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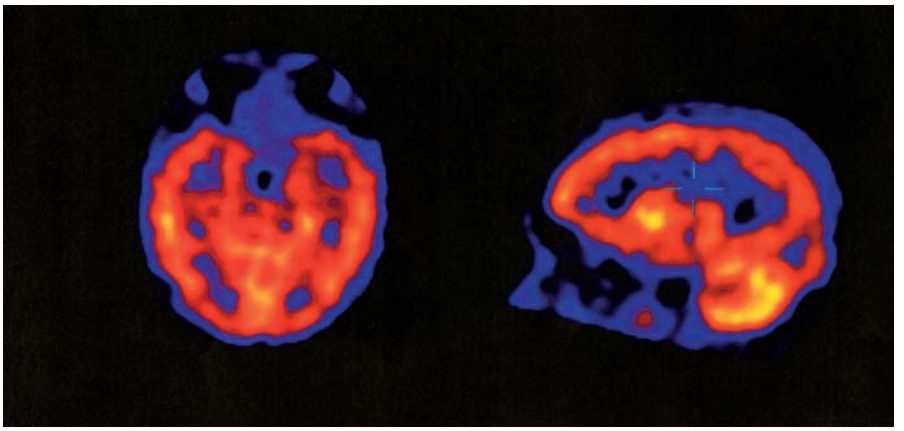

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2008

Cover picture Transverse and sagittal SPECT images from patient #19 at follow-up

Printed by Intellecta Docusys, Västra Frölunda, Sweden 2008
ISBN 978-91-628-7415-5

ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and more than 80% of the patients are cured today. Treatment might cause side effects and central nervous system (CNS) irradiation has been replaced by systemic high-dose methotrexate (MTX) and intrathecal (IT) MTX due to the risk of late effects. However, treatment without CNS irradiation is also neurotoxic and might cause brain damage.

Three patients developed subacute neurotoxicity, one after IT MTX and two after HDMTX including IT MTX. All showed impaired regional cerebral blood flow (rCBF) when examined by single photon emission computed tomography (SPECT). The patients improved within a few days during treatment with the Ca^{2+}-channel blocker nimodipine and all recovered completely. Another three patients, without neurological symptoms, were examined at different phases of ALL treatment and all had disturbances in rCBF. The heterogeneous cerebral hypoperfusion was however less pronounced than in the patients with symptoms.

Twenty-five patients were examined during remission induction with prednisolone, doxorubicin, vincristine and IT MTX. Sixteen of these patients were first examined before start of treatment and nine during the first week. None had any neurologic symptoms but rCBF had deteriorated in all patients when re-examined after four weeks. The nine patients examined during the first week had heterogeneous cerebral hypoperfusion already at the first examination but to a lesser degree than at four weeks when the two groups showed similar results. Fourteen of the twenty-five patients were re-examined seven years later, i.e. five years after cessation of treatment. Eleven had normalized rCBF, one had improved, one was unchanged and the last one had sequelae after a stroke.

Impact on CNS can also be studied by analyzing neurochemical markers of brain damage in cerebrospinal fluid (CSF). Samples were collected before start of treatment, at day 8, at day 15 and at day 29. The levels of three brain specific proteins increased during remission induction indicating damage to neurons and glia cells. Neuron-specific enolase (NSE), a marker of neurons, reached the highest level at day 8. Glia fibrillary acidic protein (GFAp), a marker of astrocytes, and the light subunit of neurofilament protein (NFp), a marker of axons, reached the highest level at day 29. Analyses of ascorbyl radical (AsR) as a marker of oxidative stress were not conclusive.

Key words: Childhood acute lymphoblastic leukemia – methotrexate – neurotoxicity – cerebral blood flow – single photon emission computed tomography – cerebrospinal fluid – neuron-specific enolase – glia fibrillary acidic protein – neurofilament – ascorbyl radical

ISBN 978-91-628-7415-5 **Gothenburg 2008**

CONTENTS

LIST OF ORIGINAL ARTICLES

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Österlundh G, Bjure J, Lannering B, Kjellmer I, Uvebrant P, Márky I. Studies of cerebral blood flow in children with acute lymphoblastic leukemia: Case reports of six children treated with methotrexate examined by single photon emission computed tomography. J Pediatr Hematol Oncol 1997;19:28-34.

II. Österlundh G, Bjure J, Lannering B, Kjellmer I, Uvebrant P, Márky I. Regional cerebral blood flow and neuron-specific enolase in cerebrospinal fluid in children with acute lymphoblastic leukemia during induction treatment. J Pediatr Hematol Oncol 1999;21:378-383.

III. Österlundh G, Kjellmer I, Lannering B, Rosengren L, Nilsson UA, Márky I. Neurochemical markers of brain damage in cerebrospinal fluid during induction treatment of acute lymphoblastic leukemia in children. Pediatr Blood Cancer 2008;50:793-798.

IV. Österlundh G, Sixt R, Uvebrant P, Márky I. Regional cerebral blood flow in children examined by SPECT five years after treatment of acute lymphoblastic leukemia. (Submitted).

ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
6-MP	6-mercaptopurine
6-TG	6-thioguanine
ADC	apparent diffusion coefficient
ALL	acute lymphoblastic leukemia
AraC	cytarabine, cytosine arabinoside
ASA	acetyl salicylic acid
ASP	Erwinia L-asparaginase
AsR	ascorbyl (or semidehydroascorbate) radical
BCNU	bischloroethylnitrosourea
BM	bone marrow
BSP	brain-specific protein
CCR	complete continuous remission
CNS	central nervous system
CPM	cyclophosphamide
CRT	cranial irradiation, cranial radiotherapy
CSF	cerebrospinal fluid
CT	computed tomography
DAMP	deficits in attention, motor control and perception
DAUNO	daunorubicin
DEXA	dexamethasone
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DOD	dead of disease
DOXO	doxorubicin
DW	diffusion-weighted
EC	enzyme commission
ECD	ethyl cysteinate dimer
EEG	electroencephalogram
EFS	event-free survival
ELISA	enzyme-linked immunosorbent assay
F	female
FDG	^{18}Fluoro-deoxyglucose
FLAIR	fluid-attenuated inversion recovery
FRFSE	fast-recovery fast spin-echo
GFAp	glial fibrillary acidic protein
GH	growth hormone
Glu	glutamate
GRE	gradient recalled echo
Gy	Gray
IQ	intelligence quotient

Hcy	homocysteine
HD	high-dose
HDAraC	high-dose cytarabine
HDMTX	high-dose methotrexate
HMPAO	hexamethylpropylene amine oxime
HR	high risk
HVA	homovanillic acid
IM	intramuscular
IR	intermediate risk
IT	intrathecal
IU	international units
IV	intravenous
M	male
MBq	mega Becquerel
Met	methionine
MRI	magnetic resonance imaging
MTX	methotrexate
NFp	neurofilament protein light sub-unit
NMDA	N-methyl-D-aspartate
NOPHO	Nordic Society of Pediatric Hematology and Oncology
NSE	neuron-specific enolase
PD	proton density
PET	positron emission tomography
PRED	prednisolone
rCBF	regional cerebral blood flow
rCMRGlc	regional cerebral metabolic rate of glucose
RNA	ribonucleic acid
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
SC	subcutaneous
SD	standard deviation
SE	spin-echo
SE	standard error
SEAA	sulfur-containing excitatory amino acid
SMN	second malignant neoplasm
SPECT	single photon emission computed tomography
SR	standard risk
TBI	traumatic brain injury
^{99m}Tc	99m-technetium
THB	tetrahydrobiopterin
THF	tetrahydrofolate
VCR	vincristine
VEP	visual evoked potentials
VHR	very high risk
WBC	white blood cell

INTRODUCTION

Background

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood and constitutes 85% of all cases of acute leukemia in children <15 years of age and 25% of all cases of pediatric malignancy. The annual incidence in the five Nordic countries in this age group is 4/100 000 [1,2].

ALL is characterized by a clonal proliferation of leukemic cells in the bone marrow, with extramedullary spread via blood vessels and often with lymphoblasts infiltrating lymph nodes, liver, spleen and other organs. Common signs and symptoms at diagnosis reflect the underlying failure of normal hematopoiesis resulting in anemia, thrombocytopenia and neutropenia, and also the extent of extramedullary spread [3]. Lymphoblasts can also reach the cerebrospinal fluid (CSF) but clinical CNS leukemia is unusual and most often asymptomatic at diagnosis [4].

Childhood leukemia was uniformly fatal within a few months until specific therapy became available. The first step towards effective treatment was when temporary remissions were achieved by the antifolate aminopterin, which was reported in 1948 [5]. This was followed in the 1950s with the development of corticosteroids [6,7] and antimetabolites (6-mercaptopurine, 6-MP) [8], and aminopterin was substituted with methotrexate (amethopterin, MTX). Combination of these drugs resulted in longer remissions and better quality of life, but the patients still relapsed and died. The addition of vincristine (VCR) and cyclophosphamide in the 1960s resulted in higher remission rates [9-11] but long time survivors were still extremely rare [12]. Along with longer survival came an increasing incidence of CNS relapse [13] and it was realized that CNS could act as a sanctuary for residual leukemic cells.

Later in the 1960s L-asparaginase, daunorubicin and cytarabine was added to the armory and the concept of "total therapy" was developed [14,15]. The different treatment phases, i.e. remission induction, intensification (consolidation), maintenance (continuation) and CNS-directed therapy were introduced and with an effective treatment to eliminate residual lymphoblasts within the CNS, cure became an option [14,16]. Craniospinal irradiation 24 Gy or cranial irradiation (CRT) 24 Gy plus five concurrent doses of intrathecal (IT) MTX reduced the CNS relapse rate to <10% [17]. CRT dose has later been reduced to 18 Gy, combined with IT MTX, without reducing survival [18].

CRT remained the golden standard of CNS-directed therapy for many years, but with the increasing number of cured children it became apparent that CRT carried the risk of development of second malignant neoplasms, i.e. brain tumors [19,20]. Furthermore, children treated with CRT showed signs of cognitive dysfunction and declines in intelligence quotient (IQ) in a prospec-

tive evaluation published in 1981 [21]. CRT can also cause neuroendocrine late effects and impaired growth [22].

With the intention to avoid these late side effects CRT has gradually been replaced by the combination of IT MTX or triple IT therapy (MTX, AraC and hydrocortisone) and high-dose (HD) systemic MTX and the good treatment results have been maintained [23-26].

Another important insight is that ALL is a heterogeneous disease and treatment has to be adapted to different prognostic factors. Some patients can be cured with less treatment and subsequently avoid side effects but other patients need more intense treatment to have a chance to be cured. The most important risk factors at diagnosis are still WBC count, age and immunophenotype. Besides them specific cytogenetic aberrations, treatment response and extension of extramedullary disease are established risk factors today [27-29].

The steady improvement of treatment results over the last four decades are truly impressive and the long-term results from 12 international study groups were published in Leukemia in December 2000 reporting 36 000 children diagnosed over 20 years [25,30-40]. Results from the "total therapy" studies are presented in Figure 1.

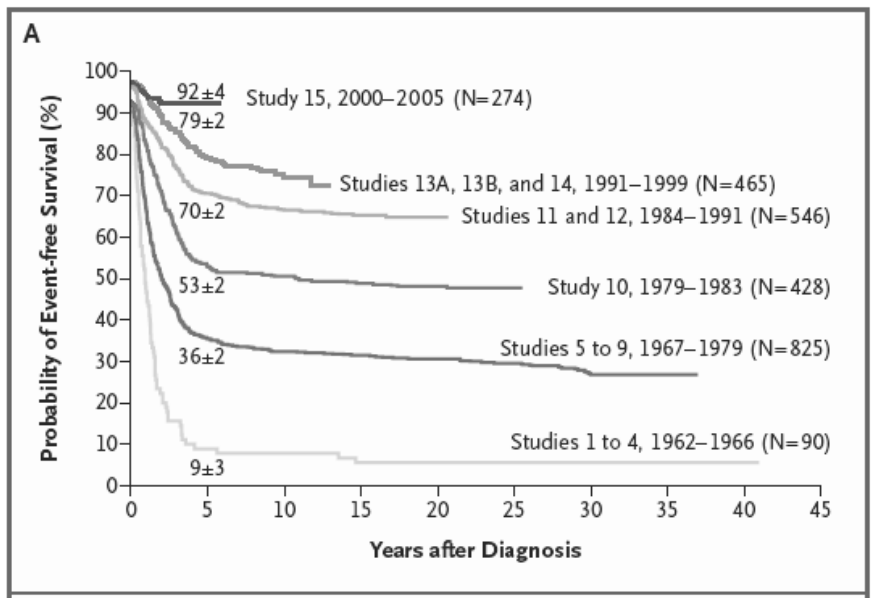


Figure 1. Improvement of treatment result in total therapy studies at St Jude Children's Research Hospital. Event-free survival ±SE (5-year EFS, except for Study 15 with preliminary 4-year EFS). Figure from [27].

The Swedish Child Leukemia Group (SCLG) was established in 1967 and managed already the following years to establish a uniform treatment for all children with ALL in Sweden [41,42]. A further step was taken with the foundation of the Nordic Society of Pediatric Hematology and Oncology (NOPHO) in 1984 although the registration of all children <15 years of age with ALL in the Nordic countries (Denmark, Finland, Iceland, Norway and Sweden) started already in 1981. Common treatment protocols were gradually developed for the different risk groups and since 1992 all children with ALL are treated with a uniform Nordic protocol, NOPHO ALL-92 [33,43].

The survival of children with leukemia has steadily improved since the introduction of common chemotherapy protocols in Sweden (Figure 2).

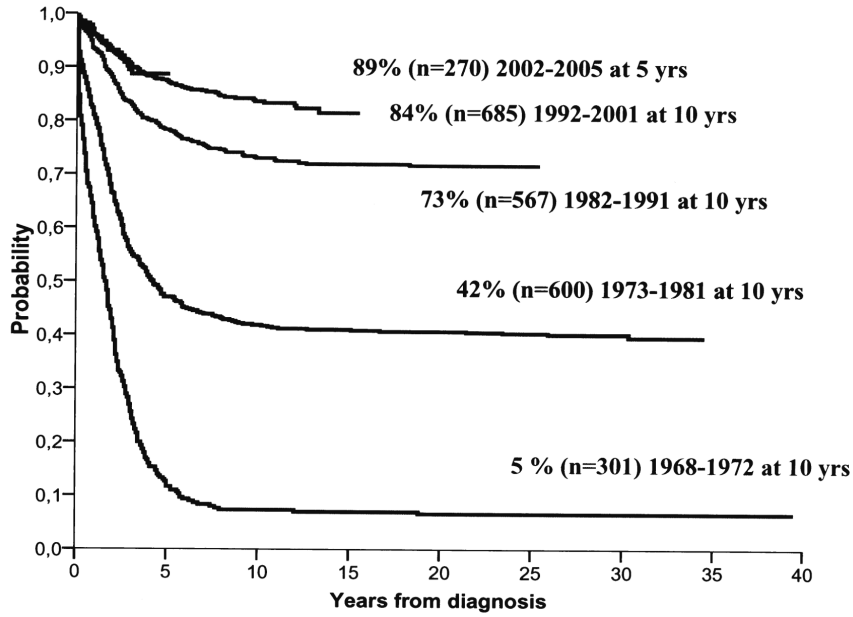


Figure 2. Overall survival for Swedish children with ALL diagnosed 1968-2005. From the Swedish Childhood Cancer Registry [44].

The patients studied in this thesis were treated with the NOPHO ALL-92 protocol, which is described in detail below (Table 3). Treatment results of the protocol are well comparable with other current protocols [1,33,43]. Remission rate was 98%, event-free survival 77% (5-year) and 74% (10-year), and overall survival 87% (5-year) and 84% (10-year). Ten percent of the patients belong to the VHR group that received cranial irradiation and the overall CNS relapse rate was 5% [1,33].

The NOPHO ALL-2000 protocol followed NOPHO ALL-92 and now the next generation, NOPHO ALL-2008, is under preparation.

NOPHO ALL-92

The NOPHO ALL-92 protocol was in use 1992–2000 and all patients in the present study were treated according to this protocol. The criteria for stratification to the different treatment groups are shown in Table 1. Infants <1 years of age and patients with mature B-ALL are treated according to other protocols and subsequently excluded.

Table 1. Risk group classification in the NOPHO ALL-92 protocol [33].

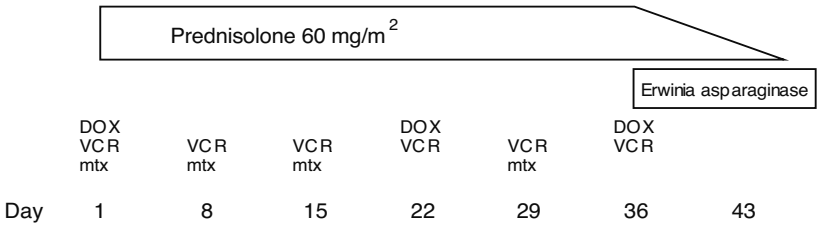
Risk group	Age (years)	Criteria
Standard	2 – <10	WBC <10 x 10^9/L No high-risk criteria
Intermediate	2 – <10 or 1 – <2 or ≥10	WBC 10 – <50 x 10^9/L WBC <50 x 10^9/L No high-risk criteria
High	≥1	and at least one of the following: WBC ≥50 x 10^9/L Mediastinal mass CNS or testicular involvement Chromosomal translocation t(9;22), 22q-, t(4;11) Slow response (day 15 M3 or day 29 M2/M3 BM) T cell leukemia
Very high	≥5	and at least one of the following: Lymphomatous features CNS involvement Slow response (day 15 M3 or day 29 M2/M3 BM) T cell leukemia with other HR criteria

M1: <5% lymphoblasts; M2: 5–25% lymphoblasts; M3: >25% lymphoblasts

Induction treatment (seven weeks) consists of oral prednisolone (PRED), intravenous vincristine (VCR), doxorubicin infusions (DOXO), and intrathecal (IT) methotrexate (MTX), followed by intramuscular Erwinia L-asparaginase (ASP). The difference between the risk groups in this phase is one extra dose of doxorubicin 40 mg/m^2 given to HR and VHR patients at day 8 (Figure 3).

