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Therapy-related Hypoglycaemia and Liver Changes Including Iron Overload in Children with Acute Lymphoblastic Leukaemia

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Distribution

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals.

I. Halonen P, Salo MK, Mäkipernaa A (2001): Fasting hypoglycemia is common during maintenance therapy for childhood acute lymphoblastic leukemia.
J Pediatr 138:428-31.

II. Halonen P, Salo MK, Schmiegelow K, Mäkipernaa A: Investigation of the mechanisms of therapy-related hypoglycaemia in children with acute lymphoblastic leukaemia. Acta Paediatr, in press.

III. Halonen P, Mattila J, Ruuska T, Salo MK, Mäkipernaa A: Liver histology after current intensified therapy for childhood acute lymphoblastic leukemia: microvesicular fatty change and siderosis are the main findings. Med Pediatr Oncol, in press.

IV. Halonen P, Mattila J, Suominen P, Ruuska T, Salo MK, Mäkipernaa A: Iron overload in children who are treated for acute lymphoblastic leukemia estimated by liver siderosis and serum iron parameters. Pediatrics, in press.

ABBREVIATIONS

ALL   acute lymphoblastic leukaemia
AML   acute myeloid leukaemia
ALT   alanine aminotransferase
ANOVA analysis of variance
ARA-C cytosine arabinoside
AST   aspartate aminotransferase
BMI   body mass index
CNS   central nervous system
E-MTX erythrocyte concentration of methotrexate polyglutamates
E-TGN erythrocyte concentration of 6-thioguanine nucleotides
γGT   gammaglutamyltransferase
HBV   hepatitis B virus
HCV   hepatitis C virus
IR   intermediate risk
6MP   6-mercaptopurine
MT   maintenance therapy
MTX   methotrexate
NOPHO Nordic Society of Paediatric Haematology and Oncology
RBC   red blood cell
SR   standard risk
sTfR   soluble transferrin receptor
TfR   transferrin receptor
TGN   thioguanine nucleotide
TIS   total iron score
TPMT   thiopurine-methyltransferase

INTRODUCTION

During the last two decades multidrug protocols, early and late treatment intensification, central nervous system (CNS)-directed chemotherapy and improved supportive care have increased the event-free survival rate in childhood acute lymphoblastic leukaemia (ALL) to 70% and almost up to 80% (Rivera et al. 1993, Reiter et al. 1994, Gustafsson et al. 2000). Nonetheless, intensifying of therapy has not been possible without cost. With increasing numbers of children cured of leukaemia there has been an increased awareness of the adverse effects and late sequelae of therapy.

In our experience children being treated for ALL according to the modern Nordic regimens (Gustafsson et al. 1998, Gustafsson et al. 2000) and receiving maintenance therapy (MT), the core of which even nowadays consists of daily oral 6-mercaptopurine (6MP) and weekly oral methotrexate (MTX), often exhibit symptoms such as tiredness and nausea, compatible with hypoglycaemia after an overnight sleep. However, at the time the present study was initiated no published data on glucose homeostasis and fasting tolerance during ALL therapy existed.

The hepatotoxicity of antileukaemic drugs was profoundly studied from the 1960´s to the1980´s and the spectrum of hepatotoxic changes has been reported to be wide (King and Perry 1995, King and Perry 2001). Furthermore, in many of the studies viral hepatitis has played an important part in the development of chronic liver disease after ALL therapy (Locasciulli et al. 1983a, Locasciulli 1983b, Locasciulli et al. 1985, Guido et al. 1991, Locasciulli et al. 1991). During the modern era of more efficient virus screening of blood products the risk for blood-born virus hepatitis has decreased. Instead, increased red blood cell (RBC) transfusion rates due to intensified chemotherapy may predispose to iron overload and liver siderosis contributing to the hepatotoxicity of antileukaemic agents. However, no studies on liver disease and iron overload after modern intensified ALL therapy in children are available.

The purpose of the present study was to determine whether MT for ALL is associated with overnight fasting hypoglycaemia and by which mechanisms, and to evaluate the severity of histological liver disease and iron overload in children and adolescents treated for ALL according to the modern Nordic therapy regimens.

REVIEW OF THE LITERATURE

1. Therapy for childhood ALL

The basic approach to therapy for ALL consists of a relatively brief remission-induction phase, followed by intensification (consolidation) treatment and then prolonged continuation therapy. Treatment for subclinical leukaemia of the CNS is initiated early and is given for varying lengths of time, depending on the treatment protocol (Pui and Evans 1998). The first goal of therapy in patients with leukaemia is to induce complete remission with restoration of normal hematopoiesis. The induction regimen invariably includes a glucocorticoid (prednisone or dexamethasone) and vincristine, as well as asparaginase and/or anthracycline. Intensive induction regimens with four or more drugs have been credited with improving outcomes in several trials in children (Niemeyer et al. 1991, Rivera et al. 1991, Gaynon et al. 1993, Reiter et al. 1994). With the restoration of normal hematopoiesis, patients whose disease is in remission become candidates for intensification (consolidation) therapy. Such treatment, administered shortly after the induction of remission, includes several drugs, most often MTX given in high doses with or without 6MP (Rivera et al. 1991, Reiter et al. 1994, Veerman et al. 1996, Mahoney et al. 1998). Another integral component of many protocols is reinduction therapy introduced early after the first induction of remission (e.g., at four months). This treatment, which relies on the same drugs that were given during the first induction phase, has improved results in both children and adults with ALL (Hoelzer et al. 1992, Reiter et al. 1994, Childhood ALL Collaborative Group 1996).

The realization that the CNS can be a sanctuary for leukaemic cells has prompted the development of presymptomatic therapy directed toward the central nervous system. Because of the concern that cranial irradiation can involve substantial neurotoxicity and can occasionally cause brain tumors, intensive intrathecal or systemic chemotherapy is instead administered early in the treatment course. The results of chemotherapy have been excellent (Veerman et al. 1996, Conter et al. 1997, Nachman et al. 1998, Pui et al. 1998).

With the exception of those with mature B-cell leukaemia, children with ALL require prolonged maintenance treatment. However, according to a meta-analysis of 42 studies, there is no advantage in prolonging treatment beyond three years (Childhood ALL Collaborative Group 1996). A combination of weekly oral MTX and daily oral 6MP constitutes the usual continuation regimen in childhood ALL. Accumulation of higher intracellular concentrations of the active

metabolites of MTX and 6MP and administration of this combination to the limits of tolerance as indicated by low leukocyte counts have been associated with an improved clinical outcome (Lennard et al. 1990, Whitehead et al. 1992, Chessells et al. 1997). The addition of intermittent pulses of vincristine and a glucocorticoid to the antimetabolite maintenance regimen has been shown to improve results (Childhood ALL Collaborative Group 1996) and has been widely adopted in the treatment of childhood ALL. The Nordic treatment regimens for standard (SR) and intermediate risk (IR) ALL also include pulses of methotrexate in high doses (Gustafsson et al. 2000).

2. Hypoglycaemia

2.1. Glucose homeostasis

Blood glucose concentration is normally maintained within a narrow range, reflecting a balance between the production and utilization of glucose. Hypoglycaemia is the result of a reduction in glucose intake or production, or excessive utilization, or a combination of these. During feeding glucose is formed principally from carbohydrate in the diet. During fasting glucose is released by the liver by glycogenolysis and gluconeogenesis. The processes involved in the maintenance of glucose homeostasis are controlled by endocrine and neural factors, and substrate availability, and are closely associated with fat and protein metabolism. The liver plays a crucial role in the control of these mechanisms. Glycogen is found particularly in muscle and liver. Muscle glycogen can only be used as a reserve substrate by the muscle itself. By contrast, hepatic glycogen can be broken to free glucose and released into the circulation. Gluconeogenesis is the process in which glycerol, amino acids and lactate are converted into glucose. Glycerol is produced from lipolysis in adipocytes, amino acids from protein catabolism and lactate from glycolysis. Via glycolysis glucose is metabolized to pyruvate which can be converted into lactate, or after decarboxylation, into acetyl co-enzyme A (acetyl −CoA). Acetyl-CoA is the substrate for the synthesis of fatty acids, which are then stored as triglycerides (reviewed by Lee and Leonard 1995).

Hormonal factors important in glucose homeostasis are insulin and counterregulatory hormones. Insulin, which is secreted in response to increases in plasma glucose and amino acid concentrations, suppresses hepatic glucose production by stimulating glycogen synthesis and inhibiting glycogenolysis (Hems and Whitton 1980). It also promotes glycolysis and suppresses gluconeogenesis (Chiasson et al. 1976). Glucose uptake by adipose tissue and muscle is stimulated. In adipocytes triglyceride synthesis is enhanced, and in muscle glycogen formation and glycolysis are favoured. The counterregulatory hormones glucagon, adrenaline, noradrenaline, cortisol and growth hormone

oppose the effects of insulin. As blood glucose level falls insulin secretion is inhibited and release of counterregulatory hormones is stimulated (Lee and Leonard 1995). Glucose metabolism in the fed state and in the fasting state is presented in Figure 1.
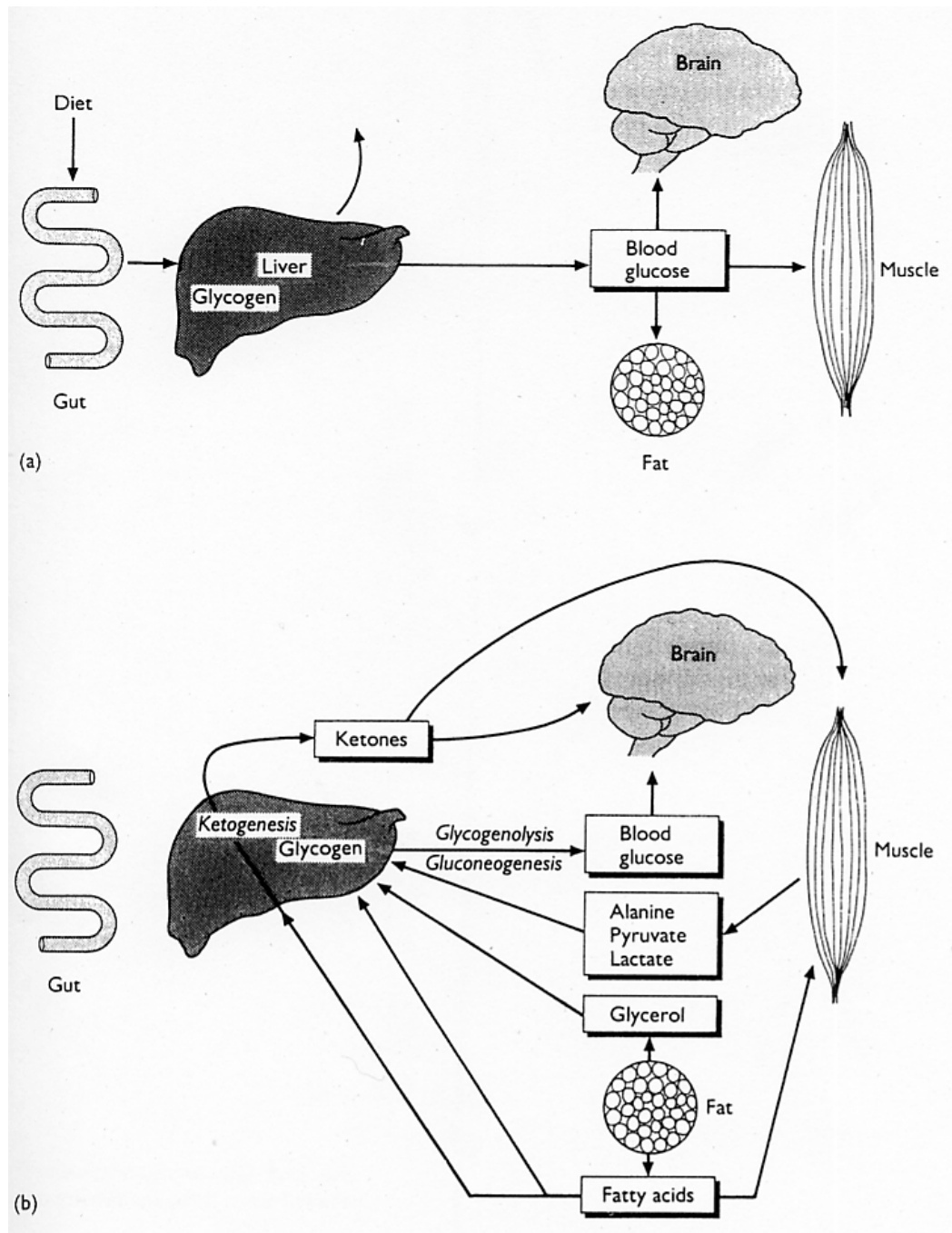
Figure 1. *Glucose metabolism (a) in the fed state, and (b) in the fasting state. (Figure from Lee PJ and Leonard JV: Hypoglycaemia. In: Clinical Paediatric Endocrinology. Ed. Brook CGD, 1995, page 681, with permission).*

