanistically linked with hereditary motor and sensory neuropathies, schizophrenia, and bipolar diseases which shows plausible role in PRES.

Another point of interest that the SNPs that showed association with SLS-DPP and with definite PRES and PRES-DPP are different.

The SNPs/genes (12 SNPs mapping on to CaSR, TUBB3, APOE genes) (**Error! Reference source not found.**) showed nominal association with the phenotype Seizures NOS. CaSR is expressed on neural cells and has shown an association with Alzheimer's disease [184,205] and methotrexate-associated neurotoxicity in Bhojwani *et al*. [51], which consolidates the association of this gene with seizures associated with methotrexate. This gene has also shown association in the PdL study with the neurotoxicity phenotype, which makes sense as seizures are a relatively large subset of the neurotoxicity cases.

TUBB3's crucial role in neuronal development, neural migration, and neural tissue matrix during brain development makes a strong case for the association of polymorphisms in this gene with Seizures NOS. This gene has been associated with vincristine neurotoxicity during the induction phase of the therapy in childhood acute lymphoblastic leukaemia [202] as seizures can be caused by a mix of drugs (methotrexate, vincristine).

APOE is responsible for producing a protein that plays a major role in transporting cholesterol and other fats in the blood [206]. The link of this gene with early-onset Alzheimer's has been reported previously [207]. Although the mechanism is unknown, mutations in APOE gene have shown association with neurocognitive outcomes in long-term cancer/lymphoma survivors [207].

The genetic polymorphisms associated with the phenotype defined in the PdL study as encephalopathy NOS were explored for the first time **Error! Reference source not found.**. The genes located nearby to the nominally significant SNPs were CRNN, MAPT, PON2, NXN, HFE and have functions in cell cycle progression, neuronal cell division, neuronal cell growth, neuronal

differentiation, and epidermal differentiation making it plausible that they might play a role in manifestation of encephalopathy NOS.

The CRNN gene reported here promotes cell proliferation, G1/S cell cycle progression and induces expression of the cell cycle regulator CCND1 [208]. Furthermore, on GWAS studies it has been observed that there is an association between genetic variants of this gene and disordered eating [209]. The association with methotrexate-induced neurotoxicity has been reported previously by Bhojwani et al. [51].

MAPT-encoded protein (tau) is abundantly present throughout the nervous system, including in neurons in the brain. It plays an important role in the formation and stabilization of microtubules, essential in the process of cell division, as well as in the transport of materials within a cell [210]. Previously reported association with Alzheimer's and dementia strengthens the link with encephalopathy NOS reported in PdL [211]. MAPT has also been previously linked with vincristine-associated neurotoxicity [105,202]. The PON2-encoded protein is ubiquitously expressed in brain tissues (cerebellum, cranial nerves and other), membrane-bound, and may act as a cellular antioxidant, protecting cells from oxidative stress [212]. PON2 has previously shown association with methotrexate-induced neurotoxicity reported by Bhojwani *et al.* [51].

NXN is a ubiquitously expressed endogenous antioxidant, member of the thioredoxin antioxidant superfamily involved in neural cell growth and cell differentiation [213]. In brain sections of mice, there is a predominant neuronal expression of NXN in septal nuclei and the hippocampus, in which its deletion is embryonically lethal, mainly due to cranial defects and deformities. It was found that immunoreactive signals associated with NXN were present in fibres in the cortex, hippocampus, and cerebellum [214]. It has previously shown association with methotrexate-induced neurotoxicity reported in the ERASE study [57].

The protein encoded by the HFE gene interacts with other proteins on the cell surface to detect the amount of iron in the body. It is also found on some immune system cells. In mouse models, it has been demonstrated that the HFE genotype impacts the expression of tyrosine hydroxylase in substantia nigra, providing additional support for the hypothesis that the HFE genotype is a disease modifier for Parkinson's [215].

It is interesting to include here that the 2 SNPs (rs12026 and rs7493), both mapping on PON2 gene showing nominal association here with encephalopathy NOS further strengthens the link

of these SNPs and PON2 with encephalopathy as they were previously reported to have shown significant association with leukoencephalopathy [51].