For all patients, except those in the SR group, induction treatment is followed by early intensification with oral mercaptopurine (6-MP), IV cyclophosphamide (CPM), AraC and IT MTX.

INDUCTION Standard risk and intermediate risk



INDUCTION High risk and very high risk

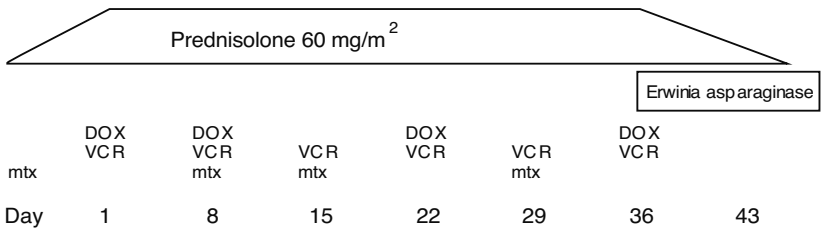


Figure 3. Induction treatment. HR/VHR patients with great tumor burden start with gradually increasing prednisolone dose.

The dose of IT MTX is 12 mg for all patients ≥3 years of age, 10 mg 2–<3 yrs and 8 mg 1–<2 yrs. Patients with CNS leukemia receive more intense IT treatment until remission, initially triple therapy (MTX, AraC, and PRED) twice weekly until the CSF has cleared, followed by weekly IT MTX for another five weeks. Only patients in the VHR group ≥5 years of age receive cranial irradiation (18 Gy).

All patients receive HD MTX; in the SR group 40 g/m^2, in the IR group 45 g/m^2, and in the HR and VHR groups 32 g/m^2 and 16 g/m^2 respectively. Furthermore, the HR and VHR patients receive 48 g/m^2 and 24 g/m^2 HD AraC respectively.

The CNS-directed therapy and the main neurotoxic chemotherapeutics are shown in Table 2 and doses and timing of all treatment in the protocol are found in Table 3.

Late intensification for all patients, except SR, includes oral dexamethasone (DEXA), VCR, daunorubicin (DAUNO), ASP, CPM, AraC, oral thioguanine (6-TG), and IT MTX.

Oral maintenance treatment consists of weekly MTX and daily 6-mercaptopurine (6-MP) and therapy is discontinued after 2.5 years for SR patients and after two years for all other patients.

Table 2. CNS-directed treatment and neurotoxic chemotherapy in the NOPHO ALL-92 protocol.

Treatment group		SR	IR	HR	VHR
HDMTX (g/m^2/24 h) IV		8 x 5	9 x 5	4 x 8	2 x 8
Injections of IT MTX*		13	17	16	18
HDAraC (g/m^2) IV		-	-	4 x (6 x 2)	2 x (6 x 2)
VCR (mg/m^2)** IV		11 x 2	14 x 2	19 x 2	20 x 2
Erwinia L-asparaginase (IU/m^2) IM		10 x 30,000	14 x 30,000	14 x 30,000	14 x 30,000
Corticosteroids	PRED	4420	4420	5820	3440
(mg/m^2) Orally	DEXA	-	250	250	250
Cranial irradiation		-	-	-	18 Gy

*) Dose according to age: 1 – <2 years 8 mg; 2 – <3 years 10 mg; ≥3 years 12 mg
**) VCR maximum single dose is 2 mg.

Table 3. NOPHO ALL-92 treatment protocol. Adapted from [26].

Treatment element/drug	Single or daily dose	Days given	Comments
All risk groups			
Induction (w 0–7)			
Prednisolone (orally)	60 mg/m^2/day	1–36/45	HR/VHR-prephase
Vincristine (IV)	2 mg/m^2 (max 2 mg)	1, 8, 15, 22, 29, 36	
Doxorubicin (IV)	40 mg/m^2 (24 h)	1, 22, 36	HR/VHR 1, 8, 22, 36
L-Asparaginase (IM)	30,000 IU/m^2 daily	36–45	
Methotrexate (IT)	8/10/12 mg (age adj.)	1, 8, 15, 29	
Standard risk (SR)			
Consolidation SR (w 8–12)			
Methotrexate (IV)	5 g/m^2 (24 h)	1, 15, 29	
Methotrexate (IT)	8/10/12 mg (age adj.)	1, 15, 29	
Maintenance (w 14–)			
6-Mercaptopurine (orally)	75 mg/m^2/day	1–until 2.5 years from diagnosis	
Methotrexate (orally)	20 mg/m^2/week	1–until 2.5 years from diagnosis	
Prednisolone (orally)	60 mg/m^2/d x 7	1, 57, 113, 169, 225	
Vincristine (IV)	2 mg/m^2 (max 2 mg)	1, 57, 113, 169, 225	
Methotrexate (IV)	5 g/m^2 (24 h)	29, 85, 151, 207, 263	
Methotrexate (IT)	8/10/12 mg (age adj.)	1, 29, 85, 151, 207, 263	

Treatment element/drug	Single or daily dose	Days given	Comments
Intermediate risk (IR)			
Early intensification (w 8–14)			
6-Mercaptopurine (orally)	60 mg/m^2/day	1–14, 29–42	
Cyclophosphamide (IV)	1000 mg/m^2	1, 29	
Cytarabine (IV)	75 mg/m^2/day	3–6, 10–13, 31–34, 38–41	
Methotrexate (IT)	8/10/12 mg (age adj.)	3, 31	
Consolidation IR (w 16–23)			
6-Mercaptopurine (orally)	25 mg/m^2/day	1–56	
Methotrexate (IV)	5 g/m^2 (24 h)	8, 22, 36, 50	
Methotrexate (IT)	8/10/12 mg (age adj.)	8, 22, 36, 50	
Late intensification (w 24–30)			
Dexamethasone (orally)	10 mg/m^2/day	1–22/29	
Vincristine (IV)	2 mg/m^2 (max 2 mg)	1, 8, 15, 22	
Daunorubicin (IV)	30 mg/m^2 (24 h)	1, 8, 15, 22	
L-Asparaginase (IM)	30,000 IU/m^2	1, 4, 8, 11	
6-Thioguanine (orally)	60 mg/m^2/day	29–42	
Cyclophosphamide (IV)	1000 mg/m^2	29	
Cytarabine (IV)	75 mg/m^2/day	31–34, 38–41	
Methotrexate (IT)	8/10/12 mg (age adj.)	31, 38	
Maintenance (w 33–)			
6-Mercaptopurine (orally)	75 mg/m^2/day	1–until 2 years from diagnosis	
Methotrexate (orally)	20 mg/m^2/week	1–until 2 years from diagnosis	
Methotrexate (IV)	5 g/m^2 (24 h)	1, 57, 113, 169, 225	
Prednisolone (orally)	60 mg/m^2/d x 7	29, 85, 141, 197	
Vincristine (IV)	2 mg/m^2 (max 2 mg)	29, 85, 141, 197	
Methotrexate (IT)	8/10/12 mg (age adj.)	1, 57, 113, 169, 225	
High risk (HR)			
Induction (w 0–7)			See Induction: w 0–7
Early intensification (w 8–14)			See IR: w 8–14
Consolidation-1 HR (w 16–26)			
Methotrexate (IV)	8 g/m^2 (24 h)	1, 43	
Cytarabine (IV)	2 g/m^2 x 2 daily x 3 days	22, 64	Total dose: 2 x 12 g/m^2
Methotrexate (IT)	8/10/12 mg (age adj.)	1, 43	
Interim maintenance (w 28–35)			
Prednisolone (orally)	40 mg/m^2/day	1–7, 29–35	
Vincristine (IV)	2 mg/m^2 (max 2 mg)	1, 29	
6-Mercaptopurine (orally)	75 mg/m^2/day	1–56	
Methotrexate (orally)	20 mg/m^2/week	1–50	

Treatment element/drug	Single or daily dose	Days given	Comments
Late intensification (w 36–42)			
Dexamethasone (orally)	10 mg/m^2/day	1–22/29	
Vincristine (IV)	2 mg/m^2 (max 2 mg)	1, 8, 15, 22	
Daunorubicin (IV)	30 mg/m^2 (24 h)	1, 8, 15	
L-Asparaginase (IM)	30,000 IU/m^2	1, 4, 8, 11	
6-Thioguanine (orally)	60 mg/m^2/day	29–42	
Cyclophosphamide (IV)	1000 mg/m^2	29	
Cytarabine (IV)	75 mg/m^2/day	31–34, 38–41	
Methotrexate (IT)	8/10/12 mg (age adj.)	1	
Consolidation-2 HR (w 44–62)			
Methotrexate (IV)	8 g/m^2 (24 h)	1, 99	
Cytarabine (IV)	2 g/m^2 x 2 daily x 3 days	22, 120	Total dose: 2 x 12 g/m^2
Methotrexate (IT)	8/10/12 mg (age adj.)	1, 99	
Prednisolone (orally)	60 mg/m^2/day	43–49, 71–77	
Vincristine (IV)	2 mg/m^2 (max 2 mg)	43, 71	
6-Mercaptopurine (orally)	75 mg/m^2/day	43–98	
Methotrexate (orally)	20 mg/m^2/week	43–92	
Maintenance (w 64–)			
6-Mercaptopurine (orally)	75 mg/m^2/day	1–until 2 years from diagnosis	
Methotrexate (orally)	20 mg/m^2/week	1–until 2 years from diagnosis	
Prednisolone (orally)	60 mg/m^2/d x 7	1, 57, 113, 169, 225	
Vincristine (IV)	2 mg/m^2/day (max 2 mg)	1, 57, 113, 169, 225	
Methotrexate (IT)	8/10/12 mg (age adj.)	1, 57, 113, 169, 225	
Very high risk (VHR)			
Week 0–42			Same as HR
CNS therapy (w 44–46)			
Cranial RT	18 Gy	1–15	
6-Mercaptopurine (orally)	50–75 mg/m^2/day	1–21	
Methotrexate (IT)	12 mg	1, 8, 15	
Maintenance LSA_2L_2 (w 48–95)			6 cycles x d 1–56
6-Thioguanine (orally)	300 mg/m^2/day	1–4	
Methotrexate (IT)	12 mg	1	
Cyclophosphamide (IV)	600 mg/m^2	5	
Hydroxyurea (orally)	2400 mg/m^2/d	15–18	cycles 1–4
Daunorubicin (IV)	30 mg/m^2 (24 h)	19	cycles 1–4
Prednisolone (orally)	40mg/m^2/d	15–22	cycles 5–6
Vincristine (IV)	2 mg/m^2 (max 2 mg)	15	cycles 5–6
Methotrexate (orally)	10 mg/m^2/day	29–32	
Carmustine (IV)	30 mg/m^2	33	
Cytarabine (IV)	150 mg/m^2/day	43–46	
Vincristine (IV)	2 mg/m^2 (max 2 mg)	47	
Maintenance (w 96–)			
6-Mercaptopurine (orally)	75 mg/m^2/day	1–until 2 years from diagnosis	
Methotrexate (orally)	20 mg/m^2/week	1–until 2 years from diagnosis	

Side effects of treatment

Any kind of treatment involves a risk of side effects, both acute and chronic. This is especially true regarding anticancer treatment when cytotoxic effect against cancer cells is a prerequisite for cure, but similar toxic effects against normal cells is an unwanted side effect. Typical acute side effects of antileukemic therapy are myelosuppression with neutropenia and infections, nausea and vomiting, alopecia and mucositis and a major contributing factor to the good treatment results of today is prompt appropriate supportive care [3]. Different chemotherapeutic drugs have different toxicity profiles and the risk for cardiotoxicity, nephrotoxicity, hepatotoxicity, neurotoxicity, et cetera has to be kept in mind [45].

Not only treatment but also the disease itself can cause side effects and side effects might not become apparent until several years after discontinuation of therapy. The term "late effect" is used for chronic or late occurring outcome that becomes apparent or persists five years after diagnosis [46,47]. Several long-term sequelae have been described in patients previously treated for ALL. Anthracyclines like doxorubicin and daunorubicin carries a risk of cardiomyopathy and even if the cumulative dose is kept relatively low there is growing concerns that subclinical cardiac damage might lead to greater risk for congestive heart failure later in life [48-51]. Neuroendocrine abnormalities, mainly involving the hypothalamic pituitary axis, and impaired growth have been described after cranial irradiation (CRT). The principal finding is blunted basal spontaneous growth hormone (GH) secretion and impaired GH response to stimulation [22,52]. Obesity is associated with dysfunctional GH secretion but there are many contributing factors, among them corticosteroids, to obesity, growth disturbances and decreased bone mineral density. Avascular bone necrosis is also associated with corticosteroids [53].

Cranial irradiation can cause second malignant neoplasms [19,20] and impaired psychosocial functioning, neurocognitive disturbances and educational difficulties after CRT are also well documented [21,54-58]. A young age, female sex and a higher dose of CRT are associated with worse outcome [59-61]. Short-term memory, speed of processing, visuomotor coordination, and sequencing ability are especially affected [56]. Deficits in psychometric intelligence can be global or focal, but decreases in performance intelligence quotient (IQ) are greater than decreases in verbal IQ. Several studies have shown deficiencies in attention and concentration ability, short-term memory, digit span, symbolic reasoning, fine motor functioning and mood stability [62]. The risk of long-term sequelae is further increased when CRT is combined with IT MTX and triple therapy and the addition of HDMTX and corticosteroids seem to increase the risk further. [57,58,63-65]. CRT is regarded the main risk factor but there are also concerns about neurotoxicity in patients treated with chemotherapy only [62,66-68].

Neuropathologically there are four distinct forms of delayed CNS toxicity; cortical atrophy, necrotizing leukoencephalopathy, subacute leukoencephalopathy and mineralizing microangiopathy [69]. The underlying mechanisms are not fully understood but they are more or less associated with vascular damage caused by CRT and/or chemotherapy. Endothelial cells are susceptible to radiation damage disrupting the blood-brain barrier leading to vasogenic edema. Radiation damage can also lead to thrombosis, hemorrhage, telangiectasias, vascular fibrosis and necrosis eventually causing hypoxic injury, white matter damage and parenchymal CNS necrosis. Furthermore oligodendroglial and progenitor cells are damaged by radiation and in the end there is a picture of vascular malformations, gliosis, demyelination and coagulative necrosis [70]. Mineralizing microangiopathy is accompanied by calcifications in brain tissue, primarily in the grey matter.

Classification of neurotoxicity and typical radiological findings [62,63,66] will be discussed in the methotrexate and neuroimaging sections. However, it is hard to explicitly determine one single cause of a specific dysfunction, as late neurological, cognitive and neuropsychological sequelae (and also other acute and late effects) have to be interpreted in the context of the complex multiagent and multimodal therapy of childhood ALL. The individual factors may interact both synergistically and antagonistically and the contribution of a specific factor will differ depending on the context. Furthermore one has to take into account the disease itself, concurrent infections and other complications, and factors like age, sex, race, individual susceptibility and genetic polymorphisms.

Chemotherapeutic drugs used in NOPHO ALL-92

Corticosteroids (prednisolone and dexamethasone)

Corticosteroids bind intracellularly to the glucocorticoid receptor and induce apoptosis. Continuous saturation of the receptor is needed for significant lymphoblast kill and administration three times daily is more efficient than intermittent scheduling indication that peak concentration is less important than a persistent therapeutic level [71]. In the NOPHO ALL-92 protocol prednisolone (PRED) is used during remission induction and also together with vincristine in re-induction pulses during maintenance, including the last two courses in the LSA_2L_2 maintenance for VHR patients. Dexamethasone (DEXA) is used during late intensification, i.e. for all patients except those in the SR group. Patients with CNS leukemia receive triple IT (MTX, AraC and PRED) until CSF has cleared of lymphoblasts. The two corticosteroids PRED and DEXA differ in protein binding and elimination half-life, and dexamethasone has better CNS penetration [71,72]. On the other hand cerebral side effects of DEXA is worse. In general, all corticosteroids have some effects on almost every organ and tissue in the body and the side effects are numerous, e.g. increased appetite, immunosuppression, peptic ulceration, precipitation of

diabetes, hypertension, mood and psychiatric disorders, etc. Glucocorticoid receptors are found throughout the brain with the highest concentration in the hippocampus and in the amygdala and in the paraventricular nucleus of the hypothalamus. These areas are important for memory and cognitive sequelae are known after corticosteroid therapy. Some studies indicate that DEXA is worse in this aspect. Corticosteroids may also modulate neurotoxicity caused by MTX and CRT; both neuroprotective and neurotoxic effects are described and might depend on dose. [45,62,64,65,73-77].

Methotrexate

MTX (amethopterin) is the most widely used antimetabolite in childhood cancer therapy and MTX substituted aminopterin in the mid 1950s. It is administered orally, SC, IM, IV and IT in different schedules and doses in several different diagnoses and treatment protocols. In NOPHO ALL-92 (as in all current ALL treatment) it is used as HDMTX, i.e. systemic and CNS directed intensification, as IT MTX (CNS directed treatment) and in oral maintenance therapy (Tables 2 and 3).

MTX is a structural analog of the vitamin folic acid and is a tight-binding inhibitor of the key enzyme dihydrofolate reductase (DHFR) that is responsible for converting folates to their active reduced tetrahydrofolate (THF) form. Folic acid is a required cofactor for the synthesis of purines and thymidine, and the active metabolites act as one-carbon donors. MTX use the same pathways and membrane-transport carrier as naturally folates. After entering the cell, MTX binds to DHFR and free intracellular drug is metabolized to polyglutamated derivatives (MTX Glu_n) that cannot efflux from the cell allowing intracellular accumulation of the drug.

MTX depletes the intracellular pool of THF and partially oxidized dihydrofolic acid is accumulated in the cell. This contributes to the inhibition of DNA synthesis together with the shortage of purines and thymidylate that is the result of THF depletion. Furthermore, MTX Glu_n inhibits DHFR stronger than unpolyglutamated MTX, and inhibits other enzymes like thymidylate synthase as well.