Early in fasting glucose is supplied by the breakdown of hepatic glycogen stores, which are depleted by fasting after 24-36 hours in adults, and after shorter periods in children (Chaussain et al. 1977). Thereafter glucose concentration is maintained by reducing the rate of glucose utilization and by gluconeogenesis from amino acid precursors, glycerol and lactate. In the fasting adult about 50% of new glucose is produced from amino acid precursors (Cahill 1970), of which alanine and glutamine are the most important (Felig 1973, Pagliara et al. 1973). Fasting activates lipolysis resulting in elevated fatty acid and glycerol levels. Free fatty acids undergo beta-oxidation to produce ketone bodies acetoacetate and beta-hydroxybutyrate, and glycerol is used for gluconeogenesis. Ketone bodies provide energy required for gluconeogenesis and also serve as additional energy sources in tissues like muscle, heart and brain (Lee and Leonard 1995). In fasting children free fatty acid and ketone body concentrations are high compared to adults (Saudybray et al. 1981), indicating an acceleration of the normal adaptive mechanisms (Bonnefont et al. 1990) and providing a greater glucose-sparing effect. During fasting normal glucose levels can be maintained in children for only 24-48 hours, whereas adults can fast for weeks (Chaussain at al. 1977, Haymond et al. 1982). In healthy children from 3 to 15 years of age, the normal lower limit for venous blood glucose (2.5th percentile) after a 14-hour fast is reported to be 3.7 mmol/l (Lamers et al. 1985).

2.2. Definition, manifestations and sequelae of hypoglycaemia

Hypoglycaemia is best documented by Whipple´s triad: symptoms compatible with hypoglycaemia, low blood glucose concentration and alleviation of symptoms after the glucose concentration is raised (Whipple 1938). In experimental studies in healthy adults, the threshold for symptoms of hypoglycaemia is on average a blood glucose level of 3.0 mmol/l (Schwartz et al. 1987, Mitrakou et al. 1991). Mitrakou et al. (1991) reported autonomic symptoms of hypoglycaemia to begin at a blood glucose level of 3.2 mmol/l, and deterioration in cognitive function tests at a level of 2.7 mmo/l. When sensory evoked potentials were measured in relation to blood glucose concentrations in infants and children with episodes of hypoglycaemia, abnormalities were noted at blood glucose levels less than 2.6 mmol/l (Koh et al. 1988). Similar changes occurred regardless of whether the children were symptomatic or asymptomatic. These abnormalities tended to subside after normalization of the blood glucose concentration. Therefore a blood glucose level of 2.6 mmol/l is at present suggested to be a criterion for hypoglycaemia (Gregory and Aynsley-Green 1993). The presence of symptoms is not necessary for diagnosis (Lee and Leonard 1995). The plasma glucose concentration at which hypoglycaemic symptoms develop varies between individuals (Snorgaard et al. 1991, Service 1993) depending upon the developmental stage and on the levels of alternative fuels, ketone bodies, free fatty acids and lactate. Symptoms and signs of

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hypoglycaemia reflect sympathetic adrenergic stimulation of autonomic nervous system and neurological disturbance. Autonomic symptoms include pallor, sweating, weakness, nausea and vomiting, abdominal pain and hunger. Neuroglycopaenic signs are confusion, irritability, headache, visual disturbance, unusual behaviour, coma and convulsions (Lee and Leonard 1995).

Glucose is an essential substrate for neuronal metabolism. In animal models, severe and sustained hypoglycaemia leads to a major structural damage, including neural necrosis with loss of dendrites. Neural damage is most likely caused by the activation of receptors for excitatory amino acids, mainly the N-methyl-D-aspartate (NMDA) receptor (Aynsley-Green 1996). An association between neonatal hypoglycaemia and adverse neurodevelopment has been reported (Sinclair 1997). In older children, acute hypoglycaemia can produce focal neurologic deficits and movement disorders. Central pontine myelinosis may be an unusual manifestation of hypoglycaemia (Rajbhandari et al. 1998). Mild, even asymptomatic, hypoglycaemia is a potential cause of neurological damage (Golden et al. 1989, Jones et al. 1990). Overall, the long-term neurologic effects of hypoglycaemia vary. The presence of concurrent medical problems and the availability of alternative fuels seem to alter the threshold for dysfunction resulting from hypoglycaemia (Aynsley-Green 1996).

2.3. Causes of hypoglycaemia

Several classifications of hypoglycaemic disorders have been proposed depending on the age and clinical characteristics of the patient (Service 1995) or based on the pathophysiology of hypoglycaemia (increased glucose use or decreased glucose production) (Haymond 1989). A classification used by Verrotti et al. (1998) is presented for the purpose of this review in Table 1. Work-up of the patient with hypoglycaemia requires a systematic approach. An algorithmic approach to the diagnosis is suggested in Figure 2 (Lteif and Schwenk 1999).

From the practical viewpoint according to Verrotti et al. (1998), the relationship between the hypoglycaemic event and last meal may provide some important help for diagnosis. When a hypoglycaemic event occurs less than 12 hours after a meal impaired glycogenolysis must be considered, whereas hypoglycaemia occurring 12 to 16 hours after the meal may be due to impaired gluconeogenesis. Among the defects of gluconeogenesis are inherited metabolic defects and endocrine causes. When hypoglycaemia occurs after long fasting it is important to find out whether it is ketotic or not. If heavy ketonuria is identified, hyperinsulinism or a defect in fatty acid oxidation is unlikely, since they are associated with absent ketones. The likely causes to be considered then are idiopathic ketotic hypoglycaemia, hormone deficiency, a glycogen storage disease or a defect in gluconeogenesis. A deficiency of gluconeogenesis is generally associated with fasting lactacidaemia caused by the counterregulatory

mobilization of gluconeogenic precursors, including alanine (Verrotti et al. 1998).

Table 1. Classification of hypoglycaemia in childhood (Verrotti et al. 1998)

Increased glucose utilization
      Hyperinsulinism
      Hypothermia
Impaired glycogen metabolism
      Glycogenosis
      Impaired glycogen synthesis (glycogen synthase deficiency)
      Impaired glycogenolysis (hepatic glycogen storage diseases)
Impaired ketogenesis and ketone body utilization
      Carnitine mediated defects
      Mitochondrial respiratory chain disorders
      Beta-oxidation defects
      Ketone body formation and utilization defects
Reduced gluconeogenesis
      Inherited metabolic defects: fructose-1,6-diphosphatase deficiency,
           phosphoenolpyruvate carboxykinase deficiency
      Endocrine causes: hyperinsulinism; GH, ACTH, epinephrine and
           glucagon deficiency; panhypopituitarism
Interference with glucose homeostasis secondary to disturbance of intermediary metabolism
      Carbohydrate disorders: galactosaemia, hereditary
           fructose intolerance
      Aminoacidopathies
      Organic acidaemias
Other causes and associations
      Liver disease: hepatitis, α_1-antitrypsin deficiency, cirrhosis,
           hepatoma, Reye´s syndrome
      Gastrointestinal causes: malabsorption, protein losing enteropathy,
           cystic fibrosis
      Neonatal associations
      Systemic disorders: sepsis, renal and heart failure, burns, shock
Iatrogenic causes
      Drug-related: salicylate, oral hypoglycaemic agents, quinine,
           pentamidine, dysopiramide
      Ethyl alcohol
Idiopathic
      Idiopathic ketotic hypoglycaemia

HYPOGLYCAEMIA

Ketotic		**Non-ketotic**	
Lactate:		*Reducing substances*	
High	*Normal*	*Negative*	*Positive*
Defects:			
PC	Type 3 GSD	Hyperinsulinism	HFI
PEPCK	Type 6 GSD	Fatty acid oxidation	Galactosaemia
G-6-P	Hormone deficiency	defect	
F-1,6-DP	Ketotic hypoglycaemia		

Abbr. PC pyruvate carboxylase, PEPCK phosphoenolpyruvate carboxykinase,

G-6-P glucose-6-phosphatase, F-1,6-DP fructose-1,6-diphosphatase,

GSD glycogen storage disease, HFI hereditary fructose intolerance

Figure 2. *Algorithmic approach to the diagnosis of hypoglycaemia. (Figure from Lteif and Schwenk, Endocrinol Metabol Clin North Am 1999;28:619-46, with permission)*

Idiopathic ketotic hypoglycaemia is the most common cause of childhood hypoglycaemia. It usually occurs between 18 months and 5 years of age and generally resolves before 8 to 9 years (Haymond 1989). It is typically triggered by intercurrent infections and during periods of caloric restriction. In affected children, fasting for 12 to 24 hours will usually lead to hypoglycaemia with ketonaemia and ketonuria (Chaussain et al. 1974). Plasma alanine concentrations have been found to be reduced both in basal and fasted states (Pagliara et al. 1972). The levels of insulin and counterregulatory hormones are appropriate. The pathophysiology of idiopathic ketotic hypoglycaemia, also called accelerated starvation of childhood, remains unknown. A defect of protein catabolism, transamination and amino acid efflux from skeletal muscle have been implicated; therefore, a decrease in muscle mass independent of a specific enzymatic defect may predispose to hypoglycaemia and ketosis when glucose requirements are high (Lteif and Schwenk 1999).

2.4. Glucose homeostasis and acute leukaemia

Only little data on glucose homeostasis during leukaemia, and very little, if any, data on the relationship of glucose homeostasis and therapy for leukaemia, are available. Collipp et al. (1967) reported a deficiency of glucose-6-phosphatase in autopsy liver specimens taken from children who had died of leukaemia. However, the report did not involve any data on those children´s glucose metabolism or tendency to hypoglycaemia during their lifetime. A few reports

of hypoglycaemia with or without lactic acidosis during leukaemia have been published (Tashima et al. 1968, Jaffe and Kim 1971, Chen et al. 1980, Al Hilali et al. 1984, Makino et al. 1985, Eadington 1988). In a report by Chen et al. (1980) hypoglycaemia and concurrent lactic acidosis in a patient with monocytic leukaemia was regarded as a result from marked leukaemic infiltration of the liver. Makino et al. (1985), who reported a case of acute leukaemia with lactic acidosis and hypoglycaemia, found that glucose–6-phosphatase activity in the patient´s liver was much the same as in the control liver.

There are two studies concerning an antileukaemic agent MTX and glucose metabolism. MTX has been reported to increase hepatic glycogenolysis in isolated perfused rat liver (de Oliveira et al. 1986), whereas in cell culture it has been observed to induce gluconeogenesis (Hudakova et al.1992).