To the best of our knowledge, this meta-analysis is the first attempt to explore the SNPs/genes associated with recurrent neurotoxicity and persistent neurological deficits, in order to better understand the neurological outcomes post-event and because they indicate a severe toxicity phenotype and therefore might have the strongest genetic association. In the combined, non-stratified analysis (including any type of neurotoxicity), when investigating recurrent neurotoxic events or long-term persistence of neurological symptoms post event, we found some of the same genes/SNPs as in the previous, stratified analysis investigating for associations with SLS, PRES and Seizures.

The nine SNPs that showed nominally significant association with recurrent neurotoxicity (**Error! Reference source not found.**) mapped near the genes COL4A2, NPY5R, SMARCA4, SHROOM3, ABCC2, MAPT, and STIM1, which have plausible roles in neuronal development, neural transcriptional role, neural cytoskeletal assembly, neuronal differentiation, neuropeptide receptor and type IV collagen synthesis. COL4A2 has been reported to play a role in Brain Small Vessel Disease 2 and Intracerebral Haemorrhage and SMARCA4 and SHROOM3 with neural tube defects and NPY5R gene has been linked with eating disorders [216-218] [219]. These candidate SNPs/genes were also previously reported in methotrexate- induced neurotoxicity/leukoencephalopathy and showing association of these SNPs/genes in PdL strengthens the evidence regarding their involvement in not only first-time occurrence of neurotoxicity but also with a recurrent neurotoxic episode [51].

MAPT has been linked with Alzheimer's and dementia [211,220], STIM1 has been reported to play a role in pregnancy-induced hypertension [220] and ABCC2 is not only involved in drug transport but has also shown role played in multidrug resistance including anti-cancers [221]. Interestingly, MAPT, STIM1 and ABCC2 genes were previously reported to be significantly associated with Vincristine-associated neurotoxicity [105,202].

There were four SNPs that showed nominally significant association with the cases that had long-term persistent symptoms (**Error! Reference source not found.**). The genes located near these SNPs were HDAC9, CAT, ASNS, ABCB1. HDAC9 plays an important role in transcriptional regulation, cell cycle progression and developmental events [222] and CAT produces catalase

enzyme, which has shown to prevent homocysteine-mediated neurotoxicity in in-vitro models [194]. ASNS encodes asparagine synthase, which is required for neurodevelopment, and deficiency in this enzyme leads to a failure of the brain to develop and severe neurological problems [223]. ABCB1 is involved in drug pharmacokinetics and mutations can lead to failure of drugs to pass through the blood–brain barrier into the central nervous system, which can lead to inadequate levels in brain of anti-depressants and antipsychotics by affecting it's elimination (risperidone, trazodone and aripiprazole enhanced elimination: olanzapine and citalopram reduced elimination) [224,225].

The genes that mapped to the SNPs showing nominally significant association were a mix of genes that had shown significant association with methotrexate and vincristine-associated neurotoxicity previously. HDAC9, CAT, ASNS gene had previously shown significant association with methotrexate-induced neurotoxicity [51]. For ABCB1, Ceppi F *et al*. reported no association between ABCB1 polymorphisms (rs4728709) and vincristine-associated neurotoxicity [226], while the PdL study finds a nominal association of a ABCB1 polymorphism with persistent neurological outcomes in neurotoxicity. This can be explained with the following considerations: 1) our study investigates a different SNP (rs10244266) mapping to ABCB1; 2) our results are from a larger cohort 3) different phenotype (long-term persistent neurological deficits vs. vincristine-associated neurotoxicity only).

The fact that most of these SNPs/genes that showed nominal association with recurrence and persistent neurological deficits, have shown association previously in PdL study with either Definite PRES (CAT gene, ABCB1 gene) or with Definite, probable and possible SLS (ASNS gene) or neurotoxicity (MAPT) strengthens the links of the variations in these SNPs/genes with neurological outcomes in neurotoxicity phenotypes i.e. recurrent neurotoxicity episode and long term persistence of neurological symptoms.

Conclusion:

None of the SNPs reached significance after adjusting for multiple testing. In some phenotypes this reflected small numbers of cases. Another possible reason could be that including the SNPs and p-value(s) together from different tests reduced the statistical power.

10 Overlapping the differently methylated genes post methotrexate-exposure with the significantly associated genes with methotrexate-induced neurotoxicity.

10.1 Background

Improving cure rates in paediatric ALL came at a cost of chemotherapy-induced neurotoxicity reported in 8-12% of cases on different arms of the UKALL 2003 study [13,47].