MTX also interferes with homocystein (Hcy) metabolism and the rate of methylation of Hcy to methionine (Met) is decreased. S-adenosyl-Met (SAM) levels decrease, S-adenosyl-Hcy (SAH) levels increase and adenosine levels will rise. Catabolism of Hcy leads to an increase of sulfur-containing excitatory amino acids (SEAA). Tetrahydrobiopterin (THB) regeneration is inhibited, which might lead to impaired biosynthesis of dopamine and serotonin [45,62,66,78].

Folate physiology and the biochemical pathways are rather complex and involve also RNA synthesis, gene regulation through DNA methylation and myelin

maintenance [45,78]. Some of these folate-mediated reactions are outlined in Figure 4.

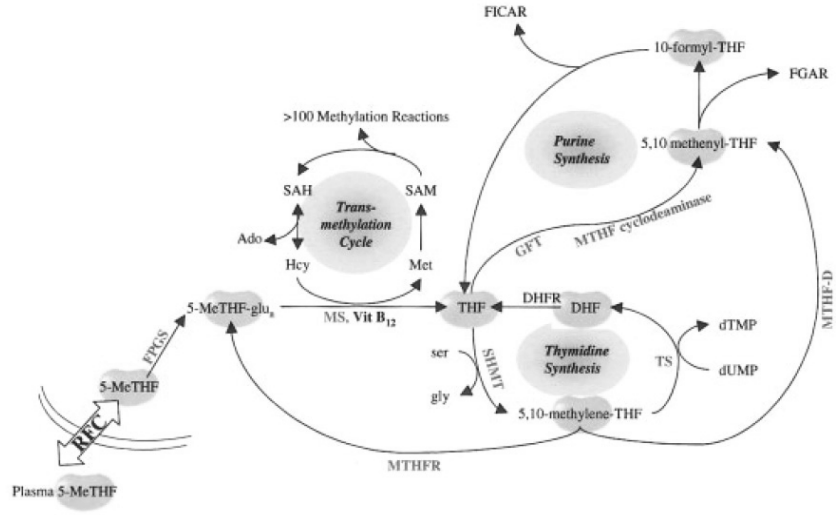


Figure 4. Some folate-mediated reactions. Reduced folates (5-methyl-THF) enter the cell through the bidirectional reduced folate carrier (RFC). Glutamate residues are added to prevent efflux and polyglutamates (5-methylTHF-glu$_n$) are formed.
The **intracellular folate pool** includes: tetrahydrofolate (THF), dihydrofolate (DHF), 5,10-methylene-THF, 5-methyl-THF, 5,10-methenyl-THF and 10-formyl-THF.
The **enzymes** indicated includes: folylpolyglutamate synthetase (FPGS), methionine synthase (MS), serine hydroxymethyltransferase (SHMT), methylene-THF reductase (MTHFR), methylene-THF dehydrogenase (MTHF-D), thymidylate synthase (TS), DHF reductase (DHFR).
Molecules in the transmethylation cycle include: homocystein (Hcy), methionine (Met), S-adenosyl-methionine (SAM), S-adenosyl-homocystein (SAH), adenosine (Ado).
Intermediates in de novo purine synthesis: formyl-glycineamide ribonucleotide (FGAR), 5-formamidoimidazole-4-carboxamide (FICAR).
Figure from [62].

MTX toxicity depends on concentration of MTX and duration of exposure. Oral maintenance is rarely associated with major toxicity but there are a few case reports on neurotoxicity in adults [79,80]. In contrast to oral low-dose MTX, HDMTX must be followed by the rescue agent leucovorin (5-formyl-THF) to prevent severe toxicities. The patients must be well hydrated and alkalinized to prevent MTX precipitation in acidic urine and serum creatinine, urine output and plasma MTX concentrations must be followed to determine the duration of leucovorin rescue. Primary toxic effects are myelosuppression

and mucositis, but nephrotoxicity is also a concern during and after HDMTX. Hepatotoxicity, dermatitis and osteopathy are reported as well [45,78].

Neurotoxicity observed after HDMTX and/or IT MTX is an issue of great importance. It was observed in increasing frequency when survival started to rise in the 1970s and at that time CNS-directed treatment generally involved both CRT and MTX. The classification (Table 4) is however generally applicable and neurotoxicity can still occur also when CRT is avoided or, as in osteosarcoma therapy, never was part of the treatment [62,63,81-83].

Table 4. Classification of (antifolate) neurotoxicity. Adapted from [62,63,66]

Neuro-toxicity	Clinical course	Clinical symptoms	Possible pathophysiology
Acute	During/within hours Transient	Headache, nausea, fever, back pain, dizziness, somnolence, confusion, disorientation, seizures	Chemical arachnoiditis, CSF adenosine ↑
Subacute	During/within 1–2 weeks Transient	Encephalopathy: hemiparesis, ataxia, pseudobulbar palsy, aphasia, confusion, affective disorders, seizures. Myelopathy: pain in the legs, sensory changes, paraplegia, bladder dysfunction	CSF homocystein ↑, causing vascular damage and excitotoxic neuronal death, through the NMDA-receptor
Delayed (Chronic)	After months to years Static or progressive	Learning disability, cognitive disturbances, decrease in intelligence. Leukoencephalopathy: confusion, somnolence, irritability, seizures, ataxia, dementia, dysphasia, tetraparesis, visual disturbances, slurred speech, coma, death	White matter damage, due to direct neuronal toxicity and impaired methylation of the myelin sheath

NMDA = N-methyl-D-aspartate

Acute neurotoxicity may occur during or within hours after HDMTX but is more common after IT MTX. It is characterized of symptoms of a chemical arachnoiditis and interpreted as an inflammatory response. Acute arachnoiditis is less common when CRT is used concomitantly due to inhibition of this inflammatory response.

Subacute neurotoxicity occurs days to weeks following treatment with MTX and the encephalopathy is also described as “stroke-like syndrome” due to the symptoms. Patients usually recover spontaneously (or during/after therapeutic measures) after a couple of days. It has been stated that it is safe to give subsequent MTX courses without increased risk of recurrence [82,84,85].

The subacute myelopathy with pain and sensory changes in the legs, paraplegia and bladder dysfunction is less common and associated with IT MTX. It might be transient or permanent [86,87].

Delayed/chronic neurotoxicity develops months to years after MTX therapy and besides neuropsychological and cognitive disturbances, the most characteristic syndrome is a leukoencephalopathy characterized by demyelination, multifocal white matter necrosis, astrocytosis and axonal damage. Especially white matter in the periventricular regions and the centrum semiovale are involved and intracerebral calcifications, cerebral atrophy and mineralizing microangiopathy have been described. Leukoencephalopathy is less uncommon and more severe in children treated with the combination of CRT, IT MTX and HDMTX [62,63,66].

The pathophysiology behind MTX neurotoxicity is not fully understood. In the context of the complexity of folate physiology and the fact that MTX interferes with a number of metabolic pathways this might not be surprising. However, two comprehensive reviews have been published the last years [62,66].

First, MTX is believed to have a direct toxic effect to the CNS [88,89]. Astrocytes seem to be the likely site for uptake and polyglutamation of MTX and neuronal disturbance, axonopathy and demyelination might be the consequence of astrocytosis. MTX can also induce signs of oxidative stress in the phospholipids of the CNS [90].

MTX-induced metabolic changes are likely to contribute to neurotoxicity and insights into pathophysiology might offer therapeutic possibilities. The main features are shown in Table 5.

Table 5. MTX-induced metabolic changes, pathogenic mechanisms, clinical symptoms and possible therapeutic options to reverse neurotoxicity. Adapted from [66].

Substance	CSF levels after MTX	Possible pathogenic mechanisms	Clinical symptoms	Therapeutic option
SAM SAH	↓ ↑	methylation capacity ↓, demethylation	leukoencephalopathy, depression, dementia	SAM
Hcy	↑	direct toxic effect to the vascular endothelium, coagulation ↑, oxidative stress ↑	cerebrovascular ischemia, mineralizing microangiopathy, focal neurological deficits	Betaine, vitamin B6, vitamin B12
SEAA	↑	excitability ↑, excitotoxicity, neurodegeneration	seizures, dementia	Dextrometorphan, Ca-channel blockers
Adenosine	↑	altered cerebral blood flow, neuronal excitability	nausea, vomiting, lethargy, headache, seizures	Aminophylline
THB	↓?	impaired biosynthesis of dopamine and serotonin	affective disturbances, hypokinesis, limb rigidity	THB L-dopa, carbidopa, and 5-hydroxytryptophan
Hcy = homocystein; SAH = S-adenosyl-homocysteine; SAM = S-adenosyl-methionine; SEAA = sulfur-containing excitatory amino acids; THB = tetrahydrobiopterin				

Since several metabolic pathways are influenced by MTX simultaneously it is hard to discriminate between the individual contributions to neurotoxicity. Folate is actively concentrated into the CNS and steady-state CSF folate is 2-3 times higher than serum levels. Patients with deficiency of reduced folates have been shown to have concurrently decreased levels of SAM, THB, homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and elevated levels of Hcy [62,66,91,92].

SAM is the methyl-donor of various molecules including catecholamines, choline, nucleic acids, phospholipids and proteins like myelin basic protein. Among other things decreased SAM levels are associated with demyelination. SAH is a strong inhibitor of methylation reactions and a lower methylation ratio (SAM/SAH ratio) was seen in two ALL patients with toxic leuko-encephalopathy [93].

The sulfur-containing amino acid Hcy may act through increased oxidative stress, altered coagulation profile and direct toxic effects to endothelial cells and the vascular intima. Hcy is believed to be involved in ischemic white matter changes, mineralizing microangiopathy and focal neurologic deficits after MTX treatment and since periventricular deep white matter is poorly vascularised these parts of the CNS is regarded more vulnerable to ischemia [66,94]. Hcy combines with adenosine (also elevated by MTX) to form SAH, which lowers the SAM/SAH ratio even more [62].

Hcy and its metabolites are sulfur-containing excitatory amino acids (SEAA) that activate receptors like the N-methyl-D-aspartate (NMDA) receptor. This stimulation can lead to seizures and influx of Ca^{2+} ions, which leads to activation of intracellular catabolic enzymes and cell death (excitotoxicity). Furthermore, SEAA can release the excitatory amino acids aspartate and glutamate and inhibit reuptake of these neurotransmitters in neuronal and glial cells. Elevated excitotoxic levels of glutamate could further damage astrocytes leading to enhanced vulnerability of glial cells and neurons. Hypoxia and ischemia due to vascular damage could lead to release of glutamate from neuronal structures further aggravating excitotoxicity. Elevated levels of SEAA have been reported in CSF from MTX treated patients with the highest levels in patients suffering from neurotoxicity. Dextromethorphan, a noncompetitive NMDA receptor antagonist has been tried to reverse symptoms of subacute neurotoxicity with promising results. Calcium channel blockers such as nimodipine are another possible treatment option [66,95-97].

Adenosine regulates cerebral blood flow and neuronal excitability through adenosine receptors. MTX can increase the adenosine levels and high levels can cause nausea, vomiting, headache, somnolence and seizures. Such symptoms of neurotoxicity have been improved after administering the adenosine receptor antagonist aminophylline [66,98,99].

THB is necessary for the hydroxylation of tyrosine, phenylalanine and tryptophan in the biosynthesis of bioamines like dopamine and serotonin. MTX inhibits the main pathway of regeneration of THB from dihydrobiopterin. This can lead to dopamine and serotonin deficiency and symptoms of hypokinesis, rigidity, trunk hypotonia and swallowing difficulties. This has led to the suggestion to try L-dopa, carbidopa and 5-hydroxytryptophan as substitutive therapy, which was successful in one case report [66,100].

Not only do the different effects of MTX interact to cause neurotoxicity; other chemotherapeutic agents and treatment modalities modulate these effects. One important example is the corticosteroids that may increase neuronal vulnerability by inhibiting glucose utilization by neurons and glia cells increasing the glutamate concentration in the hippocampus and elsewhere. This might lead to excessive stimulation of NMDA receptors causing excitotoxic neuronal death by apoptosis [62,75].

An essential part of HDMTX therapy is leucovorin rescue to prevent unacceptable toxicity and some treatment protocols also employ leucovorin rescue to replace depleted CSF folate and reduce subacute neurotoxicity [101]. Folate is actively concentrated into the CNS and too much and too early rescue might reduce the antileukemic effect in the CNS and the cure rate [62,102]. This may also be true for other interventions to decrease toxicity, at least until the pathophysiology has been clarified.

Vincristine

Vincristine (VCR) is a vinca alkaloid that binds to tubulin resulting in disruption of the intracellular microtubular system, which leads to inhibition of mitotic spindle formation and cell cycle arrest. Furthermore, maintenance of the cytostructure, transport of neurotransmitters, hormones and proteins, and transmission of receptor signals are affected. Neurotoxicity is the dose-limiting toxicity and affects predominantly peripheral and autonomic nerves through axonal degeneration and decreased axonal transport. Typical symptoms are loss of deep tendon reflexes, neuritic pain, paresthesias, bilateral ptosis, and constipation. Also paralytic ileus, urinary retention and orthostatic hypotension can occur. The only known treatment of VCR neurotoxicity is discontinuation of the drug or reduction of the dose or frequency of treatment. VCR is administered IV and the total single dose is usually capped at 2 mg. CSF concentration is 3–5% of the corresponding plasma concentration and accidental IT administration of VCR is lethal [103]. Encephalopathy is rare but seizures, ataxia and confusion have been described. In NOPHO ALL-92 VCR is used during induction and late intensification, and as re-induction pulses together with prednisolone during maintenance, as well as in the LSA_2L_2 maintenance for VHR patients [45,104-107].

Anthracyclines (doxorubicin and daunorubicin)

The anthracyclines doxorubicin (DOXO) and daunorubicin (DAUNO) intercalate into DNA and induce topoisomerase II-mediated single- and double-strand breaks in DNA. There are also other mechanisms contributing to the antileukemic effect among them nonintercalative topoisomerase II-mediated DNA cleavage, blockage of helicase-catalyzed dissociation of duplex DNA, oxidation of DNA bases and not at least formation of free radicals causing oxidative stress in the cell. These free radicals are presumed to be responsible for the cardiotoxicity that is the major disadvantage of anthracyclines [48-51,108]. Acute toxicities are myelosuppression, mucositis, nausea and vomiting, diarrhea and alopecia. The CSF/plasma ratio is very low and DOXO and DAUNO are not associated with any neurotoxicity. In NOPHO ALL-92 DOXO is used in remission induction and DAUNO is used in late intensification and also in the LSA_2L_2 maintenance (first 4 cycles) for VHR patients [45,109].

L-asparaginase

The nonessential amino acid L-aspargine is synthesized from aspartic acid and glutamine in most tissues and the enzyme L-aspargine synthase catalyzes the reaction. Normal cells are able to up-regulate this enzyme, when needed, but sensitive lymphoblasts lack this ability and are subsequently dependant on circulating L-aspargine for protein synthesis. The bacterial enzyme L-asparaginase (EC 3.5.1.1) (ASP) rapidly depletes the circulating pool of aspargine by catalyzing the degradation to aspartic acid and ammonia. Native L-ASP is derived from Escherichia coli or Erwinia carotovora and is administrated IV, IM or SC. Principal toxicities are allergic reactions due to sensitization to the bacterial protein and decreased protein synthesis, mainly in the liver. Deficiencies or imbalances in clotting factors can lead to clotting and hemorrhagic complications, including stroke [110-113]. Hyperammonemia can lead to encephalopathy and decreased serum levels of insulin, albumin and lipoproteins, as well as hepatotoxicity and pancreatitis, have been described. L-ASP is used in remission induction day 36–45 in the NOPHO ALL-92 protocol for all patients and also in late intensification (for all patients except SR) [45,114,115].

Cytarabine

The prodrug cytarabine (cytosine arabinoside, AraC) is an arabinose nucleoside analog of deoxycytidine. The active metabolite cytarabine triphosphate (AraCTP) blocks DNA polymerase α and is incorporated into DNA-strands during replication, which leads to DNA-strand breaks and induction of apoptosis. In NOPHO ALL-92 it is used in conventional dose (four consecutive days) in early and late intensification, and also in the LSA_2L_2 maintenance for VHR

patients. High-dose AraC is used to overcome cellular drug resistance and to achieve therapeutical drug levels in CNS and is used in NOPHO ALL-92 in consolidation for HR and VHR patients. Primary toxicities are myelosuppression, nausea and vomiting, and gastrointestinal mucosal damage. The so called AraC syndrome is characterized by systemic inflammatory symptoms such as high fever, malaise, myalgia, bone, joint or chest pain, rash and conjunctivitis and is not uncommon during HDAraC treatment [116,117]. Neurotoxicity is mainly associated with HDAraC and more common in adults than in children. An acute cerebellar syndrome 3–8 days after start of therapy is most common but seizures and encephalopathy have also been described. The symptoms usually resolve within a couple of days or a few weeks but long-term neurotoxicity has also been reported. AraC is also used in triple IT therapy (MTX, AraC and PRED), which in the NOPHO protocol only is used for patients with CNS leukemia [45,118-123].

6-mercaptopurine and 6-thioguanine

The thiopurines 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) are thiol-substituted derivatives of the naturally occurring purine bases hypoxanthine and guanine. These prodrugs have to be converted intracellularly to phosphorylated thioguanine nucleotides that inhibit de novo purine synthesis and purine interconversion and are incorporated into DNA, which leads to apoptosis. The thiopurines are metabolized via two different enzymatic pathways; via xanthine oxidase and via thiopurine methyltransferase (TPMT). TMPT activity is controlled by a common genetic polymorphism and one in 300 patients is deficient of TPMT activity and subsequently extremely sensitive to the cytotoxic effects. Myelosuppression is the principal toxic effect of the thiopurines and 6-MP is also associated with hepatic dysfunction and mucositis. They are not known for any neurotoxicity. In the NOPHO ALL-92 protocol 6-MP is used in early intensification, consolidation (IR), and in maintenance (daily orally together with weekly MTX). In the VHR group it is also used concurrently with CRT. 6-TG is used in late intensification and in the LSA_2L_2 maintenance [8,45,124].