3. Liver disease and childhood ALL

3.1. Etiology of hepatotoxicity

In children treated for ALL the major causes of liver disease are hepatotoxic chemotherapeutic agents (Colsky et al. 1955, Hutter et al. 1960, Einhorn and Davidsohn 1964, McIntosh et al. 1977, Parker et al. 1980) and hepatitis viruses (Locasciulli et al. 1983a, Guido et al. 1991, Locasciulli et al. 1991, Locasciulli et al. 1997).

3.1.1. Antileukaemic agents

Many of the chemotherapeutic agents used in the treatment of ALL are potentially hepatotoxic. The spectrum of hepatotoxic changes is reported to be wide, including parenchymal cell injury with fatty change, hepatocellular necrosis or fibrosis, ductular injury with cholestasis, vascular lesions such as veno-occlusive disease, and hepatic neoplasms (reviewed by King and Perry 1995, King and Perry 2001). Nonetheless, systematic data on the hepatotoxic effects of chemotherapy are scant, and the mechanisms have been established for few agents. Recognizing the severity of hepatic injury is problematic, since the estimation of liver injury is based on serum liver parameters, and is therefore indirect (King and Perry 2001)

Of antileukaemic agents 6MP and MTX are frequently implicated as hepatotoxic during ALL therapy. Elevated serum transaminase levels and cholestatic jaundice are associated with 6MP (Einhorn and Davidsohn 1964, Shorey et al. 1968). MTX-induced hepatotoxicity involves hepatic fibrosis and cirrhosis (Colsky et al. 1955, Hutter et al. 1960, Nesbit et al. 1976, McIntosh et al. 1977, Parker et al. 1980), even the development of hepatoma (Ruymann et

al.1977). Hepatotoxicity has been reported to follow chronic low-dose administration of MTX not only in patients with ALL but also in patients with psoriasis and rheumatoid arthritis (Dahl et al. 1972, Zachariae and Sogaard 1987, Kremer et al. 1989). While long-term low-dose MTX may lead to fibrosis or cirrhosis, high-dose MTX therapy results in acute serum aminotransferase elevation (reviewed by King and Perry 2001).

Hepatic toxicity is quite frequent with L-asparaginase. The mechanism is uncertain, but probably involves impaired protein synthesis from asparagine depletion. Liver steatosis, likely from decreased lipoprotein synthesis, has been found in autopsy in 42% to 87% of patients (Haskell et al. 1969, Capizzi et al. 1970, Oettgen et al. 1970). Decreased levels of serum albumin as well as coagulation factors II, VII, IX, X and fibrinogen are common (Capizzi et al. 1970). The partial thromboplastin time rises progressively. Moderate elevations of serum aminotransferase, bilirubin and alkaline phosphatase levels also occur (reviewed by King and Perry 2001). Treatment with L-asparaginase has also been implicated in significant reduction of plasma levels of antithrombin III, predisposing patients to thromboembolic complications. L-asparaginase most probably affects plasma levels of antithrombin III by interfering with the translation and/or secretion of the protein in liver cells (Bushman et al. 2000). At high doses L-asparaginase may also induce liver cell necrosis (McDonald and Tirumali 1984).

Corticosteroids are associated with fatty infiltration of the liver (Hill and Rude 1966, Scheimberg et al. 1995). Cyclophosphamide is an uncommon cause of hepatotoxicity. Only few reports of elevated hepatic enzymes and of liver necrosis are attributed to the drug (Aubrey 1970, Goldberg and Lidsky 1985, Shaunak et al. 1988, Snyder et al. 1993). The etiology is likely an idiosyncratic reaction rather than a direct toxicity (King and Perry 2001). Cytosine arabinoside (ARA-C) is associated with reversibly abnormal liver function tests after continuous infusion of high-dose medication (Donehower et al. 1986). However, drug-induced cholestasis (Pizzuto et al. 1983, George et al. 1984) and liver cell necrosis (McDonald and Tirumali 1984) have also been reported. Hepatotoxicity from doxorubicin is rare. In a series by Aviles et al. (1984) six patients with ALL were treated with induction therapy using vincristine, prednisone and doxorubicin and shortly after administration, increases in aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin were observed with focal infiltration by inflammatory cells and steatosis on liver biopsies. This was considered an idiosyncratic reaction. The spindle inhibitor vincristine is excreted primarily by the liver but has seldom been implicated as a hepatotoxic agent. It has produced hepatotoxicity when used in combination with radiation (reviewed by King and Perry 2001). 6-thioguanine, another antipurine used in therapy for childhood ALL in addition to 6MP, has been implicated in the production of hepatic veno-occlusive disease (Griner et al. 1976, Gill et al. 1982, Satti et al. 1982), and in single case of peliosis hepatis (Larrey et al. 1988).

3.1.2. Hepatitis viruses

Viruses that infect liver primarily (e.g., hepatitis A, B, C, and the delta-agent) and those that infect liver secondarily (e.g., herpes viruses including Epstein-Barr virus, cytomegalovirus, and varicella zoster virus; Coxsackie B virus, and adenovirus) are potential causes of hepatitis in paediatric cancer patients (Alexander et al. 2002). In the 1970´s when no effective virus screening of blood products was available up to 25% of children treated for leukaemia were positive for hepatitis B virus (HBV) surface-antigen in Italy (Locasciulli et al. 1983a, Locasciulli et al. 1983b, Locasciulli et al. 1985). In a few more recent Italian studies antibodies for hepatitis C virus (HCV) have been detected in about 30% of the children treated for ALL (Arico et al. 1994, Dibenedetto et al. 1994). In a study by Cesaro et al. (1997) 18% (117/658) of the patients who had completed therapy for paediatric malignancy were seropositive for HCV, and 35% of these (41/117) were also seropositive for HBV with or without delta virus. There are a lot more reports of primary viral hepatitis than of liver disease associated with viruses that involve the liver secondarily. Furthermore, hepatic damage attending the secondary virus infections is usually less severe (Alexander et al. 2002).

3.1.3. Other causes

In addition to antileukaemic drugs and hepatitis viruses there are also other factors involved in the genesis of liver disease during therapy for ALL. Leukaemia infiltration, pre-existing medical problems, immunosuppression, other infections than virus hepatitis (especially fungal infections), nutritional deficiencies or total parental nutrition, and reactions to antibiotics, analgesics, antiemetics and other medication all may contribute to liver damage (reviewed by King and Perry 2001).

3.2. Manifestations of hepatotoxicity

3.2.1. Clinical liver disease

Ascites, hepatomegaly and jaundice have been reported to be clinical signs of liver dysfunction during ALL therapy (Hutter et al. 1960, Nesbit et al. 1976, Farrow et al. 1997). In a recent study by Farrow et al. (1997) only 5 out of 239 children (2.1 %) receiving therapy for ALL developed clinical liver disease. Moreover, each of them had an infectious etiology. Hepatomegaly was found in 4 and hyperbilirubinaemia in 2 children.

3.2.2. Abnormal liver tests

Serum aminotransferase elevations are common during therapy for ALL (Hersh et al.1966, Nesbit et al.1976, Topley et al. 1979, Parker et al. 1980, Locasciulli 1983b, Bessho et al. 1994, Farrow et al. 1997). The most recent and largest paediatric series to characterize serum aminotransferase elevations during therapy for childhood ALL showed that 66.5% (159/239) of the patients had an ALT level $\geq$ 180 IU/L during therapy and 18% (28/239) had one or more values $\geq$ 720 IU/L (Farrow et al. 1997). A third (53/239) of the patients had only one episode of ALT elevation over 180 U/L, 44% (70/239) had between two and five episodes, and 23% (36/239) had more than five episodes. ALT elevations were seen throughout the 2.5 years of therapy. A quarter (54/239) of the patients had ALT elevations $\geq$ 180U/L in the consolidation phase, 48% (114/239) in the first year of maintenance, and 40% (96/239) in the second year of maintenance therapy. In a study by Bessho et al. (1994) more than half of the 27 patients with ALL had ALT values more than three times the upper limit of the normal range in 25% of the measurements, but serum bilirubin, serum albumin and prothrombin time were all within the normal limits during therapy. Weber et al. (1987) measured serum aminotransferase levels in 24 children who received six courses of extremely high-dose parental MTX (33.6 g/m^2). Elevations were present in all of the children following the sixth course and were $\geq$300 U/L in 22 cases. However, the values returned to normal 1 to 2 weeks later.

3.2.3. Histopathological liver findings

3.2.3.1. Fibrosis

The first observations of histological liver disease were the observations of fibrosis in leukaemic patients treated with folic acid antagonists aminopterin or amethopterin, and date from the 1950´s (Colsky et al. 1955). Hutter et al. (1960) reviewed the incidence of hepatic fibrosis in 273 leukaemic children autopsied during the years 1940-1957. Three quarters of the patients had some degree of fibrosis. More than half (58%) of those with fibrosis had only slight and about one third (35%) intermediate fibrosis. Fourteen patients had severe fibrosis (Table 2). Prior to the use of chemotherapy consisting of steroids and folic acid antagonists in 1948, 31% had hepatic fibrosis which was only slight in all but one case. After chemotherapy was introduced 80% had some degree of fibrosis. There was also an increase in the severity of fibrosis at this time. Clinical liver disease was recorded in 11 out 14 patients (79%) with severe fibrosis. All 14 children with severe fibrosis received folic acid antagonists and steroids, and 10 out of 14 also received purine antagonists. Leukaemic infiltration was almost invariably noted but it was only slight in the patients with severe fibrosis.

Table 2. Histopathological liver findings in children with ALL.

No. of patients	LIVER HISTOLOGY	AUTHORS
5	Fibrosis in 3/3, fatty change in 2/3 (liver histological examination performed on 3 post mortem)	Colsky et al. 1955
273 autopsies	Fibrosis in 206 (75%): mild in 120/206 (58%), intermediate in 72/206 (35%), severe in 14/206 (7%), (the exact numbers of fatty change or leukaemic infiltration not given)	Hutter et al. 1960
216 children in the study, 80 with elevated liver enzyme levels	Fibrosis in 9, cirrhosis in 5 (the total number of liver histological examinations not given)	Nesbit et al. 1976
7 (8)	Mild portal fibrosis in 4 (5), fatty change in 1 (with no other findings), in addition to fibrosis: mild focal necrosis in 3, mild inflammation in 1 and mild central degeneration in 1	McIntosh et al. 1977
21 consecutive	Portal fibrosis in 3/16 (biopsied between treatment weeks 108-130); portal inflammation $\pm$ fatty change in 3/15, mild fatty change in 1/15, focal necrosis in 1/15 (biopsied between treatment weeks 95-130)	Topley et al. 1979
7 with clinical or laboratory abnormalities	Fibrous tissue in portal tracts in 2, micronodular cirrhosis in 1, fatty change in 4, mild focal chronic inflammatory cell infiltrate in 1	Parker et al. 1980
11 consecutive	Portal fibrosis in 2, mild steatosis in 3, nuclear vacualization in 1 (out of 10 biopsies which were adequate for light microscopy)	Harb et al. 1983
72 consecutive	Fibrosis in 67 (93%) (fibrosis without cirrhosis or hepatitis in 39 or 54%), cirrhosis in 2, hepatitis in 26	Guido et al. 1991

Several small studies in the 1970´s and 1980´s have reported hepatic fibrosis in association with therapy for childhood ALL. McIntosh et al. (1977) found mild portal fibrosis in 4 out of 7 children who had received intravenous MTX for 1.5 to 2.5 years, and in one child who had received oral MTX for 6.5 years. Topley et al. (1979) obtained liver biopsies from 21 consecutive children on one or two occasions during continuing chemotherapy for ALL consisting of 6MP and MTX. An early portal fibrosis was detected in 3 out of 16 patients who were biopsied between 108 and 130 weeks of treatment. In a study by Parker et al. (1980) 8 out of 36 children receiving oral maintenance chemotherapy for ALL (7 patients) or non-Hodgkin´s lymphoma (1 patient) underwent liver biopsies on the basis of clinical abnormalities and/or elevated serum enzyme levels. One of the 7 patients with ALL had micronodular cirrhosis and 2 had a mild increase in fibrous/connective tissue in portal tracts. Harb et al. (1983) studied hepatic

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ultrastructure in leukaemic children treated with MTX and 6MP by performing liver biopsies on 11 consecutive children with ALL within one month after completion of 24 to 30 months of combination chemotherapy. Fibrosis was found in all 11 patients by electron microscopy. It was mild in 6, moderate in 3 and severe in 2 patients. However, light microscopy revealed slight portal fibrosis in only 2 out of 10 patients (Table 2).