DNA methylation, one of the epigenetic markers that can modulate gene expression, is altered more easily by a variety of more subtle exposures than DNA sequence itself [106,107]. Evidence suggests that DNA methylation may play an important role in chronic neurotoxicity such as cognitive impairments [108]. Altered DNA methylation of brain/central nervous system cells could be one mechanism involved in methotrexate treatment-related neurotoxicity and late neurocognitive effects in ALL survivors [109]. Moreover, epigenetic reprogramming is the process by which an organism's genotype interacts with the environment to produce its phenotype [110,111]. A recent study has also focused on identifying the possible mechanisms between drugs interaction, environmental factors and neurotoxicity [66].

It is clear from the literature that a complete mechanism explaining the link between genomic factors (SNPs), epigenetics (DNA methylation status) and neurotoxicity is largely missing. A study on developing rats showed post-methotrexate exposure epigenetic changes affecting neurogenesis and myelination processes [112]. It is not known how DNA methylation affects genetic polymorphisms and how these interacts with the outcome (neurotoxic events). Therefore, mapping methylation changes post-exposure to MTX with genetic variations associated with neurotoxicity may shed some light on the interplay that leads to expression of neurotoxicity. It would be interesting to investigate if genes that showed altered methylation in neuronal cells post exposure to methotrexate may have overlap with genes identified using GWAS analysis of patients with methotrexate-induced neurotoxicity.

To see if the genes that showed altered methylation in neuronal cells in response to methotrexate exposure had any overlap with genes identified using GWAS analysis of patients with methotrexate induced neurotoxicity.

10.2 Aims

1) Identify genetic loci that were present in both the datasets and see if loci (or annotated associated genes) with significant differential response in methylation were enriched in neurotoxicity vs no neurotoxicity cohort

2) Identify any individual genes that were significantly associated with neurotoxicity and significantly differentially methylated in response to methotrexate treatment.

10.2.1 Material and methods

For this purpose, two secondary datasets were obtained. Dr Foster contributed from in-vitro study conducted on hybrid neuronal cell lines and methylation assay ran post methotrexate treatment (Dataset 1). Dr Bhojwani contributed a dataset on genetic variations (SNPs) associated with neurotoxicity and leukoencephalopathy in paediatric ALL cases (Dataset 2).

In vitro, in vivo and in silico models were used for these investigations. (Explained in detail in the chapter material and methods chapter 2).

10.3 Results:

10.3.1 Aim 1: To identify genetic loci that were present in both the datasets and see if loci (or annotated associated genes) with significant differential response in methylation were enriched in neurotoxicity vs no neurotoxicity cohort

Steps of analysis

10.3.1.1 Target position (SNP/ CpG) based mapping:

a) In order to identify a link between SNPs and DNA methylation, an exact match would explain a possible gene regulating activity link between epigenetics and genomic variations. This would

indicate a direct relationship as there is evidence that DNA methylation can also positively correlated to gene transcription when found in gene bodies.[227] However, a thorough scanning for a match between SNPs and CpG sites, with the help of R- script, no exact match was observed.

b) Similarly, mapping the methylation variation with genetic variation on nearby locations might point to a link between methylation variation and genetic variation in expression. For this purpose, CpG site positions and SNP positions up to 50 base-pair distance from each other were explored with an R- script. There were no SNPs and CpG target position IDs within 50 – base pair distance range when searched with an R- script.

c) Search for a nearest location:

The SNPs between the two files (Dataset 1 and Dataset 2) that are nearest to each other were explored. The resultant distance of base-pairs found was - 5996 base pairs, as the nearest located SNP with CpG targets between the methylation variation and genetic variation datasets. Then it was decided to adapt a model of mapping based on gene-to-gene matching between methylation variation and genetic variations to explore an association between the two.

10.3.2 Aim 2: To identify any individual genes that were significantly associated with neurotoxicity and significantly differentially methylated in response to methotrexate treatment.

10.3.2.1 Genes based matches between the two datasets:

There were 4 gene matches between the two datasets:

1) PDE4B
2) BCL11A
3) ASTN2
4) CD14

Out of these only two, PDE4B and ASTN2, had the consistently hypermethylated response to all drug doses of methotrexate and had also shown significant association with neurotoxicity.