Cyclophosphamide

Cyclophosphamide (CPM) belongs to the oxazaphosphorines; a group of alkylating agents that are derived from nitrogen mustard. CPM is used in early and late intensification, i.e. for all patients except SR, and also in the LSA_2L_2 maintenance for VHR patients. It is a prodrug that is activated in the liver by cytochrome P450 and binds to a nucleophile (electron-rich atom) preferably on DNA-strands. Nitrogen mustards are bifunctional and can form DNA-DNA intrastrand and interstrand cross-links inactivating DNA eventually inducting apoptosis. Alkylating agents are myelotoxic, emetogenic, mutagenic and carcinogenic. CPM can cause hemorrhagic cystitis, nephrotoxicity and cardio-

toxicity, especially in high doses. However, in contrast to ifosfamide, it is not known for significant neurotoxicity. [45,125]

Carmustin

Carmustin (bischloroethylnitrosourea, BCNU) belongs to the nitrosourea lipid-soluble alkylating agents and is used in the LSA_2L_2 maintenance for VHR patients. It is highly reactive and by rapid spontaneous chemical decomposition the reactive alkylating intermediate is formed that forms monoadducts with DNA and then cross-links between DNA-strands or between DNA and proteins. An isocyanate moiety is also formed that is believed to be responsible for the main toxicities but also to inhibit DNA repair. BCNU cross the blood-brain barrier easily but is not known for neurotoxicity in the relatively low dose used here. Main toxicity in this setting is delayed myelosuppression [45,125].

Hydroxyurea

In NOPHO ALL-92 hydroxyurea is only used in the first four cycles of the LSA_2L_2 maintenance, i.e. only for VHR patients. Hydroxyurea is an inhibitor of the enzyme ribonucleotide diphosphate reductase that is essential for DNA synthesis and subsequently selectively kills cells in S phase. Main toxicity is myelosuppression [126].

Imaging of the brain

Since subcortical calcifications associated with ALL therapy were first observed on conventional plain radiographs in the 1970s [127,128] neuro-imaging have undergone a dramatic development. Nowadays different sophisticated anatomical and functional imaging techniques are available for the study of the CNS and the impact of disease, trauma and potentially neurotoxic treatment. All techniques have advantages and disadvantages and such aspects and the underlying basic and physical principles are discussed in references [129-131].

Computed Tomography, CT

Computed tomography was the first cross-sectional method available to detect changes in the brain and a typical finding after ALL therapy was dystrophic calcifications in subcortical white matter and the basal ganglia reflecting

mineralizing microangiopathy. This was mainly seen after treatment that included CRT. Other findings include cortical atrophy visible as ventricular dilatation and widening of the subarachnoid space, and focal white matter hypodensities indicating localized edema and/or demyelination. No clear-cut correlation between neuropsychological outcome and CT findings has been possible to establish [132-143].

Magnetic Resonance Imaging, MRI

MRI is more sensitive than CT in identifying CNS changes related to neurotoxicity except in the case of calcifications. Different studies have reported early and late white matter changes, vascular malformations, calcifications, atrophy and hemorrhage. Some of the white matter changes seem to be transient and attempts to correlate neuroimaging and neuropsychological performance have not been conclusive [143-152].

Besides anatomical MRI there are nowadays several dynamic MRI techniques, among them MR angiography, diffusion MRI, perfusion MRI, blood oxygen level dependent (BOLD) MRI and MR spectroscopy. Cerebral vasospasm and diffusion abnormalities indicating cerebral dysfunction and cytotoxic edema have been reported after chemotherapy. The use of these new techniques has just begun to further examine the pathophysiology of antileukemic treatment in the CNS [66,85,153-161].

Positron Emission Tomography, PET

Brain function and cerebral perfusion is tightly interconnected in most situations. The homeostasis hypothesis states that blood flow mirrors the underlying metabolic demands of neuronal and supportive tissue. Vasoactive substances probably further regulate local and regional blood flow. Regional cerebral metabolic rate of glucose utilization (rCMRGlc) and regional cerebral blood flow (rCBF) appear to be closely coupled under both resting and active conditions [129,153,162,163]. Examinations with ^{18}Fluorodeoxyglucose (FDG)-PET have shown decreases in glucose metabolism in the cerebral cortex, in white matter and in the thalamus in ALL patients [164-167]. One small study of long-term survivors showed that cerebral white matter glucose metabolism was reduced in patients treated with CRT but not in patients treated with IT chemotherapy (MTX ± AraC). However the cortical and subcortical grey matter rCMRGlc pattern were different and rCMRGlc were significantly lower in the thalamus in former ALL patients compared to control subjects [168]. On the other hand, in another study there were no major differences in glucose utilization or in neurocognitive performance between the patients who had received CRT and those who had not. A high WBC count at diagnosis was inversely associated with cerebral glucose utilization [169].

Single Photon Emission Computed Tomography, SPECT

SPECT is a valuable tool for measuring cerebral blood flow [170] and more available than PET due to longer half-life of the radionuclides used and no need for an on-site cyclotron. Two tracers are available, hexamethylpropylene amine oxime (HMPAO) and ethyl cysteinate dimer (ECD). The tracer cross the blood-brain barrier easily, distribute proportionally to rCBF and remain trapped in the brain sufficiently long time to allow scanning. Both tracers are labeled with ^{99m}Technetium (^{99m}Tc) with a half-life of 6 hours and HMPAO washout from CNS is about 2% per hour [129]. SPECT has been used to map cerebral hyper- and hypoperfusion in epilepsy, traumatic brain injury (TBI) and psychiatric disorders [171-176]. Studies regarding rCBF disturbances during or after ALL treatment are however few. In one study eleven patients out of twenty-five showed perfusion defects; eight of these had not been treated with CRT [177]. In another study nine patients out of twelve, showed hypoperfusion during leukemia treatment, mainly with HDAraC due to acute myelogenous leukemia [122]. In one patient with akinetic mutism after HDMTX a global, frontal dominant profoundly abnormal perfusion pattern in both gray and white matter was seen on SPECT [178]. Thirty-two ALL patients were examined five years after end of therapy and 8 of 17 irradiated patients and 5 of 15 patients treated with chemotherapy alone showed abnormal cerebral perfusion [179]. The findings did not correlate with MRI findings and neuropsychological deficits. Finally, in nineteen patients examined after end of treatment SPECT showed small perfusion defects in five of seventeen patients not visible by perfusion MRI [152].

Neurochemical markers of brain damage and oxidative stress in cerebrospinal fluid

Cerebrospinal fluid (CSF) is the compartment closest to the brain accessible in clinical practice. Neurotoxic impact and degenerative processes in the CNS are associated with an increased cell membrane turnover resulting in release of cell membrane fragments, degradation products of transmitter substances and markers of cell destruction and inflammation into the intercellular space, which is in direct contact with the CSF where these products can be detected. Some of these substances are brain-specific proteins (BSP) typical for different cells or parts of cells. Others are indirect signs of oxidative stress, immune system activation, damage to the blood-brain barrier, metabolites from drugs, etc.

Myelin basic protein, a constituent of myelin, has previously been studied in patients with ALL [91,180] and during the last years there are reports of elevated levels of tau protein, a marker of axonopathy, during remission induction [181-183].

In the present study we have analyzed three brain-specific proteins (BSP): neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAp), and the neurofilament protein light sub-unit (NFp), markers of different parts of the CNS and also a marker of oxidative stress; the ascorbyl (or semidehydro-ascorbate) radical (AsR).

Neuron-specific enolase, NSE

NSE, an isoenzyme of the glycolytic enzyme enolase (EC 4.2.1.11), is predominantly present in the neuronal soma but is also present to a lesser degree in axons. It can be found in neuroendocrine cells and to some extent in oligodendrocytes. Extracellular presence of NSE is attributed to cell destruction [184]. Outside CNS it is found in non-neural tissues and is sometimes used as a tumor marker. Other enolase isoenzymes are present in red blood cells and platelets and cross-react with the antibodies in the commercially available assays. Hemolysis will therefore cause elevated levels. NSE in CSF is a reliable marker of neuronal damage. Peak levels were reported 12 hours after hypoxic-ischemia.

Glial fibrillary acidic protein, GFAp

GFAp is expressed almost exclusively in astrocytes, constituting a major part of their cytoskeleton. Damage to the CNS, irrespective of cause, activates the astrocytes to proliferate and form filaments. In CSF, normal GFAp levels increase slowly with age, probably reflecting age-dependent astrogliosis. In many chronic brain disorders GFAp increase is due to astrogliosis [185,186]. On the other hand, after acute brain injury in adults and after full-term perinatal asphyxia, GFAp in CSF rises considerably during the first 48 hours. This is attributable to astrocyte destruction and the release of intracellular components [184,185,187].

Neurofilament protein, NFp

The neurofilament is the major structural element of axons and is composed of a triplet protein. The light sub-unit (NFp) is the essential component of the neurofilament core. Although neurofilaments are also found in the cell bodies and dendrites, elevations of NFp in CSF mainly reflect axonal damage [187-189].

Ascorbyl radical, AsR

Increased levels of BSPs in CSF reflect damage to different cells and structures in the CNS but do not explain the mechanisms behind this damage. Several studies suggest that free radicals could have an important role in vascular and neuronal brain damage after traumatic brain injury (TBI) and in hypoxic-ischemic encephalopathy, especially during the reoxygenation/reperfusion phase. This damage is probably further aggravated by secondary inflammatory processes where free radicals also play a major role. The antioxidant defense is made up of several substances, including glutathione, ascorbate and alpha-tocopherol. The water-soluble ascorbate is strongly concentrated in brain tissue and CSF through active transport. Generation of AsR is regarded as a marker of free radical-mediated oxidative stress and levels of ascorbate have been shown to decrease after severe TBI [190-193].

Introduction to the present studies (I-IV)

To summarize, the importance of CNS-directed treatment in preventing CNS relapse of ALL is generally recognized since cure became an option for children with ALL. However, after the introduction of cranial irradiation in the 1960s, it has gradually became apparent that this treatment modality involves a considerable risk of development of second malignant neoplasm, neuroendocrine and neuropsychological dysfunction and other symptoms of brain damage, especially in the youngest children [19-22,46,69,70,194].

Consequently, irradiation has been substituted with other treatment modalities, among them and most common, repeated injections of IT MTX and HDMTX [23]. However, this approach has also been suspected of carrying the risk of adverse acute and late effects and severe acute and subacute neurological symptoms have been reported [68,81,83].

The implementation of the NOPHO ALL-92 protocol, where HDMTX and IT MTX replaced CRT for all risk groups except VHR patients (by definition ≥5 years of age), offered an opportunity to study prospectively the effects of HDMTX and IT MTX on CNS functions. In the present studies we have focused on the first month of induction treatment, when only four chemotherapeutics were used, and on long-term follow-up.

AIMS OF THE STUDY

The general aim of the study was to investigate side effects on the central nervous system in children with acute lymphoblastic leukemia treated with a new protocol including intrathecal and systemic high-dose methotrexate but without cranial irradiation (for the vast majority of patients).

The specific aims were to study:

- Children with, or without, neurological symptoms with SPECT at different phases of acute lymphoblastic leukemia treatment.
- Changes in regional cerebral blood flow during induction treatment with SPECT before/at the beginning of treatment and once again after 4 weeks of treatment.
- If the changes in regional cerebral blood flow during induction treatment were transient or permanent, i.e. follow-up SPECT examinations were performed five years after treatment was completed.
- Brain specific proteins in the cerebrospinal fluid during induction treatment as markers of neuronal and astroglial damage.
- Signs of free radical-mediated oxidative stress in the brain during induction treatment by analyzing ascorbyl radical in the cerebrospinal fluid.

PATIENTS AND METHODS

Patients

The Queen Silvia Children's Hospital, Sahlgrenska University Hospital is the regional hospital for Western Sweden and referral centre for pediatric hematology and oncology with 70–90 new cases each year. The mean population <18 years of age for the time period 1991–2000 was 530 000 children. 155 cases of acute lymphoblastic leukemia were diagnosed during the period June 1992 – January 2000.

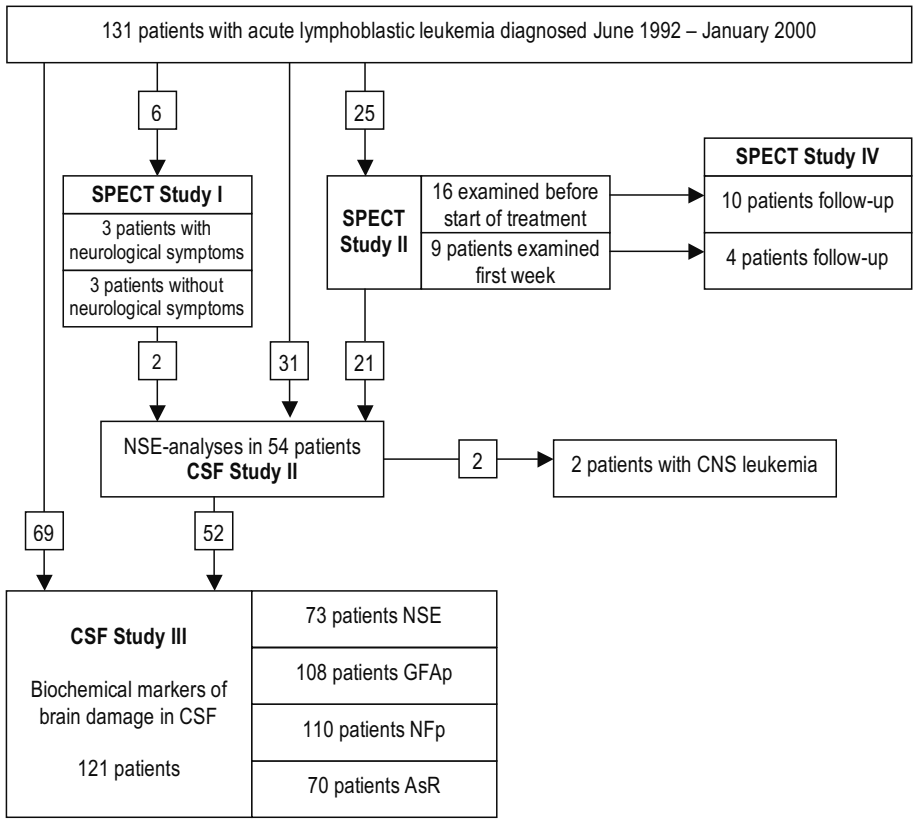


Figure 5. Number of patients who took part in one or more of the studies.

Three patients were examined by cerebral SPECT because of acute neurological symptoms at different time points during NOPHO ALL-92 treatment. Another three were examined with the intention to investigate regional cerebral

blood flow in patients without neurological symptoms as a pilot study. The SPECT examinations were performed August 1992–October 1996 and all patients were boys 4–17.9 years of age at diagnosis (Study I).

Twenty-five children were examined by SPECT during induction treatment in the time period June 1995 – October 1997. The examinations were performed at the beginning of therapy (16 untreated patients, 9 during the first week) and after four weeks, i.e. at treatment day 29. None of the patients had any neurological symptoms during the induction although one girl (#12) had slight deficits in attention, motor control and perception (DAMP) diagnosed 1.5 years before the ALL diagnosis. Another patient (#4) had CNS leukemia and received more intense intrathecal chemotherapy during induction treatment, but no irradiation because of age <5 years. (Study II).

Table 6. Sex, age at diagnosis, treatment group, day of first SPECT examination and time to follow-up of patients in Study II (n=25) and Study IV (n=14).

Patient	Sex	Age (yrs)	Treatment group	1st examination (treatment day)	Time to follow-up (years)	
					From diagnosis	After cessation
1	M	3.6	IR	0	8.3	6.3
2	F	2.9	SR	0	DOD	
3	M	3.4	HR	0	Declined	
4	F	1.5	HR	0	7.9	5.9
5	F	2.0	SR	0	7.9	5.4
6	F	6.6	SR	0	7.8	5.3
7	M	14.9	IR	0	7.9	5.9
8	M	2.5	SR	0	Declined	
9	F	4.3	IR	0	DOD	
10	F	2.7	SR	0	Declined	
11	M	4.7	IR	0	7.2	5.2
12	F	12.9	IR	0	7.1	5.1
13	F	8.8	VHR	0	DOD	
14	M	4.0	IR	0	6.6	4.6
15	F	4.3	SR	0	6.3	3.8
16	M	4.2	IR	0	6.2	4.2
17	M	11.7	IR	4	8.0	6.0
18	M	12.8	HR	5	DOD	
19	M	15.5	IR	7	8.0	6.0
20	M	10.8	IR	1	Declined	
21	F	6.0	SR	4	7.8	5.3
22	F	15.0	IR	3	7.9	5.9
23	M	6.6	SR	8	Declined	
24	F	2.1	SR	2	Declined	
25	F	7.4	SR	8	Declined	
Median		**4.7**			**7.8**	**5.3**

Fourteen of the 25 children in study II were re-examined in June 2003–April 2004, i.e. 5.4 years (mean, range 3.8–6.3) after cessation of therapy. Seven children without any neurological or other symptoms declined participation in the follow-up study and four had died of their disease. The only one who has shown any neurological signs or symptoms during treatment and follow-up was a then 12-year-old boy (#17) who suffered from generalized seizures caused by a left parietal cerebral infarction in late intensification (Study IV).

From October 1993 to March 1997 CSF samples were collected from 54 patients (including 21 examined by SPECT and 2 patients with CNS leukemia) and analyzed for NSE content (Study II).

The CSF sampling and the number of analyses were then extended and CSF samples were finally collected from 123 patients between October 1993 and February 2000. None of the patients showed any neurological symptoms. Two patients with CNS leukemia were then excluded due to more intense intrathecal chemotherapy and lack of a sufficient number of samples for statistical analysis (Study IV).

Table 7. The number of patients and their sex, age and risk group distribution in the different studies.