Guido et al. (1991) reported perivenular and/or porto-periportal fibrosis as the most common histological feature in children treated for ALL between 1979 and 1988. It was detected in altogether 67 out of 72 liver biopsies (93%) performed within 3 months of completing chemotherapy. Fibrosis without cirrhosis and hepatitis was present in 39 cases (54%). Cirrhosis was found in two patients. No liver biopsy showed leukaemic infiltration. Fibrosis was thought to be drug-related. In conclusion, the estimates of the prevalences of fibrosis detected by light microscopy in children with ALL have been 20% to 93% in previous studies (Table 2).

3.2.3.2. Fatty change

The early study of Hutter et al. (1960) on liver histopathology in acute leukaemia revealed fatty change in addition to fibrosis. Fatty change appeared as fine vacuoles in parenchymal cells distributed diffusely throughout the lobule in the livers with severe fibrosis. More marked fatty change was seen in the livers of the patient groups with no or slight or intermediate fibrosis. However, it did not appear to be consistently related to fibrosis, nor was there a predilection for a particular zonal distribution. The exact number of patients with fatty change or the degree of the fatty change in the liver cells were not reported in the study.

McIntosh et al. (1977) found fatty changes after three years of complete remission in one out of 8 ALL children who had received chemotherapy including methotrexate. Topley et al. (1979) reported mild fatty change in 1 out of 15 patients and portal inflammation with or without fatty change in 3 out of 15 patients who had undergone a liver biopsy during treatment weeks 95 to 130. The fatty change was usually centrilobular. In a study by Parker et al. (1980) a liver biopsy was performed only on the patients with clinical or enzyme abnormalities of the liver. Mild fatty change was detected in 4 out of 7 children receiving maintenance chemotherapy for ALL. One of these children had increased connective tissue on portal tracts in addition to fatty change. Harb et al. (1983) detected mild steatosis in 3 out of 10 patients using light microscopy (Table 2). However, lipid inclusions of varying sizes were detected in 6 out of 10 patients using electron microscopy. The most recent available study on liver histopathology in children with ALL did not report fatty change among the histological liver findings (Guido et al. 1991). In summary, fatty change has been detected to occur in approximately 10% to 60% of children with ALL.

3.2.3.3. Inflammatory changes and histological hepatitis

The aforementioned small studies have also reported inflammatory changes in the liver during therapy with MTX or MTX and 6MP, and mostly in association with portal fibrosis or fatty change. Guido et al. (1991) found histological features of hepatitis in 26 out of 72 children (36%) (Table 2). Acute hepatitis was detected in 9 and chronic hepatitis in 17 out of 72 patients. Hepatic viruses were the main causes of hepatitis.

3.2.3.4. Other histopathological findings

In the earliest liver studies on children with acute leukaemia (Hutter et al. 1960) leukaemic infiltration was one of the histological findings in liver biopsies. In more recent studies leukaemic infiltration has not been found (McIntosh et al. 1977, Topley et al. 1979, Parker et al. 1980, Harb et al. 1983, Guido et al. 1991). Mild focal necrosis and degenerative changes (Topley et al. 1977, Parker et al. 1980) have been described in a few children treated for ALL (Table 2).

3.3. Long-term sequelae of hepatotoxicity

Routine liver tests normalize promptly after cessation of therapy for ALL in the majority of patients (Hersh et al. 1966, Bessho et al. 1994). Farrow et al. (1997) reported that after the completion of therapy for childhood ALL, only 17 of 104 assessable patients (16%) had one or more elevated ALT value. Eight of these 17 patients (47%) were HCV seropositive. The remaining 9 children had subsequent normal or near normal ALT values, and none had clinical evidence of liver disease. Bessho et al. (1994), who studied liver function in 27 children with ALL, observed that by 3 months after the completion of maintenance therapy serum ALT levels had normalized in all patients and remained normal in all but two patients during the observation periods of 1 to 7 years. However, there were definite abnormalities in serum bile acid levels and in the distribution of various bile acid metabolites in the study patients, none of whom was positive for tests for HBV or HCV.

The most serious problems in long-term survivors of ALL have been chronic active and inactive persistent hepatitis. In the majority of patients HBV and HCV have been etiologic agents (Rossetti et al. 1992). In Italy 78% of the children who were treated for ALL during the 1970´s and who were HBV surface-antigen positive developed chronic hepatitis (Locasciulli et al. 1985). More recently, the Italian researchers reported that up to 46% of the patients with chronic liver disease following leukaemia therapy had evidence of HCV infection (Locasciulli et al. 1991). Dibenedetto et al. (1994) detected chronic liver disease in 17% (5/28) of HCV seropositive children with ALL. In an American study on HCV infection among survivors of childhood cancer (not only survivors of ALL) 6.6% (77) of the patients transfused between 1961 and 1992 and tested were found to

be infected with HCV. Chronic active hepatitis was found in 28 (80%), fibrosis in 25 (71%), and cirrhosis in 3 (9%) out of the 35 HCV-infected patients who had undergone a liver biopsy for abnormal liver function (Strickland et al. 2000). Locasciulli et al. (1997) followed up 114 patients cured of childhood leukaemia for 13 to 27 years after cessation of chemotherapy. At chemotherapy withdrawal 49% (56/114) were HCV-RNA positive in serum. At the end of the observation period, a persistent transaminase elevation was detected in only 4 HCV-RNA positive and anti-HCV positive cases. No patient developed signs or symptoms of decompensated liver disease.

Hepatocellular carcinoma has occasionally been described in patients treated for ALL (Ruymann et al. 1977, Kumari et al. 2000, Strickland et al. 2001). Strickland et al. (2001) quite recently reported hepatocellular carcinoma in two patients more than 20 years after therapy for childhood ALL. Serologic testing, done at the time hepatocellular carcinoma was diagnosed, revealed antibodies for HCV, suggesting that chronic HCV infection contributed to the development of the subsequent neoplasm.

During the follow-up after therapy for childhood ALL progressive histological liver disease has not been reported to occur without hepatitis infections. Of liver histological findings steatosis is thought to be reversible in most cases.

4. Iron overload

4.1. Definition and causes

Iron overload can be defined as an increase of body storage iron. In men the normal concentration of iron in the storage pool is 13 mg/kg and in women 5 mg/kg. Excess iron can gain access in three ways: the enteral route, through absorption of dietary iron, the parenteral route, through transfusions or injections of iron-containing compounds, and placental route during fetal life. Iron overload may be either primary, resulting from a deregulation of intestinal iron absorption as in hereditary haemochromatosis or secondary to other congenital or acquired conditions (Piperno 1998).

4.2. Assessment of iron overload

4.2.1. Biochemical and histological estimation of liver iron content

At present the most accurate way of estimating iron overload is by direct measurement of iron concentration in the tissues. The liver is the most accessable

for this (Gabutti and Borgna-Pignatti 1994). Different methods have been proposed for chemical determination of liver iron concentration (Barry and Sherlock 1971, Walker et al. 1971, Kreeftenberg et al. 1984). A measurement done by atomic absorption spectrometry on samples obtained by needle biopsy correlates well with the total amount of blood transfused (Gabutti and Borgna-Pignatti 1994). Since biochemical estimation of hepatic iron concentration is not always available, alternative procedures for assessing iron load have been sought. Liver iron quantification can be performed histologically (Brissot et al. 1981). Deugnier et al. (1993) have presented a histological liver iron grading system to evaluate patients with genetic haemochromatosis. From slides routinely processed and stained with Perl´s stain iron deposits are assessed according to their size and cellular and lobular locations in Rappaport´s acinus, leading to three different scores: hepatocytic, sinusoidal and portal scores. The sum of these scores defines the total iron score TIS (range from 0 to 60). Based on TIS a histological hepatic iron index is defined and calculated as the ratio of TIS to age in adult patients. It correlates well with the biochemical hepatic iron index, defined as the ratio of hepatic iron concentration (expressed as μmoles per gram dry weight) to age (Bassett et al. 1986).

4.2.2. Serum markers for iron overload

Three conventional serum markers for iron overload can be considered: serum iron, serum transferrin iron saturation and serum ferritin. The main limitation in the diagnostic usefulness of the serum iron concentration is its instability; sudden large changes occur even in healthy subjects (Cavill 1982). Acute infection, inflammation and minor injuries all precipitate a rapid fall in serum iron concentration (Cavill et al. 1986). Transferrin iron saturation corresponds to the ratio of serum iron and total iron-binding capacity, and, being influenced by the changes of serum iron concentration, may be equally variable (Cavill et al. 1986, Piperno 1998). Total iron-binding capacity is a derivative of transferrin, the production of which mainly occurs in the liver (Aisen 1984). The main determinant for the production of transferrin is the availability of iron; bone marrow iron depletion is associated with an increase and repletion with a decrease in production (McKnight et al. 1980, Idzerda et al. 1986). If there is an abundance of iron, the saturation of transferrin is increased. However, irrespective of the prevailing iron status, inflammation decreases transferrin concentrations (Huebers and Finch 1987, Ponka et al. 1998). Moreover, transferrin iron saturation may be high without iron overload in the presence of liver dysfunction, due to increased serum iron (through hepatocellular necrosis) and decreased transferrin synthesis (through liver failure) (Bonkovsky et al. 1996, Deugnier et al. 1997).

Ferritin is the major iron storage protein used in iron homeostasis to achieve reversible accumulation of iron in non-toxic forms in the tissues. Small amounts of ferritin are also secreted into the plasma (Jacobs et al. 1972). Normally the

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amount of plasma ferritin synthesized and secreted seems to be proportional to the amount of cellular ferritin produced in the internal iron storage pathway (Jacobs et al. 1972, Cook et al. 1974, Harju et al. 1984). The plasma/serum ferritin concentration is thus directly related to the magnitude of the iron stores of the body. In normal individuals it correlates with liver iron measured directly by liver biopsy. In the absence of liver damage it also correlates with transfusional iron overload (reviewed by Gabutti and Borgna-Pignatti 1994). However, being an acute-phase reactant, serum ferritin is increased in inflammation, malignancy and infections (Konijn and Hershko 1977, Siimes et al. 1977, Birgegard et al. 1978). Accordingly, patients with active liver disease may have disproportionately high values. De Virgiliis et al. (1981) demonstrated that for a given level of directly measured iron concentrations, serum ferritin was significantly higher in patients with chronic hepatitis than in those with liver siderosis only. On the other hand the final stages of hepatic fibrosis may be associated with decreased serum ferritin concentrations (Mazza et al. 1995). In iron overloaded situations serum ferritin is usually higher than the upper normal limits adjusted for sex and age, and is most often associated with high transferrin iron saturation (Halliday and Powell 1982, Piperno 1998). In haemochromatosis, ferritin concentration above 1000 µg/l suggests liver damage (fibrosis or cirrhosis) (Deugnier et al. 1992). The same level is associated with an increasing risk of developing iron-induced complications in thalassaemia major (Olivieri and Brittenham 1997).