10.3.2.2 PDE4B

This gene is a member of the type IV, cyclic AMP (cAMP)-specific, cyclic nucleotide phosphodiesterase (PDE) family. The encoded protein regulates the cellular concentrations of cyclic nucleotides and thereby play a role in signal transduction [228].

Phenotype significance:

Altered activity of this protein has been associated with schizophrenia and bipolar affective disorder.[229-231]There is also evidence from mouse models about involvement in dopamine-associated and stress-related behaviours.

Genetic variation significance:

SNP ID: rs6696880 (source Dataset 2)

The significance of association with clinical neurotoxicity: p value = 0.032

Methylation variation significance:

Methylation variation: 20 nM:6%, 30 nM:7%, 50 nM:8 %

10.3.2.3 ASTN2:

This gene encodes a protein that is expressed in the brain and may function in neuronal migration. A deletion at this locus has been associated with schizophrenia and age at onset for Alzheimer's [232,233].

Phenotype significance:

It has known association with ADHD widely studied on different studies and age ta the onset for Alzheimer's [233-235].

Genetic variation significance:

SNP ID: rs12379211 (source Dataset 2)

Significance of association with clinical neurotoxicity: p value = 0.00003

Methylation variation significance:

Methylation variation: 20 nM: 5%, 30 nM: 6%, 50 nM: 6%

10.3.3 Validation

The result of this analysis as a gene of interest in neurotoxicity PDE4B, which already came up as nominally significantly associated with definite, probable, and possible PRES in genotyping meta-analysis, (Chapter 9 – Table 9.4).

10.4 Discussion:

This study aimed to explore the association between methylation and genetic variations for neurotoxicity post methotrexate exposure. For this purpose, a computational mapping technique was adapted to look for exact and/or in-approximation matches of SNPs or their annotated genes (in-vitro model) with CPG target IDs with their annotated genes (in-vivo model).

There were two genes, PDE4B and ASTN2, found both significantly associated with clinical neurotoxicity and had significant methylation variation. CD14 and BCLA11 did not show significant genetic association with neurotoxicity. PDE4B shows significant association with schizophrenia and bipolar affective disorder across different ethnicities [21, 22] ASTN2, has also shown a significant association with neurological diseases like ADHD and Alzheimer's [25-27].

These genes (PDE4B and ASTN2) that were consistently hypermethylated in response to all three doses (20, 30 and 50 nM) of methotrexate. Also, same genes had a significant genetic association with clinical neurotoxicity in paediatric ALL cases post methotrexate exposure. Moreover, as these differentially methylated sites are within 500 base-pairs distance with gene bodies, increasing the significance of this finding. More importantly, these genes are encoding for proteins involved in neurogenesis, hypermethylation on these genes sites, logically, resulted in decreased expression and transcription. Thus probably, increasing the risk of neurotoxicity by disrupting normal developmental physiology of neuronal cells in paediatric ALL patients.

It is also important to consider here that known association of PDE4B with schizophrenia and bipolar disorder might be more relevant for steroid psychosis type of neurotoxicity, with different aetiology and causative agent (corticosteroids)rather than methotrexate induced neurotoxicity [236,237]. Similarly, as seven of 14 patients with clinical neurotoxicity in SNP polymorphism associated with neurotoxicity cohort were also diagnosed with ADHD (four before ALL diagnosis, three post-therapy). Of these seven patients, six had inherited the risk allele (C) in rs12379211 in ASTN2 [238]. It might have falsely increased the chances of ASTN2 in this study for finding association between methylation variation and genetic variation.

Despite having two large datasets, only 4 genes matched. The plausible explanation of this might be

1) One limitation was that they come from different data sets and don't cover the entire genome but only a small subset of it.

2) methodological differences between the two datasets. Whereas both datasets were obtained post exposure to methotrexate, the concentration of methotrexate in the two were different. High doses (20, 30 and 50 nM) of methotrexate were used for treating the cell cultures whereas the physiological CSF concentration of methotrexate in ALL patients from the second dataset would be significantly lower [239,240]. This might have resulted in falsely high differentially methylated sites as compared to actual paediatric ALL cases on methotrexate treatment. The selection of hybrid cell line (M03.13- oligodendrocytes with rhabdomyosarcoma) and higher doses of methotrexate treatment might be one of the reasons of having less than expected gene matching in the methylation variation and genetic variation datasets.