Study		No	Sex M/F	Age at diagnosis		Treatment group			
				Mean	Median (range)	SR	IR	HR	VHR
I	CBF pilot	6	6/0	10.3	9.9 (4.0-17.9)		3	2	1
II	SPECT	25	12/13	6.8	4.7 (1.5-15.5)	10	11	3	1
	before	16	7/9	5.2	4.1 (1.5-14.9)	6	7	2	1
	first week	9	5/4	9.8	10.8 (2.1-15.5)	4	4	1	
IV	SPECT	14	7/7	7.6	5.3 (1.5-15.5)	4	9	1	
	declined	7	4/3	5.1	3.4 (2.1-10.8)	5	1	1	
	DOD	4	1/3	6.9	6.6 (2.9-11.7)	1	1	1	1
II	CSF	54	32/22	6.1	4.6 (1.3-16.8)	18	22	10	4
III	CSF	121	74/47	6.4	4.7 (1.3-16.8)	37	58	15	11

Single photon emission computed tomography

The radiotracer ^{99m}Tc-hexamethylpropylene amine oxime (HMPAO) (Ceretec™, GE Healthcare Medical Diagnostics, Little Chalfont, Bucks, UK) is distributed in accordance with rCBF within seconds of an intravenous injection and then remains stable in the cerebral tissue for several hours. It was used in

all the studies and the patients were awake, unsedated, and not noticeably upset or anxious during the injection. Fourteen MBq ^{99m}Tc-HMPAO per kilogram of body weight was given through a central venous catheter at the SPECT examinations before and during induction treatment and through a peripheral venous catheter at follow-up. The patient was lying supine with the head in a low-attenuation head holder.

The elliptical tomographic acquisition before and during induction treatment were performed using a single-head General Electric 400 AT gamma camera (GE Healthcare, Chalfont St. Giles, Bucks, UK) equipped with a parallel-hole, low-energy, high-resolution (LEHR) collimator. The acquisitions were made over 360° in 64 projections (128 x 128 matrix, 30 s per frame) with an energy window of 20% centered at 140 keV. Transaxial, coronal, and sagittal slices (1 pixel = 3 mm thick) were reconstructed from the pre-filtered projection data (two-dimensional Hanning filter, cutoff frequency 0.88 cm^{-1}) by the filtered back-projection algorithm using a ramp filter. To ascertain good image quality, a new uniformity correction map was acquired the day before or on the same day as the investigation. Energy correction maps are made once a month (Studies I and II).

At the follow-up the acquisition was performed using a Picker Prism 3000 triple-head rotating gamma camera (Marconi Medical Systems Inc., Cleveland, OH, USA) equipped with parallel-hole, low-energy, high-resolution (LEHR) collimators. Data was acquired in a 128 x 128 matrix with a magnification of 1.6 in 128 projections with the camera running in a continuous mode with a total acquisition time of 30 minutes. The acquired data were reconstructed and filtered in three dimensions post-reconstruction (Metz filter). The reconstructed volume was repositioned in a standardized way and transaxial, coronal and sagittal slices with a thickness a 2.2 mm were obtained. New uniformity and energy correction maps were obtained on a regular basis (Study IV).

The SPECT findings were assessed visually on the basis of the relative blood flow in each lobe. At the department of Pediatric Clinical Physiology and Nuclear Medicine at the Queen Silvia Children's Hospital more than 700 children and adolescents had been examined with SPECT using this procedure when the studies were started. Even though no clearly normal group of patients had been examined, for ethical reasons, the experience acquired from this large group of young patients (mostly with neurologic disorders of some kind) led to the development of reference values at the department. The reduction in blood flow according to visual inspection has been confirmed by EEG analysis and by findings of abnormal brain tissue in corresponding regions in conjunction with epilepsy surgery.

All the SPECT images in the first two studies were analyzed by two experienced researchers who remained blind to the status of the patients and to the order of the examinations. A reduction in rCBF was diagnosed in a consensus

fashion. A previous study, which did not include the patients in the present study, has shown good interobserver reliability for this parameter, with complete agreement between the two readers. The same procedure was used in study IV, although as time had passed, one of the two reviewers had to be substituted.

In the first paper the results were presented as case reports in a descriptive manner and in the following papers II and IV according to a scoring system developed at the department.

The SPECT findings were assessed on the basis of the relative blood flow in each lobe as: normal = 0, subtle hypoperfusion = 1, mild hypoperfusion = 2, moderate hypoperfusion = 3, severe hypoperfusion = 4 and maximum hypoperfusion = 5. The number of affected lobes (0–10) was counted (frontal, temporal, parietal, occipital, cerebellar, right and left) and the SPECT score (0–50) was calculated by adding up the scores for the separate lobes (Figure 6).

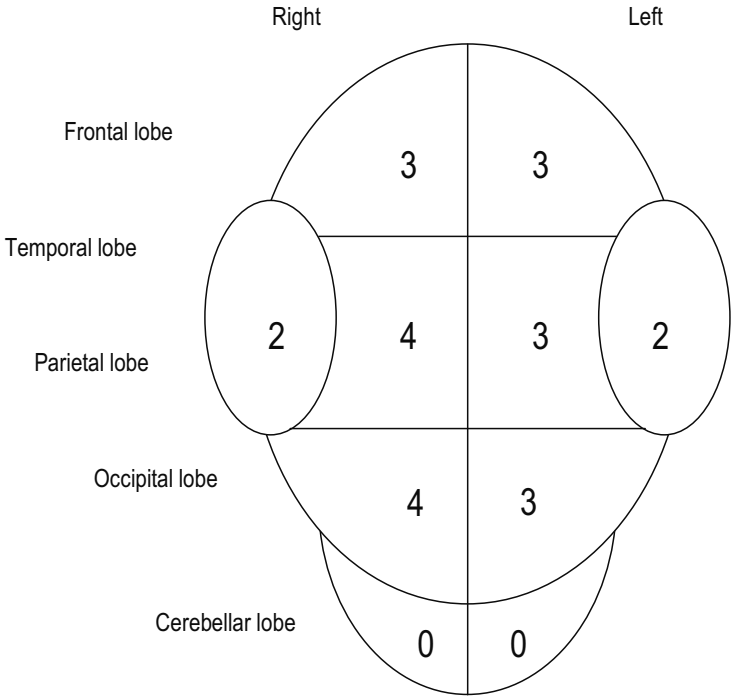


Figure 6. Example of SPECT findings (patient #5, day 29): 8 affected lobes, SPECT score 24.

Computed Tomography

In study I the computed tomography (CT) examinations were performed with and without intravenous contrast on a Siemens Somatom Plus (Siemens AG, Munich, Germany) in the first case and on a Philips Tomoscan LX (Philips Healthcare, Eindhoven, The Netherlands) in the following three cases.

Magnetic Resonance Imaging

In study I the magnetic resonance imaging (MRI) were performed on a Siemens Magnetom Impact (Siemens AG, Munich, Germany) in the first case and on a Philips Gyroview-HR scanner (Philips Healthcare, Eindhoven, The Netherlands) in the following three cases. The examinations included T1-, T2-, and proton density (PD)-weighted pulse sequences, some with gadolinium contrast enhancement.

In study IV a General Electric Signa HD 1.5T MR scanner (GE Healthcare, Chalfont St. Giles, Bucks, UK) was used for the MRI examinations and T1-, T2-, and Diffusion-Weighted (DW) images were acquired in spin-echo (SE), gadolinium contrast enhanced (+C), fluid-attenuated inversion recovery (FLAIR), fast-recovery fast spin-echo (FRFSE), and gradient-recalled echo (GRE) sequences. Also apparent diffusion coefficient (ADC) maps were made.

Cerebrospinal fluid sampling

Lumbar punctures were performed according to the NOPHO ALL-92 protocol before the start of therapy (i.e. day 0) and on days 8, 15 and 29 during induction treatment. Whenever possible, 2 mL of CSF was sampled (before injection of intrathecal MTX), chilled, divided into separate tubes and immediately frozen and stored at -70°C until analyzed.

Cerebrospinal fluid analyses

Because of the small volumes of fluid in some samples, CSF could not be analyzed at every point in time in every patient for all four markers. The highest numbers of samples analyzed were 73 for NSE, 108 for GFAp, 110 for NFp and 70 for AsR, all at day 0 (Study III).

Neuron-specific enolase, NSE

The NSE content was determined using a commercial radioimmunoassay kit (Pharmacia Diagnostics AB, Uppsala, Sweden). Calibration against pure NSE,

prepared according to the method developed by Påhlman [184], was carried out during each series of measurements. The method measures concentrations in the range of 2-200 µg/L with a detection limit less than 2 µg/L. Hemorrhagic CSF samples were excluded.

Glial fibrillary acidic protein, GFAp

GFAp concentrations were measured using an ELISA described previously but slightly modified [185,195]. In short, microtest plates were coated with hen anti-GFAp. CSF samples or reference GFAp were added and incubated in sequence with rabbit anti-GFAp, horseradish peroxidase-conjugated donkey anti-rabbit IgG and *o*-phenylenediamine. The color reaction was developed using H_2O_2. The optical density was measured at 490 nm using a computerized ELISA reader (Molecular Device Corp, Sunnyvale, CA, USA). The concentrations of GFAp were interpolated from the standard curves using log-log transformation. The standard curves ranged 16-8000 ng/L.

Neurofilament protein, light chain, NFp

NFp concentrations were measured with an ELISA similar to the one used for the GFAp analyses [188]. The standard curves ranged 125-64000 ng/L.

Ascorbyl radical, AsR

The intensity of the AsR signal was measured using a Bruker ECS 106 EPR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) as described previously [196]. Spectrometer settings were as follows: field center: 3478.5 Gauss; modulation amplitude: 1.0 Gauss; microwave power: 10 mW; microwave frequency: 9.74 GHz; scan range: 10 Gauss; scan rate: 60 Gauss/min; time constant: 0.02 s. The intensity of the signal is displayed as arbitrary units and is proportional to the amount of radicals in the sample.

Statistics

Significance was tested on paired samples using Student's paired t-test with Bonferroni correction except for the NFp results where Fisher's exact test was used. Spearman's ρ was used for correlation testing. SPSS software (SPSS Standard version 11.0.4 for Mac OS X. SPSS Inc., Chicago, IL, USA; 2005) was used for the statistical analyses except for study II where StatView software (StatView 4.5 for Mac OS. Abacus Concepts, Inc., Berkeley, CA, USA; 1995) was used.

Ethical approval

The Research Ethics Committee at the Faculty of Medicine, University of Gothenburg, Sweden has approved the study and patients were included after informed consent was obtained from the children and their parents.

RESULTS

SPECT examinations of regional cerebral blood flow.

Patients with subacute neurological symptoms (Study I).

A six-year-old boy developed déviation conjugée and a somnolent nightmare-like state six days after the second IT injection of MTX during remission induction. The following morning he had recovered and received his third IT MTX as scheduled. The same evening he experienced somnolence, hallucinations, déviation conjugée, nystagmus, and amaurosis fugax. Brain CT showed diffuse low-attenuating areas in several parts of the cortex but no edema and EEG showed signs of general encephalopathy. CSF was normal. He developed hypertension and received propranolol besides antibiotics. Six days later he experienced fluctuating somnolence, headache, nausea, vomiting, but showed no focal neurological signs. Diazepam and paracetamol had no effect, but a certain improvement was noted 20 min after acetylsalicylic acid (ASA). The following day the neurological symptoms had resolved. Cerebral SPECT (Fig 7, case 1a) showed multifocal hypoperfusion, which was more pronounced in the right hemisphere. Nimodipine was added to ASA and captopril replaced propranolol. Visual evoked potentials (VEP) were normal three days later. MRI showed multiple subcortical areas with altered signaling patterns in several parts of the brain, mainly in the white matter, with the radiological appearance of progressive multifocal leukoencephalopathy. Most of these changes had disappeared when MRI was repeated one month later. HDMTX and IT MTX was substituted by HDAraC and IT AraC plus PRED, respectively. Cerebral perfusion had improved markedly at SPECT re-examination eight months later (1b). Only discrete changes remained in some areas, still mainly in the right hemisphere. The boy is in CCR and has not had any further neurological symptoms.

Another boy experienced short periods of numbness, paresthesia and impaired motor control six weeks into ALL treatment. Brain CT was normal, EEG showed discrete but nonspecific changes, and SPECT found only a slight rCBF asymmetry (2a). The symptoms resolved spontaneously within a week and treatment continued without complications. One week after the fifth HDMTX, he suddenly developed increasing weakness, loss of sensibility and difficulty swallowing. Consciousness impaired gradually to the extent that he needed intensive care. Nimodipine treatment was started, initially with very good, but short lasting effect. SPECT performed when he had symptoms on the right side of the body, found hypoperfusion in the left hemisphere, more severe in the parietal lobe and in the anterior parts of the frontal lobe (2b). Brain CT and MRI showed normal results, as did CSF examinations. During the following days consciousness and symptoms fluctuated but gradually improved and finally disappeared during nimodipine treatment. He recovered without

sequelae and treatment was completed without parenteral MTX and further complications.

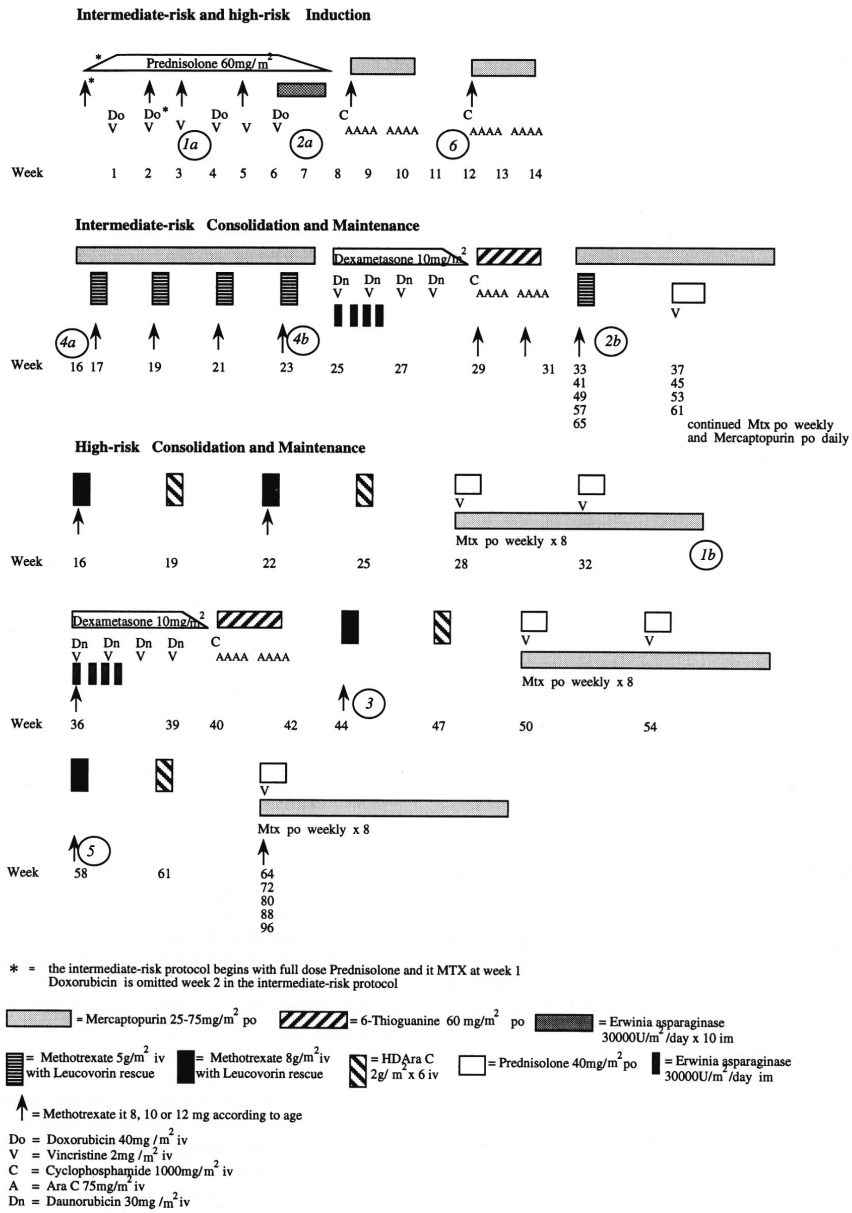


Figure 7. Timing of the SPECT examinations (case number within the circles) in relation to treatment given according to the NOPHO ALL-92 protocols (Study I).

The third patient experienced increasing numbness and weakness in his right arm two weeks after the third HDMTX. There was no impact on consciousness or any signs of CNS infection and both CT scan and EEG were normal. SPECT showed impaired rCBF (3) in the left hemisphere, in posterior parts of the frontal lobe and in anterior parts of the parietal lobe. Local hyperperfusion was also registered in other parts of the same areas. MRI was normal, except for a diffuse area deep in the white matter in the frontoparietal parts of the left hemisphere in proton density (PD)- and T2-weighted sequences. Nimodipine treatment was started the same day, and his condition completely normalized. No further HDMTX was given.

Patients without neurological symptoms (Study I).

Before the first HDMTX, but after six injections of IT MTX, local hypoperfusion was noted in the right frontal lobe and in both temporal lobes, more pronounced on the left side (4a). Brain MRI performed at the same time as the SPECT examination, and cerebral CT at diagnosis were both normal. Two months later this impairment of rCBF was slightly enhanced (4b). Hypoperfusion was noted in the same areas as before, primarily in the left temporal lobe. Furthermore, slight hypoperfusion was also noted in the left occipital lobe and in the right parietal lobe and finally the rCBF was very slightly impaired in the left frontal lobe.

SPECT was performed in another boy during the excretion phase after his fourth and last course of HDMTX (5). Multifocal disturbance of cerebral perfusion was noted 21 h after the infusion was stopped (22 h after IT MTX). Hypoperfusion was seen to an intermediate degree in the right temporal and occipital lobes and to a less pronounced degree in the temporal and parietal lobes, in the left hemisphere, and in medial parts of the cerebellum. In some local areas, the perfusion was slightly enhanced. The excretion was normal.