In patients who have fully saturated transferrin levels, a significant proportion of iron may be present in serum unbound to transferrin (Hershko et al. 1978, Ahmed et al. 1986). Sensitive methods for measuring non-transferrin-bound iron in serum have been developed (Jakeman et al. 2001), but are so far not widely used in clinical practice. The benefits of serum ferritin iron measurement have been found to be marginal in patients with iron overload disease (Nielsen et al. 2000).

Few recent studies have investigated whether serum soluble transferrin receptors (sTfR) can be used in evaluating increased iron content of the body. Khumalo et al. (1998) detected sTfR to be decreased in the presence of iron overload. Looker et al. (1999) reported increased serum transferrin saturation to be associated with lower serum TfR concentration and suggested that sTfR may be useful for assessing high iron status in populations.

TfRs are present on virtually all mammalian cell surfaces except on mature red cells, and mediate the flow of transferrin-bound iron into cells by receptor-mediated endocytosis (Bomford and Munro 1985, Trowbridge and Shackelford 1986, Huebers and Finch 1987, Huebers et al. 1990, Ponka 1997). The synthesis of TfR is tightly regulated by the amount of transferrin-bound iron available to the cell. A reduction in transferrin iron saturation if body iron stores are depleted up-regulates TfR synthesis, while abundance of iron decreases synthesis. Small amounts of circulating, soluble TfR (sTfR) have been reported in human serum (Kohgo et al. 1986). This circulating receptor is a truncated form of the receptor, and lacks its cytoplasmic and transmembrane domains (Shih et al. 1990). About

80% of sTfR originates from the erythropoietic cells of the bone marrow and the circulating reticulocytes that eventually shed their receptors during maturation (Iacopetta et al. 1982, Beguin et al. 1993).

The amount of sTfR is directly proportional to the total amount of tissue receptors. STfR has been introduced as an effective and specific measure of functional iron stores, since it correlates directly with the erythropoietic activity and inversely with the amount of iron available for erythropoiesis (Huebers et al. 1990, Ferguson et al. 1992, Beguin et al. 1993). Unlike ferritin, sTfR retains its specificity to changes in iron status and is insensitive to acute-phase responses (Huebers et al. 1990, Skikne et al. 1990, Cazzola and Beguin 1992, Ferguson et al. 1992, Cook et al. 1993, Beguin et al. 1993, Punnonen et al. 1994, Punnonen et al. 1997). STfR is reported to accurately portray not only manifest but also subclinical (Suominen et al. 1998), and complicated (Suominen et al. 1997, Suominen et al. 2000) iron deficient conditions.

4.2.3. Non-invasive imaging methods

Of non-invasive imaging methods computed tomography and nuclear magnetic resonance imaging have been used in evaluating body iron stores (Gabutti and Borgna-Pignatti 1994). The methods based on measurement of hepatic magnetic susceptibility, either using superconducting quantum interference device susceptometry or magnetic resonance susceptometry, have been reported to be the most promising for assessment of iron stores in patients with sickle cell anaemia and transfusional iron overload (Brittenham et al. 2001).

4.3. Toxicity of iron and clinical sequelae of iron overload

The tissue damage associated with iron overload is believed to result primarily from free radical reactions mediated by iron. The toxicity of non-transferrin-bound iron is much higher than that of transferrin-bound iron as judged by its ability to promote hydroxyl radical formation, which results in peroxidative damage to membrane lipids and proteins, and consequently irreversible tissue damage (Hershko et al. 1998). Specific proteins are synthesized and used to store iron in non-toxic forms in the tissues. Normal cells store iron mainly in ferritin molecules. In conditions of iron excess some of it is shunted into another storage form known as hemosiderin which in states of pathological iron overload may accumulate in large quantities in almost all tissues of the body (Harrison and Arosio 1996). Hemosiderin may, in fact, be a degradation product of ferritin which is taken up by lysosomes where it undergoes partial dissolution of the core and formation of insoluble hemosiderin (Ponka et al. 1998).

The liver is a principal target for iron toxicity because it is chiefly responsible for taking up and storing excessive amount of iron. The major hepatic toxicities of iron overload include damage to multiple cell types (hepatocytes, Kuppfer

cells, hepatic stellate cells) and to multiple subcellular organelles (mitochondria, lysosomes, and smooth endoplasmic reticulum). Heavy iron overload in the liver occuring in both hereditary and secondary forms of haemochromatosis may cause fibrosis or cirrhosis, and even liver failure and hepatocellular carcinoma (reviewed by Bonkovsky and Lambrecht 2000). Iron has been shown in both human studies and animal models to be associated with lipid peroxidation and hepatic stellate cell activation (Houglum et al. 1994, Farinati et al. 1995, Houglum et al. 1997, Paradis et al. 1997, Ramm et al. 1997), and to be a contributory factor in the development or progression of alcoholic liver disease, non-alcoholic liver steatosis, chronic viral hepatitis, porphyria cutanea tarda, and perhaps, in α1-antitrypsin deficiency and end-stage liver disease regardless of cause (Bonkovsky and Lambrecht 2000).

Recently, hereditary haemochromatosis heterozygosity has been identified as an independent risk factor for myocardial infarction and cardiovascular mortality. It is suggested that iron, namely non-transferrin-bound iron, may play an important role in atherogenesis by catalysing peroxidation of low-density-lipoprotein (LDL), an essential step in atherogenesis (de Valk et al. 2000).

Iron overload and associated cardiac, endocrine and hepatic abnormalities are well-known complications of RBC transfusions (Schafer et al. 1981, Barton 1989). The relation between transfusional iron overload and organ complications has been widely documented in thalassaemia patients (reviewed by Olivieri and Brittenham 1997). The natural history of haemosiderotic cardiac disease includes first subclinical dysfunction. Subsequently, cardiomegaly and left ventricular deterioration progress to congestive heart failure. Arrhytmias can cause sudden death (Ehlers et al. 1980). Low stature, delayed puberty and hypogonadism, hypothyroidism, hypoprolactinaemia, reduced ACTH reserves, low cortisol levels, hypoparathyroidism and diabetes are reported to be associated with iron overload of endocrine glands (reviewed by Olivieri and Brittenham 1997 and Olivieri 1999). Diabetes results from a combination of insulin deficiency and insulin resistance (DeSanctis et al. 1988). In the studies by De Virgiliis et al. (1981) and by Aldouri et al. (1987), hepatic iron was related to the degree of necroinflammatory acitivity. Substantial liver fibrosis has been found to occur during the first decade of life in thalassaemic children receiving inadequate chelating therapy, and histologically proven liver cirrhosis in adolescence in most of them (Jean et al. 1984). Hepatocellular carcinoma has also been reported in a patient with thalassaemia major and with iron overload (Borgna-Pignatti et al. 1986). However, in many previous studies viral infections together with iron overload have played an important role in the genesis of liver disease in thalassaemia patients.

4.4. Transfusional iron overload in haematological malignancies

Chemotherapy for acute leukaemia routinely includes RBC transfusions. The more intensive the chemotherapy is, the more RBCs are needed in the course of prolonged cytopenias. So far no published articles on iron overload in children after treatment for haematological malignancies are available. However, recent studies have revealed that 15-20% of adults with acute leukaemia in long-term remission after routine chemotherapy, with or without bone marrow transplantation, develop iron overload, often with abnormalities in serum liver tests (Harrison et al. 1996, Barton and Bertoli 2000). Harrison et al. (1996) studied 38 patients (18 with AML, 7 with ALL, the rest with lymphomas or plasmacytoma) at least three years after the end of treatment. Ten had consistently raised serum AST activities. Four out of 6 patients with no obvious reason for elevated AST activities had non-transferrin bound iron detectable in their serum, pointing to iron overload as a cause of their liver damage. McKay et al. (1996) reported elevated serum AST and/or ALT levels in 41% (31/76) of survivors of allogeneic and autologous bone marrow transplantation at least one year post-transplant. Most patients had AML or ALL but a few had lymphoma or some other haematological disease than leukaemia. In half of those with abnormal serum AST/ALT levels, the abnormalities were not explained by viral hepatitis, veno-occlusive disease or graft-versus-host disease, suggesting that iron overload may be an important contributing factor to liver disease in the stable post-transplant setting. This view was supported by the observation of improving liver tests in 10 patients after a trial of venesection therapy. In the aforementioned studies transfusional iron overload after therapy was diagnosed by elevated serum ferritin values.

No cardiac, endocrine or rheumatologic abnormalities have been reported so far in adult patients with transfusional iron overload after treatment for acute leukaemia or other haematological malignancies (Lichtman et al. 1999, Barton and Bertoli 2000).

AIMS OF THE STUDY

The purpose of the present study was to investigate fasting tolerance and liver disease associated with therapy for childhood ALL.

The specific aims were as follows:

1. To study whether hypoglycaemia is a potential adverse effect after an overnight fast during MTX/6MP maintenance therapy for childhood ALL

2. To study the mechanisms of the proposed fasting hypoglycaemia during ALL therapy

3. To study liver function and histological liver changes after modern intensified therapy for childhood ALL

4. To study iron overload and its course in children treated conventionally for ALL and to assess whether serum iron parameters including serum transferrin receptor (TfR) are useful markers for increased body iron stores

PATIENTS AND METHODS

1. Patients

The study was a single-centre, prospective study and carried out in the Department of Paediatrics at Tampere University Hospital between 1995 and 2000. The study population consisted of children and adolescents with ALL. All of them were being treated or had been treated according to the NOPHO –92 (Nordic Society of Paediatric Haematology and Oncology) protocols for standard risk (SR) or intermediate risk (IR) ALL or high risk (HR) ALL **(**Table 3**)** (Gustafsson et al. 2000). The NOPHO ALL risk classification is presented in Table 4.

The original studies on fasting hypoglycaemia and its mechanisms (I, II) included 35 consecutive children and adolescents receiving MT for ALL consisting of oral daily 6MP and oral weekly MTX (Table 2). Sixteen were SR and 19 IR ALL patients. Nineteen of the patients were girls and 16 boys. The median age was 5.6 years (range 2.4 to 17.4 years) at the time of the study. The study was conducted between September 1995 and December 1998. Fifteen children with hypoglycaemia were re-studied 3 to 4 months after completion of therapy, between December 1995 and July 1999.

The study on liver histology after ALL therapy (III) comprised 27 consecutive children and adolescents treated for SR ALL (n = 13) or IR ALL (n = 14). Sixteen of the study patients were girls and 11 boys. The median age was 6.8 years (range 3.5 to 17.6 years) at the time of the study. The study was carried out between January 1996 and April 1999.

The study on iron overload after ALL therapy (IV) included 30 consecutive children and adolescents treated for SR ALL (n = 13), IR ALL (n = 14), or HR ALL (n = 3, all below the age of 5 years). There were 18 girls and 12 boys. The median age was 6.6 years (range 2.6 to 17.6 years) at the time of the study. Twenty-two of the 30 patients were followed up and re-studied 1 to 3 years after cessation of therapy. The study was conducted between January 1996 and April 2000.

Table 3. Nordic treatment protocols for standard risk (SR), intermediate risk (IR) and high risk (HR) (<5 years of age) ALL.