Fewer number of cases in genetic variation cohort 14 with clinical neurotoxicity and 76 with leukoencephalopathy, where it was difficult to draw Genome-Wide Association Studies (GWAS) association, might also be a reason for less matches [106].

Conclusion:

A novel approach of compared two large datasets and statistically analysing genomic and epigenetic factors was undertaken. Two genes (PDE4B and ASTN2) were identified which could potentially be related to neurotoxicity. Although this study design and its findings are novel in trying to map genetic variation and methylation variation patterns associated with methotrexate induced neurotoxicity in ALL cases.

However, more studies on the similar concept, on larger cohorts, with GWAS and differentially methylated patterns reported in chemotherapy induced neurotoxicity ALL cases are required.

Overall summary and future directions:

Overall, this study has identified clinical risk factors of neurotoxicity (UKALL 2003) and developed a differential scoring system for PRES and SLS. There were several sub-studies that stemmed from the Ponte di Legno neurotoxicity cases dataset including:

1) Two intercalated projects were deputy supervised by the author of this thesis of BSc (Med Sci) Clinical Medicine student's intercalated research project exploring the deep phenotyping of the neurotoxicity cases with metabolic imbalances and of the steroid psychosis.

2) Dr Tania Christoforaki, Uppsala University, Sweden carrying out a PhD project using the PdL database under Chris's joint supervision.

Next steps for this research include:

1) Validation of differential diagnostic scoring system for PRES and SLS in collaboration with St. Jude's hospital. Dr Inaba and Dr Raja Khan.

2) Publications.

3) Genotyping (held up by the Covid-19 lab shutdown) is underway on the identified true cases from the PdL cohort.

4) The database with neurotoxicity cases due to CVST was shared with Dr Marion to include in the venous and/or arterial chemotherapy- induced thromboembolism study.

5) The database containing steroid psychosis cases was shared with Dr Deepa Bhojwani as this section was included in the PdL questionnaire on her special interest and request.

6) The database to explore the heterogeneity of protocols is near completion and will be assigned to a student to identify the variations in treatment administration and its effect on patterns and types of neurotoxicity.

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17 July 2017

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Dear Dr Halsey

Study title:	**An international retrospective investigation of central neurotoxicity related to therapy in children with acute lymphoblastic leukaemia/ lymphoma**
REC reference:	**17/LO/1258**
Protocol number:	**n/a**
IRAS project ID:	**231162**

The Proportionate Review Sub-committee of the London - Westminster Research Ethics Committee reviewed the above application on 17 July 2017.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact hra.studyregistration@nhs.net outlining the reasons for your request. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Favourable opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see

"Conditions of the favourable opinion").

Approved documents

The documents reviewed and approved were:

Document	*Version*	*Date*
Covering letter on headed paper [Covering letter]		23 June 2017
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [insurance policy]		23 November 2016
Letter from funder [GCHC award letter]		30 November 2016
Non-validated questionnaire [Data capture form]	v1.0	23 June 2017
REC Application Form [REC_Form_07072017]		07 July 2017
Referee's report or other scientific critique report [Reviewers comments]		30 November 2016
Research protocol or project proposal [Neurotoxicity protocol]	v1.0 230617	23 June 2017
Summary CV for Chief Investigator (CI) [C Halsey CV]		23 June 2017
Summary CV for student [Cv Dr Wahid]		29 June 2017
Summary CV for supervisor (student research) [Robin Young]		23 June 2017

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:

http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

17/LO/1258	**Please quote this number on all correspondence**

Yours sincerely

Mr Robert Goldstein Chair

Email: nrescommittee.london-westminster@nhs.net

Enclosures: List of names and professions of members who took part in the review

"After ethical review – guidance for researchers"

Copy to: Ms Sheena McGowan, University of Glasgow

Dr Melissa McBride, NHS Greater Glasgow and Clyde

London - Westminster Research Ethics Committee

Attendance at PRS Sub-Committee of the REC meeting on 17 July 2017

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mr Robert Goldstein	Chair - Economist	Yes	Meeting Chair
Mr Christopher Mellor	Barrister	Yes	
Mr Michael Puntis	Vice-Chair - ICU Anaesthetist	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Ms Rachel Katzenellenbogen	REC Manager