After five IT MTX doses, but before the start of HDMTX, SPECT revealed hypoperfusion to a varying extent in both hemispheres in a 15-year-old boy (6). Impairment of rCBF was most severe in the right hemisphere, except for the frontal lobe, where the perfusion was only very slightly impaired, as it was in the frontal and occipital lobes on the left side.

Impairment of rCBF during induction treatment (Study II).

Twenty-five patients without any neurological symptoms were examined by SPECT during induction treatment (Table 8).

The sixteen patients examined before the start of any treatment and at day 29 had an increase of the number of affected lobes from median 4 (range 1-7) to 8 (5-10). The SPECT score increased from median 6 (range 2-13) to 17.5 (8-24). The patient with the highest increase of number of affected lobes (from 1 to 10) and in SPECT score (from 2 to 21) was the girl with CNS leukemia who received the most intensive IT treatment (patient #4). The girl with the second highest rise in SPECT score (from 6 to 24) had standard-risk leukemia and no adverse events (patient #5).

The nine patients who were first examined during the first week of treatment had higher SPECT scores (median 8) than the 16 patients not yet treated at the first examination and a wider range (2-24). The effect on rCBF was enhanced at the second examination (median 20, range 6-23), even if two of the nine patients had improved, with lower SPECT scores. The number of affected lobes increased from median 6 to 8.

Table 8. Number of affected lobes and SPECT score in the 25 patients first examined in study II and in the 14 patients re-examined in study IV.

Patient	Sex	Age (yrs) Diagnosis	Treatment group	Affected lobes				SPECT score				Notes / MRI Follow-up
				Day 0	Week 1	Day 29	Follow-up	Day 0	Week 1	Day 29	Follow-up	
1	M	3.6	IR	1	-	7	2	2	-	12	3	-
2	F	2.9	SR	4	-	7		8	-	11		DOD
3	M	3.4	HR	4	-	5		5	-	8		Declined
4	F	1.5	HR	1	-	10	1	2	-	21	1	-
5	F	2.0	SR	4	-	8	3	6	-	24	3	-
6	F	6.6	SR	5	-	7	4	7	-	13	6	-
7	M	14.9	IR	4	-	8	3	6	-	19	4	-
8	M	2.5	SR	7	-	8		13	-	17		Declined
9	F	4.3	IR	2	-	8		2	-	13		DOD
10	F	2.7	SR	3	-	8		5	-	18		Declined
11	M	4.7	IR	5	-	8	4	9	-	23	7	Normal
12	F	12.9	IR	7	-	8	6	8	-	20	9	Normal
13	F	8.8	VHR	4	-	8		5	-	16		DOD
14	M	4.0	IR	4	-	6	2	5	-	13	5	-
15	F	4.3	SR	4	-	8	2	8	-	18	3	Normal
16	M	4.2	IR	7	-	8	4	12	-	19	6	-
17	M	11.7	IR	-	8	9	8	-	14	20	25	Abnormal
18	M	12.8	HR	-	9	9		-	18	23		DOD
19	M	15.5	IR	-	9	8	8	-	23	20	20	-
20	M	10.8	IR	-	6	8		-	8	20		Declined / Normal
21	F	6.0	SR	-	6	8	6	-	6	20	13	Normal
22	F	15.0	IR	-	10	8	5	-	24	14	7	-
23	M	6.6	SR	-	5	8		-	7	16		Declined
24	F	2.1	SR	-	4	5		-	6	6		Declined
25	F	7.4	SR	-	2	7		-	2	12		Declined
Median		**4.7**		**4**	**6**	**8**	**4**	**6**	**8**	**18**	**6**	

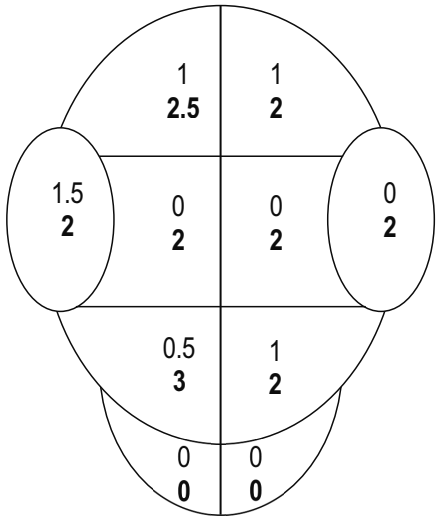


Figure 8. Median SPECT findings in the different lobes in the 16 patients that were examined before start of treatment (above), and at **day 29** (below), respectively.

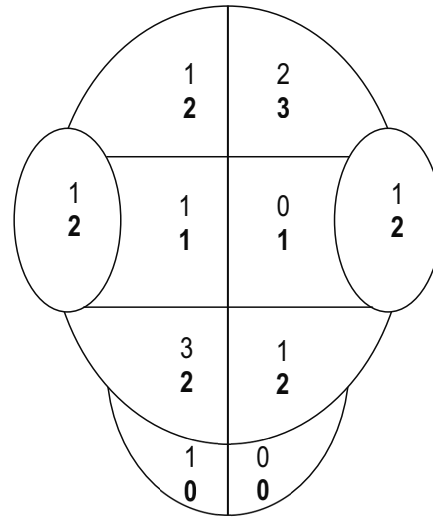


Figure 9. Median SPECT findings in the different lobes in the 9 patients that were examined the first week of treatment (above), and at **day 29** (below), respectively.

The hypoperfusion was heterogeneous and global without any preference for any hemisphere. The cerebellum was not affected. In the group of patients who were untreated at the first examination, the parietal lobes displayed the highest increase in SPECT score (from 7 to 56) at the second examination, but they still had the lowest score, compared with the temporal lobes (SPECT score 65), the frontal lobes (72) and the occipital lobes (66).

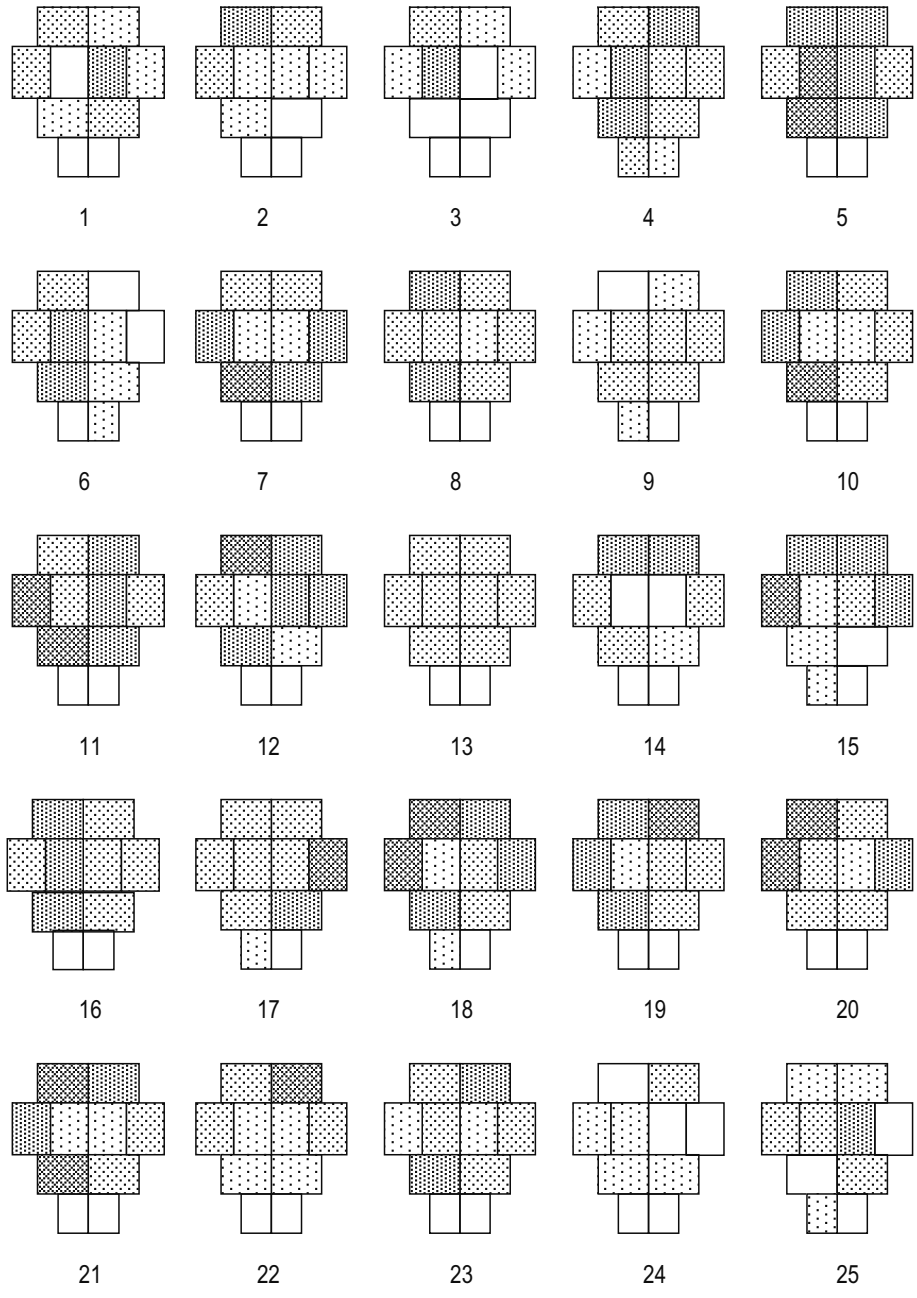


Figure 10. Lobar distribution of impaired rCBF at day 29. SPECT score range 0–5 (normal–maximal hypoperfusion) in each lobe and patient.

Table 9. Lobar distribution of SPECT findings before diagnosis (n = 16 patients) or during the first treatment days (n = 9), and after 29 days. (Study II)

	Right frontal	Left frontal	Right temporal	Left temporal	Right parietal	Left parietal	Right occipital	Left occipital	Right cerebellar	Left cerebellar
Affected lobes Day 0	9/16	12/16	10/16	7/16	2/16	4/16	8/16	9/16	4/16	1/16
Affected lobes Day 29	15/16	15/16	16/16	15/16	14/16	14/16	15/16	13/16	3/16	2/16
SPECT score Day 0	14/80	20/80	19/80	10/80	3/80	4/80	13/80	14/80	5/80	1/80
SPECT score Day 29	39/80	33/80	35/80	30/80	29/80	27/80	40/80	26/80	4/80	2/80
Affected lobes Week 1	5/9	7/9	8/9	7/9	6/9	3/9	8/9	8/9	5/9	2/9
Affected lobes Day 29	8/9	9/9	9/9	7/9	9/9	8/9	8/9	9/9	3/9	0/9
SPECT score Week 1	12/45	17/45	16/45	13/45	6/45	4/45	20/45	12/45	6/45	2/45
SPECT score Day 29	22/45	24/45	22/45	18/45	13/45	13/45	19/45	17/45	3/45	0/45

Table 10. Lobar distribution of SPECT findings before diagnosis (n = 10 patients) or during the first treatment days (n = 4), after 29 days (n = 14), and at follow-up (n = 14). SPECT score range 0–5 (normal – maximum hypoperfusion) in each lobe and patient. (Study IV)

	Right frontal	Left frontal	Right temporal	Left temporal	Right parietal	Left parietal	Right occipital	Left occipital	Right cerebellar	Left cerebellar
Affected lobes Day 0	5/10	7/10	5/10	5/10	1/10	3/10	6/10	6/10	3/10	1/10
Affected lobes Week 1	3/4	3/4	4/4	4/4	4/4	2/4	4/4	4/4	3/4	1/4
Affected lobes Day 29	14/14	13/14	14/14	13/14	12/14	13/14	14/14	13/14	3/14	2/14
Affected lobes at follow-up	5/14	7/14	4/14	11/14	6/14	6/14	8/14	11/14	0/14	0/14
SPECT score Day 0	8/50	12/50	10/50	7/50	2/50	3/50	9/50	9/50	4/50	1/50
SPECT score Week 1	7/20	10/20	9/20	9/20	4/20	3/20	11/20	8/20	4/20	2/20
SPECT score Day 29	37/70	36/70	34/70	30/70	23/70	26/70	38/70	26/70	4/70	2/70
SPECT score at follow-up	11/70	10/70	12/70	21/70	8/70	11/70	17/70	22/70	0/70	0/70

Table 11. Number of affected lobes and SPECT score in the 14 patients with follow-up: Ten patients (#1-16) first examined by SPECT before any treatment was given and four patients (#17-22) first examined during the first treatment week. (Study IV).

Patient	Sex	Age (yrs) Follow-up	Treatment group	Affected lobes				SPECT score				MRI Follow-up
				Day 0	Week 1	Day 29	Follow-up	Day 0	Week 1	Day 29	Follow-up	
1	M	11.8	IR	1	-	7	2	2	-	12	3	-
4	F	9.3	HR	1	-	10	1	2	-	21	1	-
5	F	9.9	SR	4	-	8	3	6	-	24	3	-
6	F	14.3	SR	5	-	7	4	7	-	13	6	-
7	M	22.7	IR	4	-	8	3	6	-	19	4	-
11	M	11.9	IR	5	-	8	4	9	-	23	7	Normal
12	F	20.0	IR	7	-	8	6	8	-	20	9	Normal
14	M	10.7	IR	4	-	6	2	5	-	13	5	-
15	F	10.6	SR	4	-	8	2	8	-	18	3	Normal
16	M	10.5	IR	7	-	8	4	12	-	19	6	-
17	M	19.7	IR	-	8	9	8	-	14	20	25	Abnormal (stroke)
19	M	23.4	IR	-	9	8	8	-	23	20	20	-
21	F	13.8	SR	-	6	8	6	-	6	20	13	Normal
22	F	22.9	IR	-	10	8	5	-	24	14	7	-
Median		**12.9**		**4**	**8.5**	**8**	**4**	**6.5**	**18.5**	**19.5**	**6**	

Improvement of rCBF at follow-up five years after end of treatment (Study IV).

The SPECT results at follow-up were compared with the previous examinations during induction treatment. On re-examination, twelve of the 14 patients showed an improved cerebral blood flow (Table 10 and Figure 11, 14 and 15). Eleven of these 12 patients had a normalized rCBF and one (#21) showed a considerable improvement (Figure 14). The normalization/improvement in the blood flow was global, like the hypoperfusion detected at day 29.

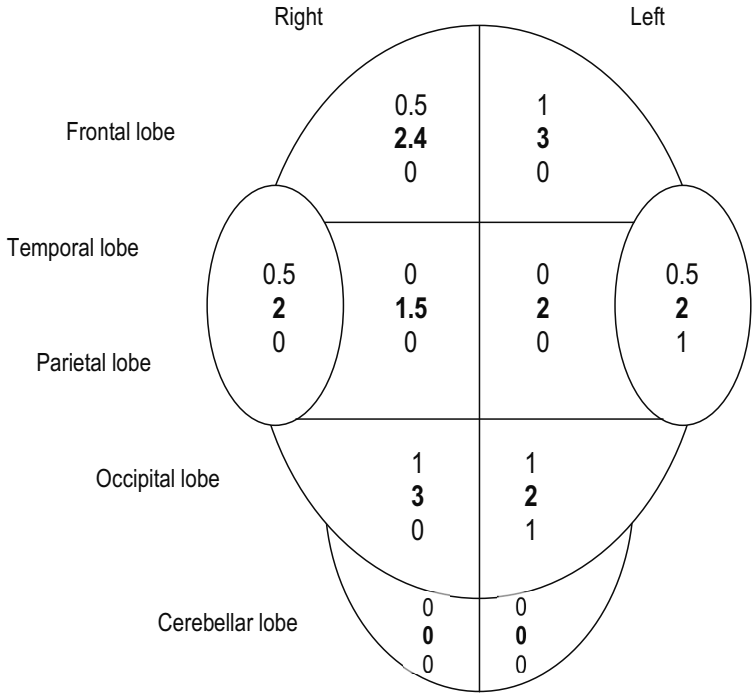


Figure 11. Median SPECT score in the different lobes in the 10 patients (#1-16) that were examined before start of treatment (above), at **day 29** (middle), and at follow-up (below) respectively.

Of the two patients who did not show any improvement, one (#19) had a SPECT score identical to the results at day 29. This patient had his first examination on day 7, i.e. no information is available about cerebral circulation before chemotherapy was started (Figure 13). Unfortunately, neither do we have any CT or MRI. He has never had any neurological deficits or symptoms.

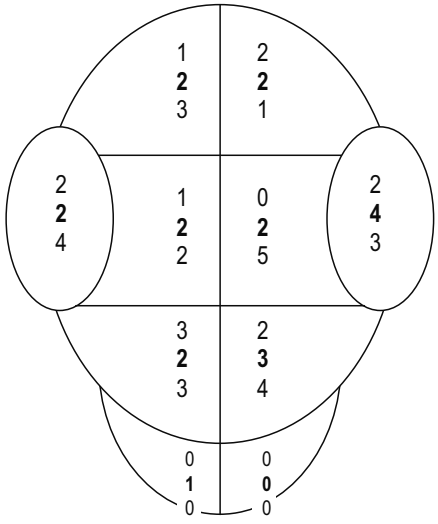


Figure 12. SPECT findings in patient 17 examined at treatment day 4 (above), at **day 29** (middle), and at follow-up (below) respectively.

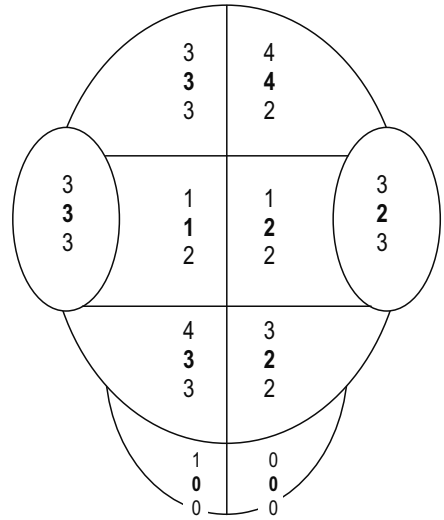


Figure 13. SPECT findings in patient 19 examined at treatment day 7 (above), at **day 29** (middle), and at follow-up (below) respectively.