	Induction I	II	Consolidation	Interim maintenance	Delayed intensification	Maintenance therapy
SR	Pred po d 1-36 Vcr iv x 6 Adr iv x 3 40 mg/m² It MTX x 4 L-Asp im x 10		HD MTX 5g/m² iv x 3 It MTX x 3			HD MTX 5 g/m² iv x 5 It MTX x 6 Vcr/Pred po pulses x 5 6MP po 75 mg/m²/d MTX po 20 mg/m²/wk **From wk 51: po 6MP/MTX**
IR	Like SR	Cyc iv x 2 AraC iv x 16 6MP po x 28 d 60 mg/m²/d It MTX x 2	HD MTX 5 g/m² iv x 4 It MTX x 4 6MP po d 1-56 25 mg/m²/d		Dexa po d 1-22 Vcr iv x 4 Dauno iv x 4 L-Asp im x 4 Cyc iv x 1 AraC iv x 8 TG po x 14 d It MTX x 2	Like SR But: It MTX x 5
HR	Like SR/IR But: Adr iv x 4 40 mg/m² It MTX x 3	Like IR But: It MTX x 1 6MP po x 22 d 60 mg/m²/d	HD MTX 8 g/m² iv x 4 It MTX x 4 HD AraC 12 g/m² x 4	Vcr iv/Pred po pulses x 4 Vcr 1.5 mg/m² Pred 40 mg/m²/d 6MP po 75 mg/m²/d MTX po 20 mg/m²/wk (6MP/MTX for 16 wks)	Like IR But: Adr iv x 3 30 mg/m² L-Asp im x 4 1000 U/kg TG po x 11 d It MTX x 1	LSA₂L₂ (Anderson et al. 1983) **From wk 65: po 6MP/MTX**

Abbr. Pred prednisolone (60mg/m²/d or as above), Vcr vincristine (2mg/m² or as above), MTX methotrexate (doses above), L-Asp L-asparaginase (30000 U/m²/d), 6MP 6-mercaptopurine (doses above), Cyc cyclophosphamide (doses above), AraC cytarabine (75mg/m² or as above), Dexa dexamethasone (10mg/m²/d), Dauno daunorubicin (30 mg/m²), TG thioguanine (60mg/m²), HD high dose, it intrathecal, im intramuscular, iv intravenous, po oral, d day, wk week

Table 4. ALL risk classification according to the NOPHO-92 protocols.

SR

        Age 2 to < 10 years
        WBC $\leq$ 10 x 10^9/L
        No HR criteria

IR

        Age 2 to < 10 years and WBC > 10 to < 50 x 10^9/L
        Age 1 to < 2 or $\geq$ 10 years and WBC < 50 x 10^9/L
        No HR criteria

HR

        WBC $\geq$ 50 x 10^9/L
        T-ALL
        Mediastinal mass
        Lymphomatous leukaemia
        CNS ALL at diagnosis
        Testicular ALL at diagnosis
        Slow response
        T(4;11), t(9;22), 22q-

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2. Methods

2.1. Overnight fasting test (I, II)

To study fasting tolerance an overnight fasting test of up to 16 hours was performed on each study patient in the Department of Paediatrics (Children´s Ward 6) at Tampere University Hospital. Fasting was routinely started at 6 p.m. (or alternatively at 4 p.m.) following normal daily food intake and normal meals. During fasting all the children had intravenous access and were observed by staff experienced in the procedure. Blood samples for immediate bedside measurements of blood glucose levels were taken every 4 hours for the first 12 hours, thereafter every 2 hours or more often if there were clinical concerns. Blood glucose levels were confirmed in a clinical chemistry laboratory. Fasting was discontinued if a child proved to be hypoglycaemic. Hypoglycaemia was defined as a blood glucose level < 2.7 mmol/l (Koh et al. 1988, Mitrakou et al.

1991), or if there were hypoglycaemic symptoms as a blood glucose level < 2.9 mmol/l. To allow characterization of hypoglycaemia, blood samples for analyses of metabolic changes and liver tests were obtained at the time of discontinuance of fasting, or at the end of fasting for 16 hours.

2.2. Liver biopsy (III, IV)

A percutaneous liver biopsy specimen was obtained from each study patient with a Menghini biopsy set (Hepafix Luer Lock, diameter 1.4 mm, Braun, B.Braun Melsungen AG, Melsungen, Germany) during intravenous general anaesthesia concurrently with a routine bone marrow examination by the paediatric gastroenterologist Tarja Ruuska. No bleeding complications were detected. All but three patients underwent a liver biopsy at the end of therapy. Two SR patients had one year and one IR patient 4 months of therapy left at the time of the biopsy.

Liver biopsy specimens contained on average five portal tracts (range 3 to 11). A piece of the biopsy specimen was reserved for a fatty stain. The liver biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin wax, and sectioned (5 μm). The sections were stained with haematoxylin and eosin, Perl´s Prussian blue for iron, periodic acid-Schiff with and without diastase digestion and Gomori´s reticulin stain. Frozen sections were stained with oil red O to confirm the presence of fat in the cytoplasm of liver cells.

2.3. Histopathological evaluation of liver biopsy specimens (III, IV)

The pathologist Jorma Mattila performed the histopathological evaluation of the liver biopsies. The liver fatty changes were quantified as 1+, 2+ and 3+ . The biopsy specimens with 1+ fatty change contained fatty vacuoles in less than one third of the cells, those with 2+ fatty change in one third or more, but in less than two thirds of the cells, and those with 3+ fatty change in two thirds or more of the cells. Liver fibrosis was graded according to Scheuer (1991). The biopsy specimens were also screened for histological hepatitis (Scheuer 1991).

2.4. Histological determination of liver iron content (III, IV)

Liver iron deposition was evaluated (by Jorma Mattila) using the scale developed by Deugnier et al. (1993). This scoring system encompasses a total liver iron score of 60, divided into the 3 compartments: hepatocytic, sinusoidal and portal iron deposition. The sum of these scores defines the total iron score TIS ranging from 0 to 60. In Study III the total iron score was subdivided into five histological grades from 0 to IV. Grade 0 included patients with TIS 0, grade I

those with TIS 1 to 14, grade II those with TIS 15 to 29, grade III those with TIS 30 to 45 and grade IV those with TIS 45 to 60.

2.5. Biochemical analyses

2.5.1. Blood glucose

Immediate bedside blood glucose levels were assessed using Hemocue (Hemocue AB, Ängelholm, Sweden) or Glucometer Elite (Bayer Diagnostics GmbH, Leverkusen, Germany). Blood glucose levels were confirmed with an enzymatic method (Cobas Integra GLUC, Roche Diagnostics, Mannheim, Germany) in a clinical chemistry laboratory.

2.5.2. Metabolites and hormones involved in glucose homeostasis

Whole blood lactate was determined spectrophotometrically by an enzymatic end-point method with a commercially available kit (Sigma Chemical Co., St Louis, MO, USA) from a deproteinized blood sample. Plasma levels of amino acids alanine and glutamine were separated by ion-exchange chromatography using an automated amino acid analyser Alpha Plus (Pharmacia Biotech, Biochrom Ltd., Cambridge, UK). Serum levels of carnitine were measured using the original radioenzymatic assay (Cederblad and Lindstedt 1972) with minor modifications as described by Holme et al. (1989). Serum free fatty acids were measured with an enzymatic colorimetric assay (Wako Chemicals GmbH, Neuss, Germany). Plasma hydroxybutyrate concentration was analysed by an enzymatic method (Sigma Diagnostics, St. Louis, MO, USA). Radioimmunoassays were used to measure the serum levels of cortisol and growth hormone (kits from Orion Diagnostica, Espoo, Finland) as well as insulin (Pharmacia Diagnostics AB, Uppsala, Sweden). Urinary levels of organic acids were determined by gas chromatography-mass spectrometry as described earlier (Remes et al. 1992).

2.5.3. Glucagon stimulation test

To study patients´ capacity for glycogenolysis glucagon was infused intravenously 0.03 mg/kg (maximum 1 mg) after 4 to 16 hours of fasting. Blood glucose levels were measured by an enzymatic method at 0, 10, 20, 30, 40 and 60 minutes from glucagon infusion.

2.5.4. Erythrocyte concentrations of MTX and 6MP metabolites

Eryhtrocyte concentrations of MTX polyglutamates (E-MTX) and 6-thioguanine nucleotides (E-TGN) were determined by radioligand assay and high performance liquid chromatography respectively (Kamen et al.1976, Bruunshuus and Schmiegelow 1989) in a laboratory of Rigshospitalet in Copenhagen.

2.5.5. Liver tests

Serum ALT, albumin, alkaline phosphatase, bilirubin and gammaglutamyl-transferase (γGT) were analysed in an accredited laboratory using routine methods. Plasma thromboplastin time (P-TT-SPA) was assessed by a nephelometric measurement of the clotting time (Stago prothrombincomplex assay SPA, Diagnostica Stago, Asnieres, France).

2.5.6. Hepatitis serology

Serological screening for HBV and HCV (determinations of a surface-antigen of HBV, antibodies for a core antigen of HBV and antibodies for HCV) was carried out using commercial Cobas[R]Core enzyme immunoassays (Hoffman-LaRoche, Basel, Switzerland).

2.5.7. Serum lipid profile

Enzymatic methods were applied to determine serum levels of total cholesterol and triglycerides (kits from Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA and Roche Diagnostics, Mannheim, Germany). Serum HDL-cholesterol level was measured by an enzymatic, colorimetric dry chemistry method after Dextran sulphate/MgCl precipitation (Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA) or by an enzymatic, colorimetric end-point method (Roche Diagnostics System, Basel, Switzerland). Serum LDL-cholesterol level was calculated according to a Friedewald formula (Friedewald et al. 1972).

2.5.8. Serum iron status

Serum iron levels were analysed using a colorimetric assay (Cobas Integra 700, Hoffman-LaRoche, Basel Switzerland), and serum transferrin levels with an immunoturbidometric assay (Cobas Integra 700, Hoffman-LaRoche, Basel, Switzerland). Serum transferrin iron saturation (%) was calculated as the molar ratio of analytes multiplied by 100 and bearing in mind that one transferrin

molecule binds two ferric ions. Serum ferritin levels were analysed by an immunoluminometric assay (Ciba Corning ACS:180 Plus Apparatus, Ciba Corning Diagnostics, Halstead, UK). STfR measurements were carried out using the IdeA sTfR IEMA assay (Orion Diagnostica, Turku, Finland).

2.6. Study protocols

2.6.1. Studies I and II

A fasting test lasting for up to maximum 16 hours was performed during MT for SR or IR ALL. MT started at treatment week 14 in the SR ALL and at treatment week 32 in the IR group. Children with SR ALL were studied from treatment weeks 26 to 130 (median 62), and those with IR ALL from treatment weeks 40 to 104 (median 64). The 15 patients (out of 35 study patients) who developed hypoglycaemia on the fasts during MT were re-fasted 3 to 4 months after cessation of therapy. Blood glucose levels and the levels of metabolites and hormones involved in glucose homeostasis were measured in association with fasting as described above. In Study II hepatic capacity for glycogenolysis in children with a tendency to hypoglycaemia was studied by determining blood glucose responses to glucagon after fasts of 4 to 16 hours. E-MTX and E-TGN were also measured at the time of the fasts, and the current doses of MTX and 6MP medication calculated as mg/m^2 per week or per day respectively.

2.6.2. Study III

Children treated for SR ALL (n=13) or IR ALL (n = 14) underwent a liver biopsy at the end (n = 24) or close to the end (n = 3) of therapy. Serum ALT levels were followed repeatedly during therapy. At the time of a liver biopsy ALT and also other serum liver tests and lipid profiles were measured, and hepatitis serology screened.

2.6.3. Study IV

A liver biopsy specimen for histological evaluation of liver siderosis was obtained at the end (n = 27) or close to the end (n = 3) of ALL therapy. In addition to the patients in Study III (13 with SR ALL and 14 with IR ALL), Study IV also included 3 children with HR ALL. Irradiated, white-cell depleted, packed RBCs had been transfused during intensive therapy phases (induction, consolidation and reinduction therapy or during the first 6 to 12 months of therapy) whenever blood haemoglobin level was below 100 g/L. The total volume of RBCs transfused during therapy was calculated for each patient as

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millilitres per kilogram. Serum iron status and sTfR were measured at the time of a liver biopsy and again 1 to 3 years after cessation of therapy. Serum liver tests were also measured at the time of a liver biopsy.