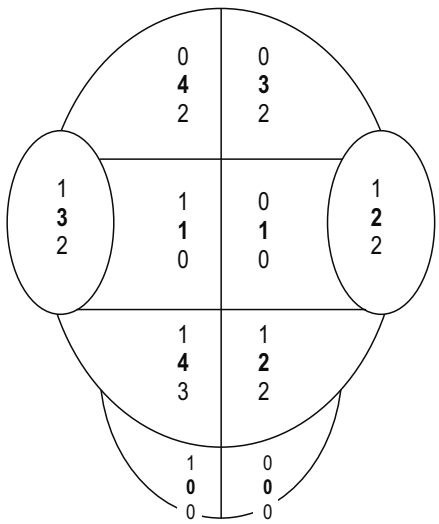


Figure 14. SPECT findings in patient 21 examined at treatment day 4 (above), at **day 29** (middle), and at follow-up (below) respectively.

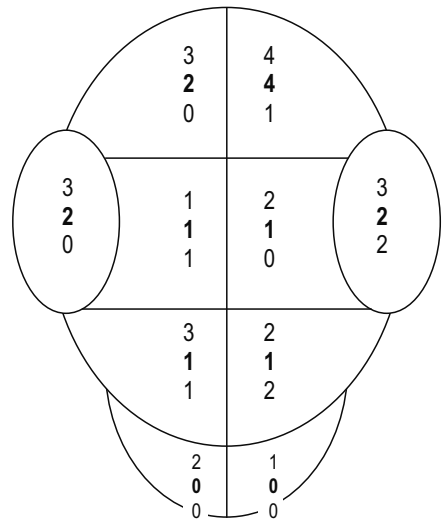


Figure 15. SPECT findings in patient 22 examined treatment day 3 (above), at **day 29** (middle), and at follow-up (below) respectively.

The second patient, (#17) showed deterioration in SPECT and MRI at follow-up, consistent with a cerebral infarction he suffered at the end of late intensification, twenty-eight weeks after start of treatment. He then had generalized seizures followed by dysphasia for a short period and weakness in his right side for a month. CT and MRI showed an infarction in the left parietal lobe with slight secondary hemorrhage. EEG was pathological and SPECT confirmed the infarction with severe hypoperfusion dorsally of a parieto-frontal area with hypoperfusion detected at day 29. Furthermore, there was impaired rCBF in the right frontal lobe and the left temporal lobe compared to previous examinations. He was put on antiepileptic treatment with carbamazepine. Two months later the symptoms had resolved and SPECT showed improved perfusion in the area of the infarction and no change in other areas. MRI eight months after the infarction showed no pathological findings except for a defect at the site of the infarction. MRI remained unchanged for two years after the infarction, i.e. eight months after cessation of therapy, and simultaneous SPECT showed improvement around the defect and in some areas although rCBF was still impaired in other areas. The final SPECT eight years after start of treatment, i.e. 7.4 years after the infarction and six years after discontinuation of ALL therapy, showed the same picture (Figure 12). He is still treated with carbamazepine.

Five of the 14 patients, plus one additional patient (#20) who later declined follow-up with SPECT, have been examined with MRI and except for the patient with the cerebral infarction (#17) the findings were normal.

Neurochemical markers of brain damage and oxidative stress in CSF

All available samples were analyzed and the number of samples of each marker analyzed at the four time-points is shown in the table below together with the number in the full series (n = 43–49) with results from all time-points. The full series is included in the larger series with more samples analyzed at each time-point (n = 73–110) although results are lacking from some time-points.

Table 12. Number of samples analyzed for brain specific proteins and AsR at each and all time-points respectively.

	Day 0	Day 8	Day 15	Day 29	Full series
NSE	73	59	66	69	44
GFAp	108	74	83	88	43
NFp	110	77	84	91	49
AsR	70	54	57	60	41

The levels and the patterns of change of the three brain specific proteins and AsR do not show any significant differences between the full series and the larger series. The results from the larger series are presented below.

CNS leukemia

Two patients had CNS leukemia and were not included in the presented groups but were analyzed separately. The levels of the markers at day 0 did not exceed the mean levels of the patients without CNS leukemia. The few samples available indicate a similar pattern of change during treatment to that of the other patients.

Neuron-specific enolase, NSE

The NSE concentrations in the CSF samples increased from 9.0 ± 3.5 (mean ±SD) µg/l at day 0 to the highest level 15.0 ± 5.3 at day 8 ($P<0.001$) and remained increased but at gradually falling levels, 13.6 ± 4.7 at day 15 ($P<0.001$) and 11.1 ± 4.3 at day 29 ($P<0.001$). The levels at day 0 were compared with those at day 8, day 15 and day 29 using Student's paired *t*-test with Bonferroni corrections.

CSF samples from 54 patients were analyzed for NSE already in study II. Although less samples and patients the pattern was the same and NSE content increased from 8.9 ± 3.6 µg/l at day 0 to the highest level 14.2 ± 5.4 at day 8 ($P<0.001$). The levels were 13.1 ± 4.3 at day 15 ($P<0.001$) and 10.8 ± 4.0 at day 29 ($P<0.05$). The levels at day 0 were compared with those at day 8, day 15 and day 29 using Student's paired *t*-test.

Twenty-one of these patients were also examined by SPECT and their NSE values were 9.0 ± 3.1 at day 0, 16.5 ± 6.4 at day 8, 13.9 ± 2.7 at day 15 and 10.4 ± 3.9 at day 29.

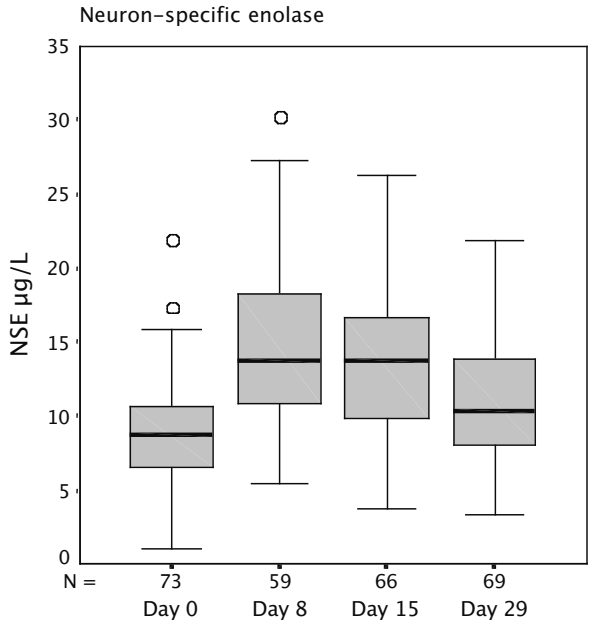


Figure 16. Box-plot diagram of neuron-specific enolase levels (NSE µg/L) in the CSF at day 0, at day 8, at day 15 and at day 29 in 73, 59, 66, and 69 patients respectively. The bold line indicates the median values and the upper and lower edge of the box indicate the quartiles. The outliers are shown with circles.

Glial fibrillary acidic protein, GFAp

The GFAp concentrations in the CSF samples increased from 177 ± 98 ng/L at day 0 to 206 ± 101 at day 8 (P<0.001), reached 200 ± 106 at day 15 (n.s.), and attained its highest level 228 ± 137 at day 29 (P<0.001).

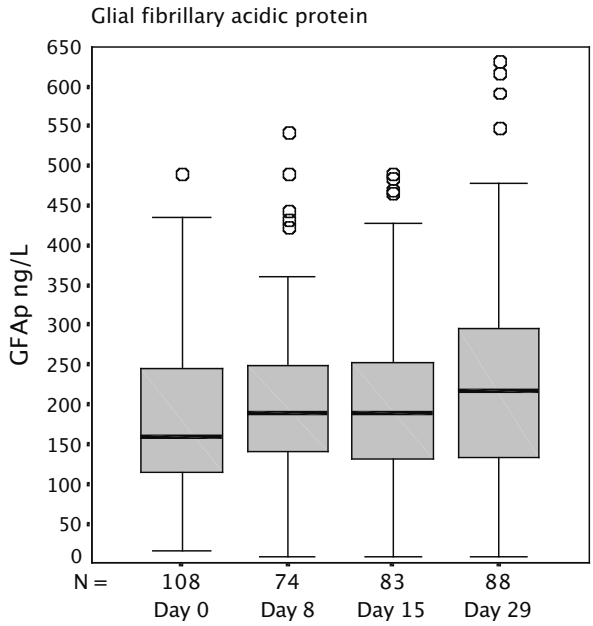


Figure 17. Box-plot diagram of glial fibrillary acidic protein levels (GFAp ng/L) in the cerebrospinal fluid at day 0, at day 8, at day 15 and at day 29 in 108, 74, 83, and 88 patients respectively. The bold line indicates the median values and the upper and lower edge of the box indicate the quartiles. The outliers are shown with circles.

Neurofilament protein (light sub-unit), NFp

The NFp concentrations were below the detection limit 125 ng/L before treatment in all 110 CSF samples analyzed and increased above the limit in 6 out of 77 samples at day 8, in 11 out of 84 samples at day 15 and in 22 out of 91 samples at day 29. The ratios of elevated samples are 0.08, 0.13 and 0.24 for days 8, 15 and 29 respectively, significant when tested with Fisher's exact test ($P<0.01$, <0.001 and <0.001 respectively).

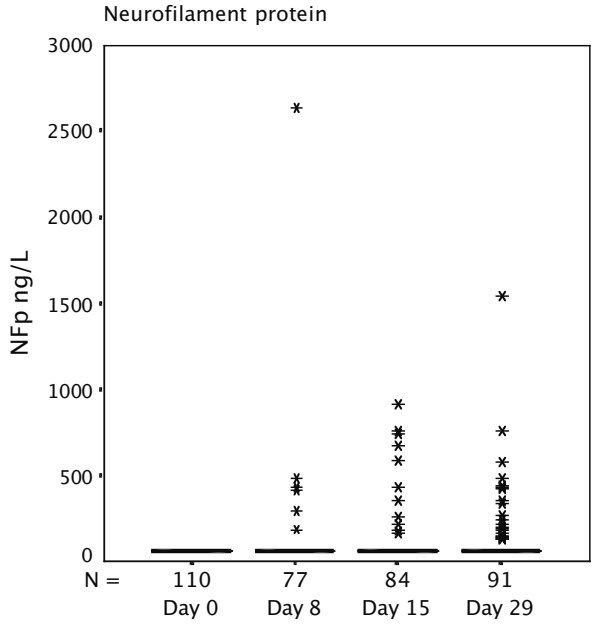


Figure 18. Box-plot diagram of neurofilament protein light chain levels (NFp ng/L) in the cerebrospinal fluid at day 0, at day 8, at day 15 and at day 29 in 110, 77, 84, and 91 patients respectively. The bold line indicates the median values and the quartiles, all below the detection limit 125 ng/L. The proportion of samples with elevated NFp (indicated by stars) was 6/77 (8%), 11/84 (13%), and 22/91 (24%), respectively.

Ascorbyl radical, AsR

The AsR content in the CSF samples did not change significantly. The mean values (±SD) were 1240 (±590) before the start of treatment, 1470 (±740) at day 8, 1200 (±570) at day 15 and 1300 (±630) at day 29.

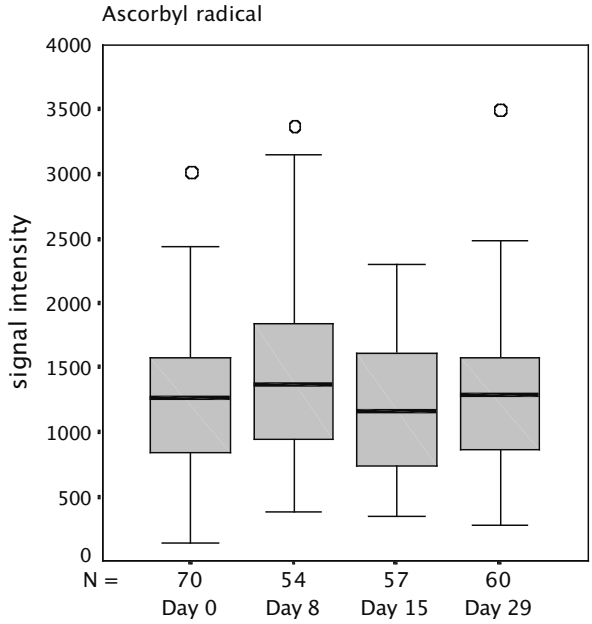


Figure 19. Box-plot diagram of levels of ascorbyl radical (AsR signal intensity) in the cerebrospinal fluid at day 0, at day 8, at day 15 and at day 29 in 70, 54, 57, and 60 patients respectively. The bold line indicates the median values and the upper and lower edge of the box indicate the quartiles. The outliers are shown with circles.

Correlations

The levels of the three brain specific proteins and AsR do not seem to correlate with age. Only NFp shows a correlation with age at day 8 and day 15 ($\rho = 0.31$ ($P<0.01$) and $\rho = 0.28$ ($P<0.01$) respectively) but not at day 0 or day 29. The other markers do not correlate with age at all.

There is a correlation between the NSE and GFAp values at day 0 ($\rho = 0.35$, $P<0.01$), at day 8 ($\rho = 0.52$, $P<0.01$), at day 15 ($\rho = 0.40$, $P<0.01$) and at day 29 ($\rho = 0.37$, $P<0.01$).

In 34 observations of simultaneous SPECT examinations and NSE sampling, there was no correlation between the number of lobes and SPECT score, respectively and the NSE levels ($r=0.26$ and 0.25).

DISCUSSION

Therapy of childhood ALL and the results of treatment have undergone an amazing development; from a uniformly fatal disease half a century ago to cure rates approaching 90% today. This is the result of basic research, numerous clinical studies and cooperation worldwide between devoted pioneers and their disciples. Today the cost of cure has become an issue of increasing importance since the cure rate now allows us to ask questions like: Is it possible to cure with less acute and late side effects?

The essential CNS directed treatment was for many years tantamount to CRT, with or without IT MTX. However, due to the side effects, i.e. SMN, cognitive, neuroendocrine and neuropsychological late effects, CRT has been substituted with HDMTX and IT therapy [17-26,63]. Furthermore, systemic HD and IT chemotherapy alone may also cause late effects to the CNS [62,63,66-69,82-84]. The introduction of the NOPHO ALL-92 protocol offered an opportunity to prospectively study ALL patients treated without CRT.

This study was first planned to focus on HDMTX, as there are several reports on stroke-like symptoms of neurotoxicity after HDMTX with, or as in the case of osteosarcoma therapy, without concomitant IT MTX [63,83,84,197]. The suspected cerebrovascular cause of these symptoms made it probable that SPECT would give valuable information. At that time PET studies reported reduced rCMRGlc in ALL patients and long-term survivors, and also in animal models [164-166,168,198].

In study I the first three patients developed subacute neurotoxicity and stroke-like clinical symptoms during treatment but in one of them (case 1) the symptoms came already during remission induction, months before HDMTX was scheduled. He actually suffered neurological deficits six days after the second IT MTX and then again, more severe, six days after the third IT MTX. SPECT showed multifocal hypoperfusion and MRI signs of leukoencephalopathy. Moreover, he developed hypertension but improved during treatment with the Ca^{2+}-channel blocker nimodipine otherwise used to treat cerebral vasospasm in subarachnoid hemorrhage [199,200]. Hypertension has previously been discussed in connection with partial complex seizures and transient bilateral cortical and subcortical white matter lesions visible at CT examinations during induction treatment. Vascular ischemia was suspected as the cause and the symptoms were often preceded by severe constipation after VCR injections [201]. Another patient (case 2) had his first transient episodes of neurological symptoms after six weeks of treatment, two weeks after IT MTX. A confounding factor, besides the other chemotherapeutic drugs (PRED, VCR and DOXO) given during induction, is that ASP treatment had also started. The symptoms resolved spontaneously and no significant changes were

found on CT, SPECT or EEG examinations. However, after 33 weeks of treatment, one week after HDMTX and IT MTX, he developed subacute neurotoxicity with stroke-like symptoms and impaired consciousness. Initially the effect of nimodipine was dramatic with amazing improvement but then the symptoms increased again. CT and MRI were normal but SPECT showed nonhomogeneous hypoperfusion in the left hemisphere consistent with more severe symptoms in the right side of the body. He gradually recovered completely during continued nimodipine therapy. The third case had shown symptoms of peripheral neuropathy during induction but did not show major neurotoxicity until two weeks after his third HDMTX. Now again CT and EEG were normal, MRI showed normal results except for a diffuse area deep in the white matter only visible on PD- and T2-weighted sequences. SPECT on the other hand showed impaired heterogeneous rCBF. This patient also recovered completely during nimodipine treatment. We have positive experience of nimodipine therapy at our centre in these and other cases but practice is not yet established and in our few cases spontaneous recovery or effect of other concurrent treatment cannot be ruled out. However, nimodipine might have neuroprotective effects besides the vasoactive effects and the possible beneficial effects of Ca^{2+}-channel blockers on inhibiting NMDA-receptor linked excitotoxicity is discussed in reference [66].

All three cases illustrate the typical symptomatology and timing of subacute neurotoxicity after treatment. The pathophysiology behind this neurotoxicity is not elucidated yet but the symptoms and findings of regional hypoperfusion suggest that an effect on the cerebral vasculature is involved. This might be a direct toxic effect on the vascular endothelium resembling the effects of radiotherapy or mediated thorough metabolic effects of MTX and/or other chemotherapeutics. Radiotherapy can cause vascular damage, focal necrosis, vascular occlusions, microinfarction and calcifications ending in mineralizing microangiopathy. This is however a slow process that is accompanied by demyelinization and if anything an example of delayed neurotoxicity. The effect of irradiation is mediated through reactive oxygen species and chemotherapy might also initiate such processes in the CNS. MTX treatment is associated with elevated homocysteine that can cause oxidative stress and direct toxic effects on the endothelium and intima. Furthermore, MTX can also increase the levels of adenosine that is involved in cerebral blood flow regulation and neuronal excitability. Increased capillary permeability, transient vasospasm and small vessel occlusion have also been suggested [62,63,66,81,83,165,166].