3. Statistical analyses

Statistical analyses were performed using SPSS versions 6.1, 9.0 or 10.1. A non-parametric Mann-Whitney U test was applied to evaluate differences in laboratory parameters between two independent subgroups, and Wilcoxon Signed Ranks test between two dependent subgroups. A Chi-Square test was used whenever the variables were categorical. Differences in laboratory values among several study subgroups were assessed with a nonparametric Kruskall-Wallis one-way ANOVA test. A Spearman rank test was applied for correlations. A two-sided p-value below 0.05 was considered to indicate significance.

4. Ethics

The study was approved by the Ethical Committee of Tampere University Hospital. Informed consent was obtained from the parents of each study patient. Adolescents and children who were old enough also consented themselves to participate in the study.

RESULTS

1. Therapy-related fasting hypoglycaemia in children with ALL (I)

Nineteen out of 35 patients (54%) developed hypoglycaemia after 11 to 16 hours of overnight fasting. In 6 out of the 19 patients (32%), hypoglycaemia occurred before 12.5 hours of fasting. Almost half (9/19) had blood glucose levels below 2.0 mmol/l. Thirteen out of the 19 children with hypoglycaemia (68%) exhibited one or more symptoms (6 vomited, 2 trembled, 2 sweated, 2 experienced nausea, and 5 were drowsy or restless) (Fig. 3).

The patients with hypoglycaemia were younger than the patients with normal blood glucose levels. Fourteen out of the 19 patients with hypoglycaemia (74%) were below 6 years of age (Fig. 4). Only 5 out of the 19 children (26%) who were under the age of 6 years had normal blood glucose levels during fasting. The children with hypoglycaemia and the children with normal blood glucose levels did not differ significantly with respect to sex, height (height SD score), body mass index (BMI), current doses of 6MP and MTX, duration of therapy, or ALL risk group (Table 5).

Fifteen out of the 19 patients who developed hypoglycaemia on the fasts during MT were re-tested 3 to 4 months after cessation of therapy. Fasting tolerance had improved in all of them (Fig. 5). It had become normal in 10 out of 15 patients (67%). In 5 patients, blood glucose levels still fell below 2.7 mmol/l (range 2.0 to 2.6 mmol/l) after 16 hours of fasting. However, none had any symptoms.
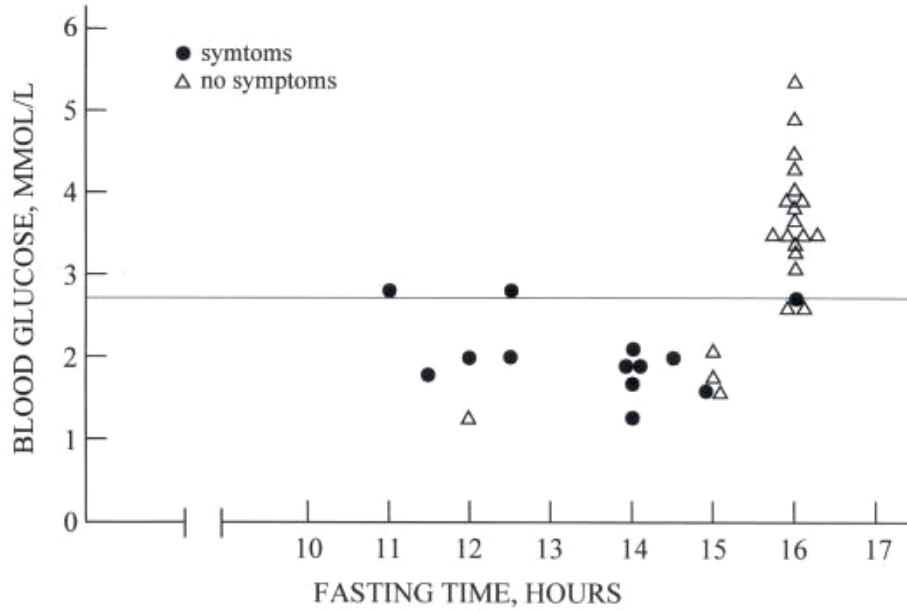
Figure 3. *Minimum fasting blood glucose concentration and duration of fasting in 35 children with ALL. The horizontal line represents a blood glucose level of 2.7 mmol/l.*
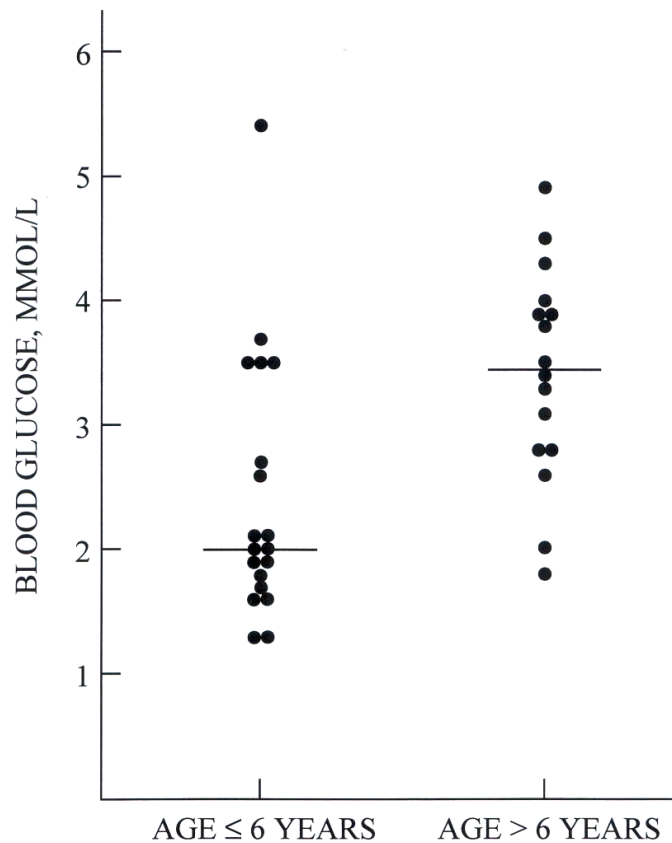

Figure 4. *Minimum fasting blood glucose concentration in children ≤ 6 years and > 6 years of age with ALL. Horizontal lines represent medians.*
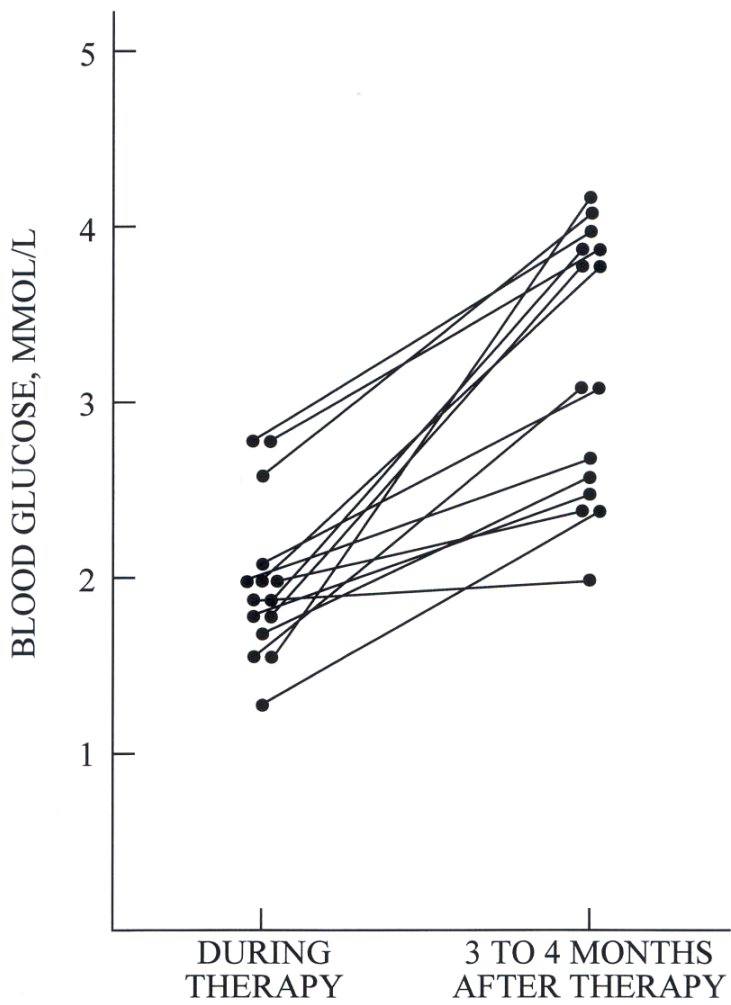
Figure 5. *Minimum blood glucose concentration in 15 children in the fasting test during maintenance therapy and 3 to 4 months after cessation of therapy*

Table 5. Clinical characteristics and laboratory parameters of the 35 children with ALL fasted for up to 11 to 16 hours during MT. Medians and ranges.

	HYPOGLYCAEMIA GROUP [a] n = 19	NORMOGLYCAEMIA GROUP n = 16	p
Girls/Boys	13/6	6/10	0.068*
SR/IR ALL	10/9	6/10	0.371*
Age, years	4.5 (2.4-11.3)	8.3 (3.9-17.4)	0.002
Number of therapy week	56 (26-130)	71 (42-130)	0.217
BMI, kg/m^2	17.4 (14.9-21.0)	18.6 (14.2-24.6)	0.243
Height, SDS	-0.3 (-1.3-1.1)	0.4 (-2.4-2.4)	0.422
Current medication			
6MP, mg/m^2/day	63 (19-132)	63 (23-102)	0.883
MTX, mg/m^2/week	17 (10-23)	18 (8-23)	0.589
B-glucose, mmol/l	2.0 (1.3-2.8)	3.8 (3.1-5.4)	< 0.001
B-pH	7.31 (7.25-7.36)	7.36 (7.33-7.39)	< 0.001
B-BE, mmol/l	-6.6 (-11.4- -0.7)	-0.8 (-4.0-1.5)	< 0.001
B-HCO$_3$, mmol/l	18.6 (14.6-25.0)	24.1 (19.5-28.2)	< 0.001
P-OH-butyrate, mmol/l	2.25 (1.00-3.94)	0.19 (0.13-1.37)	< 0.001
S-free fatty acids, mmol/l	3.09 (1.59-4.54)	1.23 (0.15-3.48)	< 0.001
B-lactate, mmol/l	1.3 (0.6-2.6)	1.0 (0.5-1.5)	0.061
S-cortisol, nmol/l	868 (173-1410)	449 (202-1418)	0.007
S-GH, µg/l	2.9 (0.9-20.3)	1.4 (0.8-19.3)	0.151
S-insulin, mU/l	3.0 (3.0-20.0)	7.5 (4.0-25.0)	< 0.001
P-amino acids, µmol/l			
Alanine	117 (73-265)	190 (86-404)	0.009
Glutamine	396 (242-757)	448 (357-761)	0.031
S-carnitine, µmol/l			
Free	20.3 (13.8-29.5)	29.8 (16.8-45.5)	0.027
Total	34.2 (24.4-44.7)	36.1 (23.1-52.5)	0.505
S-albumin, g/l	43 (35-50)	43 (34-51)	0.945
S-ALT, U/l	172 (23-614)	116 (38-293)	0.286
P-TTSPA, %	70 (44-89)	81 (50-92)	0.037
E-MTX, mmol/nmolHb	6.4 (1.7-16.0) (n=16)	6.5 (1.8-10.5) (n=9)	0.671
E-TGN, mmol/nmolHb	147 (42-536) (n=16)	191 (147-595) (n=9)	0.070
U-dicarboxylic acids			
High in	14/16 (88%)	2/14 (14%)	< 0.001*

Mann-Whitney U test, * Chi Square test

a) Blood glucose level below 2.7 mmol/l or below 2.9 mmol/L with symtoms

Abbr. ALT alanine aminotransferase, BMI body mass index, BE base excess, GH growth hormone, E- MTX/TGN erythrocyte concentration of methotrexate polyglutamates/ 6-thioguanine nucleotides, SDS standard deviation score, P- TT-SPA plasma thromboplastin time.