Besides the three patients with symptomatic neurotoxicity we report three patients without any neurological signs or symptoms examined by SPECT at different points of time during ALL treatment. All showed impaired rCBF but to a lesser degree than the patients with symptomatic neurotoxicity. Moreover, we have in a pilot study examined further patients (unpublished data) at other points of time also showing subclinical hypoperfusion. The fact that the first

three patients experienced neurotoxicity already in remission induction, i.e. before the administration of HDMTX, and the complexity of multiagent ALL therapy with many confounding factors and little known pathogenesis made us focus on the first four weeks of induction treatment in the next study (II).

This study design made it easier to interpret the findings. SPECT (and PET) has the advantage over conventional CT and MRI in producing functional images instead of showing manifest anatomical changes. However, one major drawback is the lack of generally accepted reference maps and good normal values, due to the fact that it would be unethical to expose completely healthy children to the small amount of, but still, ionizing radiation needed for the examination. There is however groups of children examined under the suspicion of neurological disease, or as part of evaluation of other disease, which has led to development of reference values at our and other institutions. By making serial SPECT examination of the same individuals this disadvantage is less problematic. Another advantage of limiting the study period to the first four weeks of treatment is that this reduces the number of chemotherapeutics and other events involved.

The short time available between diagnosis and start of treatment made it impossible to have all patients examined before any chemotherapy was given. However, this anticipated problem resulted in two groups of patients and the finding that patients examined after a few days of treatment (n=9) had disturbances in cortical cerebral perfusion compared to untreated patients (n=16). This implicates a fairly rapid effect of the chemotherapeutics in question, i.e. PRED, DOXO, VCR and IT MTX. Moreover, rCBF in all twenty-five patients had further deteriorated when re-examined at day 29 and the results in the two groups were the same. No areas of predilection were found and the impairment of rCBF appears to be a generalized and diffuse effect. This interpretation of hypoperfusion is corroborated by the fact that none of the patients had any focal neurologic symptoms.

Of the four drugs involved DOXO is not associated with neurotoxicity. VCR is well known for peripheral and autonomic neurotoxicity although encephalopathy is extremely rare and CNS penetration is low. The most likely causative agent is IT MTX and the effects are probably further modulated by PRED as stated in previous discussions [62,63,66]. The pathogenic mechanisms behind the subclinical hypoperfusion are not elucidated yet and might involve both direct vascular effects and metabolic changes. Anyway, the cause is most probably multifactorial.

Following remission induction there is 2–2.5 years of further therapy including other potentially neurotoxic chemotherapy. One of the patients suffered an ischemic stroke with slight secondary hemorrhage at the end of late intensification in treatment week 28. The recently received treatment included DEXA, DAUNO, VCR, IT MTX, and ASP and in this case ASP is the main suspected

causative agent with DEXA and MTX as possible contributing risk factors. CT, MRI and SPECT findings were consistent with the infarction located dorsally of an area with hypoperfusion detected on SPECT at day 29. When reviewed, the SPECT findings from day 29 did not correspond with the infarction area to be. This is the only patient with neurologic symptoms and sequelae of the twenty-five examined during remission induction. Fourteen of the patients were reexamined five years after cessation of therapy and the majority (eleven) had completely normalized their cerebral perfusion. Of the remaining three, one had improved, one was unchanged and the last one was the case with a stroke. This result can be compared with the few other studies published reporting persistent hypoperfusion in 8 of 17 irradiated patients and 5 of 15 non-irradiated patients five years after cessation of therapy [179] and in 3 of 13 treated with CRT and in 8 of 12 treated with chemotherapy alone at the end of therapy or one year later [177]. In another study SPECT could detect small defects in 5 of 17 patients examined at the end of therapy or 4–8 years later. Perfusion MRI could not detect these defects [152]. The strength of our small study is that the patients are examined longitudinally and that in 10 of 14 subjects we know the rCBF before any chemotherapy had been given. A weakness is that only one third have MRI examinations to compare the SPECT results with, but on the other hand other studies have not been able to show any relation between SPECT and MRI findings or between imaging and neurological and cognitive outcome. The facts that rCBF did not deteriorate in any patient except for the stroke case and that rCBF improved in 12 out of 14 are quite encouraging.

We have been able to detect subclinical uneven cerebral hypoperfusion during remission induction that in the majority of cases have normalized five years after treatment. However, we do not know the clinical significance of these observations yet. Neither do we know if regional hypoperfusion leads to relative ischemia causing oxidative stress or aggravates direct toxic effect of the chemotherapy. In an effort to come closer to the cellular mechanisms and to which cells therapy may have an impact on, we have analyzed three brain specific proteins (NSE, GFAp and NFp) and the ascorbyl radical (AsR) as a marker of oxidative stress. These markers represent different cellular compartments in the CNS and have previously been shown to signal brain damage after hypoxic-ischemic and traumatic injury. They have been analyzed in various neurodegenerative, inflammatory and infectious diseases, both in blood and in CSF [185-189,195,202-209]. To be able to detect elevated levels in blood there must be severe damage to the CNS. This is not likely to occur during induction treatment. CSF is in direct contact with the intercellular space and the cells in the CNS, which makes it possible to detect lower levels and less pronounced effects on neurons, astrocytes and other cells.

We found that the levels of all three BSPs analyzed increased during remission induction. NSE levels reached their highest levels at day 8, then remained increased but at gradually falling levels at day 15 and day 29. The absolute level at day 8 (mean 15.0 μg/l) is not as high as in more severe brain damage

like asphyxia but significantly above the results at day 0 (9.0 μg/l) and the control group (10.0 μg/l) in a study of asphyxiated newborns [210]. Reference values in the literature vary depending on the assay used [211]. The level at day 29 was still significantly elevated compared to the level at day 0. The GFAp content increased also but with the highest levels detected at day 29. NFp were below the detection level in all samples at day 0 and increased during induction treatment, but again seemed to reach the highest levels at day 29. The results from the two patients with CNS leukemia indicate that lymphoblasts in the CSF do not cause elevated BSPs.

The CSF results indicate damage to neuronal and astroglial cells during induction therapy. The time interval between IT MTX and CSF sampling is one week for samples obtained at day 8 and day 15 and two weeks for samples obtained at day 29. Hence, the CSF levels could be higher during the first hours and days after the MTX injections and our results might underestimate the impact of chemotherapy. This could be especially true for NSE and AsR. The GFAp results indicate an astroglial reactivity, which in view of the high levels observed at day 29 may be interpreted as signs of gliosis. The NFp values are in line with the GFAp results with more pronounced findings at day 29, indicating axonal damage. This seemingly late increase is in agreement with the known kinetics of this marker after damage to the CNS [188,206,212]. Although the increase in CSF levels of the BSPs studied is moderate compared to situations with more severe impact on the brain, e.g. asphyxia and stroke, the findings must be interpreted in the light of the recent findings that similar levels of NFp are often observed in young males after amateur boxing and in HIV [208,209].

The level of AsR did not change significantly during the period studied, at least not when the samples were taken. Consequently, we have not been able to show any oxidative stress or ongoing inflammatory reaction. The ascorbyl radical is fairly stable but other free radicals are highly reactive and short-lived. There may not be any effect on AsR levels in CSF during induction treatment but there might as well be changes that we are unable to detect with this study design. For ethical reasons it is difficult to take more frequent CSF samples which leaves us to search for other methods and/or animal models. Signs of an inflammatory component of the subclinical brain damage could be investigated by analyzing pro- and anti-inflammatory cytokines in CSF. Several other neurochemical markers of damage to different cells and compartments of the CNS are available but kinetics, treatment schedule and availability of CSF has to be considered when designing the next study.

CONCLUSIONS

Three patients developed subacute neurotoxicity (encephalopathy), one after IT MTX and two after HDMTX including IT MTX. All had impaired rCBF consistent with the symptoms when examined by SPECT. All improved within a few days during treatment with the Ca^{2+}-channel blocker nimodipine. All recovered completely without any sequelae.

Another three patients, without neurological symptoms, were examined at different phases of ALL treatment. All had disturbances in rCBF but impairment was less pronounced than in the patients with symptoms.

Twenty-five patients were examined after four weeks of remission induction treatment with PRED, DOXO, VCR and IT MTX. None had neurologic symptoms but in all patients had rCBF deteriorated compared to previous examination.

Nine of these patients had earlier been examined after a few days of treatment and had already then heterogeneous cerebral hypoperfusion unlike the sixteen patients examined before start of any treatment.

Fourteen of the twenty-five patients were re-examined five years after cessation of treatment. Eleven had normalized rCBF, one had improved, one was unchanged and the last one had sequelae after a stroke.

The levels of three brain specific proteins in CSF increased during remission induction. NSE, a marker of neurons, reached the highest level at day 8. GFAp, a marker of astrocytes, and NFp, a marker of axons, reached the highest level at day 29. Analyses of AsR were not conclusive.

To summarize:

- All patients had disturbances in rCBF during ALL treatment.
- The hypoperfusion was reversible in the vast majority of cases.
- The results of the CSF-analyses indicate damage to neurons and glia cells during induction treatment.

SAMMANFATTNING PÅ SVENSKA

Akut lymfatisk leukemi (ALL) är den vanligaste barncancersjukdomen och idag botas mer än 80 % av patienterna. Ett avgörande framsteg var när profylaktisk strålbehandling mot CNS infördes för att förhindra återfall på grund av kvarvarande lymfoblaster i CNS. Strålbehandling medför dock seneffekter i form av neuropsykologiska och kognitiva störningar, hormonpåverkan och risk för sekundära maligniteter. Därför har man ersatt strålbehandling med intensifierad cytostatikabehandling, högdos metotrexat (HDMTX) och upprepade intratekala metotrexatinjektioner (IT MTX). Emellertid kan även metotrexat, och andra cytostatika, ge akuta och sena biverkningar men kunskaperna om långtidseffekter och verkningsmekanismer är begränsade. Därför är det viktigt att undersöka detta hos patienter som behandlas med enbart cytostatika.

Tre patienter med ALL fick olika neurologiska symtom (hallucinationer, nystagmus, övergående blindhet, medvetandepåverkan, sväljningssvårigheter, svaghet, känsel- och motorikpåverkan) vid olika tidpunkter under behandlingen. Symtomen gav med sig under behandling med en Ca^{2+}-kanalblockerare, nimodipin, och ingen har några kvarvarande neurologiska besvär. Patienterna undersöktes med EEG, datortomografi, magnetresonanstomografi (MR) och gammakamera (SPECT, single photon emission computed tomography). SPECT visade att samtliga patienter hade störningar i hjärnans blodflöde (rCBF, regionalt cerebralt blodflöde). Vid kontroll efter 8 månader hade rCBF förbättrats påtagligt hos patienten med mest uttalade symtom. Ytterligare tre patienter utan neurologiska symtom undersöktes med SPECT och samtliga uppvisade blodflödesstörningar (hypoperfusion), men mindre uttalat än hos patienterna med symtom. Hypoperfusion och symtom påvisades redan innan HDMTX givits.

För att närmare studera blodflödespåverkan under induktionsbehandlingen (de första veckorna med prednisolon, doxorubicin, vinkristin och IT MTX) gjordes en studie med SPECT av 25 patienter med ALL. Sexton undersöktes före behandlingsstart och nio under de första dagarna efter att den första cytostatikadosen getts. Efter fyra veckor gjordes en förnyad undersökning av rCBF som visade att samtliga patienter hade diffust spridd hypoperfusion. Blodflödespåverkan poängsattes (0 = normalt – 5 = maximal påverkan) i tio områden (lober) och räknades samman till SPECT-score. Medianscore steg i den första patientgruppen från 6 före behandlingsstart till 17,5 efter fyra veckor och i den andra gruppen från 8 första behandlingsveckan till 20 efter fyra veckor. Ingen av patienterna hade några neurologiska symtom. Dessutom tillvaratogs cerebrospinalvätska (CSF) från ALL-patienter vid fyra tillfällen i samband med att de erhöll IT MTX. Hos 54 patienter analyserades nivåerna av en nervcellskademarkör (NSE, neuronspecifikt enolas) i CSF. Nivåerna steg signifikant

tidigt under behandlingen med en topp dag 8 följt av successivt lägre, men fortsatt förhöjda värden dag 15 och 29.

Analyserna av CSF utvidgades till följande fyra skademarkörer: NSE (nervceller), GFAp (surt gliafibrillärt protein, astrocyter), NFp (neurofilamentprotein, nervcellernas axon) och AsR (askorbylradikal, mått på fri radikalbildning). För analyserna användes RIA-metodik för NSE, ELISA för GFAp och NFp samt elektronspinresonans för AsR. Prov från behandlingsdag 8, 15 och 29 analyserades för de fyra markörerna och jämfördes med nivåerna före behandlingsstart (dag 0) hos totalt 121 patienter. NSE i prov från 73 patienter visade samma mönster som ovan, högst nivå dag 8 och sedan successivt lägre men signifikant förhöjda nivåer. GFAp hos 108 patienter steg successivt till högsta nivå dag 29. NFp låg under detektionsgränsen hos 110 patienter dag 0 och uppvisade förhöjda nivåer hos 6 av 77 dag 8, hos 11 av 84 dag 15 och hos 22 av 91 dag 29. Förändringarna är statistiskt signifikanta vilket AsR-variationerna inte är.

För att se om blodflödesstörningarna var bestående gjordes en uppföljningsstudie. Fjorton av de 25 patienterna ovan undersöktes ånyo med SPECT fem år efter avslutad behandling. Elva uppvisade en normalisering av rCBF, en hade förbättrats påtagligt och ytterligare en visade samma bild som under första behandlingsveckan. Dessa patienter hade inga neurologiska symtom och MR av de 4 som även MR-undersöktes visade normalfynd. Den återstående patienten fick en stroke efter 7 månaders ALL-behandling och uppvisade hypoperfusion motsvarande resttillståndet efter hjärninfarkten. Medianscore hos patienterna var 6, d.v.s. samma som innan ALL-behandlingen inleddes.

Sammanfattningsvis kan störningar i hjärnans blodflöde påvisas tidigt under cytostatikabehandling av ALL även hos patienter utan neurologiska symtom. Patienter med symtom har mer uttalad hypoperfusion. Fem år efter avslutad behandling har blodflödesstörningarna försvunnit hos majoriteten av patienterna. Om hypoperfusionen beror på kärlspasm och/eller underliggande nervcellspåverkan är ännu ej känt. Förhöjda nivåer av tre hjärnskademarkörer tyder på skada på nervceller och astroglia tidigt under induktionsbehandlingen innan HDMTX getts. Om detta beror på blodflödesstörningen och/eller direkt toxisk effekt av cytostatika är ännu okänt. Oxidativ stress i form av bildning av fria radikaler har inte kunnat påvisas med detta studieupplägg.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to **everyone** who has helped me through the years to carry out the work with this thesis.

I am particularly grateful to:

All patients who have participated in the studies and their families.

Ildikó Márky, tutor, professor and former head of the department of pediatric hematology and oncology. Thank you for friendship, guidance, patience, and many other things, including bringing me into the world of pediatric oncology.

Ingemar Kjellmer, co-tutor, professor, former head of the department of pediatrics, University of Gothenburg. Thank you for introducing me into research in the field of brain damage markers and for excellent co-authorship.

Birgitta Lannering, co-worker, head of the department of pediatric hematology and oncology. Thank you for invaluable help when recruiting patients to study II and for constructive criticism and co-authorship.

Paul Uvebrant, Rune Sixt and the late Jan Bjure for reviewing SPECT images, for co-authorship and generous co-operation and support.

Lars Rosengren and Ulf Nilsson, co-authors, for CSF expertise and improving the manuscript.

Jonas Abrahamsson, Margareta Bergkvist, Klas Blomgren, Marianne Jarfelt, Lotta Mellander, Karin Mellgren, Mirka Pinkava and Magnus Sabel, friends and colleagues at the pediatric oncology ward 322. Thank you for all the CSF you have collected, for helpful and pleasant clinical collaboration and not at least, doing all the clinical work for a long time while I was finishing this thesis.

All the staff at ward 22/305/322 and 321 through the years for always being helpful and generous, for remembering test tubes and CSF samples and giving extra time for SPECT examinations, and always giving the patients the best care. Especially I would like to thank Agneta Landerberg and Carina Fondin for helping me to keep the CSF samples in order and Carina Hallberg for coordinating everyday work and treatments with the SPECT study. I would also like to thank Anna Schröder-Håkansson for help with patient records etc.

All colleagues and staff at the county hospitals in Borås, Halmstad, Jönköping, Karlstad, Skövde, Trollhättan and Uddevalla for all CSF samples you have collected and kept in the deep freezers and sent on dry ice through the years.

Your help has been invaluable.

Maria Nyholm and Anna Lindblom at the department of Physiology who kept all CSF samples in order in the deep freezer and performed the NSE and some of the AsR analyses. Shirley Fridlund at the department of Neurochemistry who performed the GFAp and NFp analyses.

Pediatric neuroradiologist Lars-Martin Wiklund who reviewed the MRI and CT scans and hospital physicist Eleonor Vestergren that assisted with the SPECT examinations. I would also like to thank psychologist Anna-Kerstin Wetterqvist for doing a lot of work that haven't been transformed into a manuscript yet.

Margareta Ryden and Marlene Mussmann at the department of Pediatrics for always being encouraging, positive and helpful.

Eva-Lotte Daxberg and Krystyna Johansson at the hospital library for kind help through the years.

This study was supported by grants from the Children's Cancer Foundation of Sweden, the Swedish Cancer Society, the Medical Research Council (grant No 2591), the Knut and Alice Wallenberg Foundation, the IngaBritt and Arne Lundberg Research Foundation, the Mary Béve Foundation for Children's Cancer Research, the Gothenburg Children's Hospital Research Foundation and the Göteborg Medical Society.

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