2. Metabolic changes and liver dysfunction associated with fasting hypoglycaemia (I, II)

Fasting hypoglycaemia was ketotic. Plasma hydroxybutyrate and serum free fatty acids were elevated in each patient with hypoglycaemia. No evidence of hyperlactacidaemia was detected. Serum free carnitine was low in the patients with hypoglycaemia. However, serum total carnitine was not decreased. The response of serum cortisol to hypoglycaemia was appropriate. Hyperinsulinism was not detected. A 16-hour fast with hypoglycaemia did not stimulate growth hormone secretion. Plasma levels of gluconeogenic amino acids alanine and glutamine were reduced in the patients with hypoglycaemia, and significantly lower in them than in the patients with normal blood glucose levels (Table 5). Plasma alanine was either at or below the lower limit of the normal range in 12 out of 18 patients with hypoglycaemia (67%) versus in 4 out of 15 patients with normal blood glucose levels (27%) ($p = 0.022$). Urinary dicarboxylic acids were also more often elevated in the patients with hypoglycaemia. There were no differences in E-MTX and E-TGN at the time of the fasts between the patients with hypoglycaemia and those with normoglycaemia (Table 5). The ketosis and metabolic changes described above had disappeared by the time of control fasts performed 3 to 4 months after cessation of therapy (Table 6).

Four out of 11 patients with hypoglycaemia (36%), but only one out of 8 patients (12.5%) with normoglycaemia exhibited a defective response in blood glucose levels to glucagon stimulation after fasts of 4 to 15 hours and after a fast of 16 hours respectively ($p = 0.243$).

There was no statistically significant difference in ALT levels between the patients with hypoglycaemia and those with normal blood glucose levels (Table 5). However, serum ALT was above 200 U/l in 9 out of 19 patients with hypoglycaemia (47%) but in only 2 out of 16 patients with normal blood glucose levels (12.5%) ($p = 0.029$). Plasma thromboplastin time (P-TT-SPA, %) was lower in the patients with hypoglycaemia (Table 5).

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Table 6. Age and laboratory values of the 15 study patients at the fasts during MT for ALL and at the control fasts 3 to 4 months after therapy. Medians and ranges.

	During therapy n = 15	After therapy n = 15	p
Age, years	4.5 (2.4-9.4)	5.1 (3.9-9.6)	0.001
B-glucose, mmol/l	1.9 (1.3-2.8)	3.1 (2.0-4.2)	0.001
B-pH	7.31 (7.26-7.36)	7.35 (7.28-7.42)	0.005
B-BE, mmol/l	-6.6 (-9.5- -3.2)	-2.6 (-8.4-0.1)	0.002
P-OH-butyrate, mmol/l	2.25 (1.03-3.30)	1.06 (0.38-2.82)	0.002
S-free fatty acids, mmol/l	3.13 (1.59-4.54)	1.54 (0.70-3.01)	0.002
B-lactate, mmol/l	1.4 (0.7-2.6)	0.9 (0.6-2.8)	0.068
P-amino acids, μmol/l			
Alanine	122 (90-265)	171 (84-218)	0.043
Glutamine	396 (242-757)	473 (281-579)	0.442
S-carnitine, μmol/l	(n = 8)	(n = 8)	
Free	21.0 (13.8-27.3)	27.9 (16.2-36.6)	0.043
Total	34.0 (24.4-40.8)	39.8 (31.1-51.1)	0.043
S-ALT, U/l	212 (23-614)	18 (1-68)	0.001
Plasma thromboplastin time			
(P-TTSPA, %)	66 (44-83)	95 (65-136)	0.001
U-dicarboxylic acids	(n = 9)	(n = 9)	
High in	9/9 (100%)	2/9 (22%)	< 0.001[*]

Wilxcoxon Signed Ranks test, * Chi-Square test

3. Liver histology and serum liver tests in children with ALL (III)

3.1. Liver histology after ALL therapy

Liver histological changes after ALL therapy, clinical characteristics and median/mean ALT levels in 27 study patients are presented in Table 7.

None of the patients had entirely normal liver histology. Panacinar fatty change was detected in 25 out of 27 patients (93%). In 5 out of the 25 patients (20%) the fatty infiltration was graded as 1+, in 7 (28%) as 2+, and in 13 (52%) as 3+ fatty change. Microvesicles were the exclusive or dominant feature in the majority of the cases. The hepatocytes were filled with small fat droplets and had centrally placed nuclei. In many cases fine fat droplets in the liver cells could

only be found in a frozen section stained with oil red O. Macrovesicles obscuring the nucleus by displacing it to the periphery of the cell were present in 8 out of the 25 cases (32%). Large fat droplets were mainly centrolobular. Concomitant microvesicles were always present. There were no differences in the degree of fatty change between the SR and IR ALL risk groups (Table 7).

Mild liver fibrosis was found in 3 out of 27 patients (11%). It was quantified as grade I (enlarged, fibrotic portal tracts) in one out of 3, and as grade II (periportal or porto-portal septa but intact liver architecture) in 2 out of 3 patients. One of the patients with fibrosis had been treated according to an SR ALL protocol for 1.5 years (patient No. 27), two had completed the therapy for ALL (patient No. 12 for SR ALL and patient No. 7 for IR ALL) (Table 7).

Liver siderosis was detected in 19 out of 27 patients (70%). Stainable iron was seen in parenchymal cells as well as in reticuloendothelial Kupffer cells. Siderosis was grade I/IV in 3 out of 19 patients (16%), grade II/IV in 10 out of 19 patients (53%), and grade III/IV in 6 out of 19 patients (32%) (Table 7). The SR and IR ALL groups did not differ significantly with respect to siderosis, even though there were 6 patients without siderosis in the SR group versus 2 patients in the IR group.

Fatty change without any other histological liver findings was detected in 8 out of 27 patients (30%). In half of these 8 patients fatty change was graded as 3+. Fatty change together with siderosis was found in 14 out of 27 patients (52%). Each of the 3 patients with fibrosis also had various degrees of fatty change and siderosis. Siderosis as a sole finding was found in 2 out of 27 patients (7%) (Table 7).

Seven out of 27 cases (26%) showed ballooning degeneration of liver cells (patients Nos. 3, 8, 10, 11, 19, 21, 23). Only one had intracanalicular cholestasis (patient No. 3). Inflammation was minimal or absent. No signs of histological hepatitis was found.

3.2. Laboratory data and liver histology

To evaluate the association between the laboratory data and liver histology the study patients were categorized into three study subgroups according to liver histology. The patients with fatty change as a sole finding were assigned to the fatty change group, the patients with siderosis and with or without fatty change to the siderosis group, and the patients with fibrosis to the fibrosis group. The three liver histology groups with laboratory data and clinical characteristics are presented in Table 8.

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Table 7. Liver histological changes and serum ALT levels in the 27 study patients treated for ALL.

Patients	Age (years)	Gender (F/M)	ALL group (SR/IR)	LIVER HISTOLOGY			Median/mean ALT (range)
				Fatty change	Fibrosis	Siderosis	
1	4.9	F	IR	none	none	II	52/52 (10-103)
2	4.2	M	IR	none	none	III	36/45 (5-210)
3	3.5	F	IR	1+ (m)	none	0	62/106 (18-275)
4	5.8	F	SR	1+ (m)	none	0	191/133 (12-631)
5	6.0	F	SR	1+ (m)	none	0	207/207 (2-544)
6	6.5	M	SR	1+ (m)	none	II	91/112 (28-352)
7	7.0	M	IR	1+ (m)	II	II	94/149 (12-856)
8	8.8	F	SR	2+ (m, M 5%)	none	0	216/258 (14-965)
9	6.6	F	SR	2+ (m, M 5%)	none	II	50/68 (11-257)
10	7.5	M	SR	2+ (m, M 10%)	none	II	108/139 (5-857)
11	15.8	M	IR	2+ (m, M 10%)	none	II	48/67 (12-380)
12	5.0	M	SR	2+ (m, M 50%)	II	II	169/262 (42-1071)
13	13.6	M	IR	2+ (m, M 50%)	none	III	106/112 (50-172)
14	17.6	M	IR	2+ (m)	none	III	141/214 (7-1635)
15	4.7	F	IR	3+ (m)	none	0	64/87 (17-398)
16	4.8	F	SR	3+ (m)	none	0	40/99 (13-593)
17	6.8	F	SR	3+ (m)	none	0	166/172 (10-386)
18	10.3	M	SR	3+ (m)	none	0	75/92 (2-413)
19	3.9	F	IR	3+ (m)	none	I	58/61 (4-155)
20	9.4	F	IR	3+ (m)	none	I	100/132 (18-501)
21	12.5	F	IR	3+ (m, M 10%)	none	I	53/99 (25-265)
22	5.9	F	SR	3+ (m)	none	II	88/123 (9-685)
23	12.4	M	IR	3+ (m)	none	II	76/85 (12-380)
24	13.7	F	IR	3+ (m, M 5%)	none	II	101/110 (10-280)
25	5.5	F	SR	3+ (m)	none	III	77/146 (2-1333)
26	17.4	M	IR	3+ (m)	none	III	104/129 (24-430)
27	7.8	F	SR	3+ (m)	I	III	332/315 (23-870)

Fatty change grading: none, 1+, 2+, 3+, Fibrosis grading: none, I, II, III, IV, Siderosis grading: 0, I, II, III, IV, m microvesicular, M macrovesicular

3.2.1. Serum liver tests

In the fibrosis group serum ALT level was 200 U/l or more in 47%, and 300 U/l or more in 33% of the serial measurements performed during total therapy. The corresponding figures were lowest in the siderosis group (13% and 4%). Serum bilirubin level at the time of the liver biopsy was significantly highest in the siderosis group and lowest in the fibrosis group. No significant differences were detected in maximal or serial serum ALT, serum albumin, alkaline phosphatase and γGT levels, or plasma thromboplastin time between the three liver histology groups (Table 8).

In 24 out of the 27 study patients (89%) the maximum ALT level peaked at least once over 200 U/l, in 12 out of 27 patients (44%) over 500 U/l, and in 7 out of 27 patients (26%) over 750 U/l during therapy.

3.2.2. Serum lipid profiles

There was a significant difference between the three liver histology groups in serum total and LDL-cholesterol levels, which were highest in the fibrosis group and lowest in the siderosis group (Table 8). When two liver histology groups were separately compared to each other, serum total and LDL-cholesterol levels were significantly higher in the fibrosis group than in the fatty change group (p = 0.036, p = 0.042 respectively), or in the siderosis group (p = 0.036, p = 0.042 respectively). However, there were no differences between the fatty change and siderosis groups. No significant differences were detected in serum HDL-cholesterol or triglyceride levels between the three liver histology groups, even though serum HDL-cholesterol level tended to be lowest and triglyceride level highest in the fibrosis group (Table 8).

3.2.3. Hepatitis serology

Serological screening for HBV and HCV was negative in all study patients.

Table 8. ALL group, gender, age, BMI and laboratory parameters in 27 ALL patients grouped according to their liver histology. Medians and ranges.

GROUP	FATTY CHANGE n = 8	SIDEROSIS ± fatty change n = 16	FIBROSIS+fatty change+siderosis n = 3	p
SR/IR	6/2	5/11	2/1	0.106[#]
Girls/Boys	7/1	8/8	1/2	0.186[#]
Age, years	5.9 (3.5-10.3)	8.4 (3.9-17.6)	7.0 (5.0-7.8)	0.233
BMI, kg/m^2	18.2 (15.0-21.8)	17.9 (14.2-23.4)	17.9 (17.0-19.4)	0.986
S-albumin, g/l	45 (43-48)	45 (40-51)	43 (34-50)	0.689
S-alkaline phosphatase, U/l	569 (315-687)	449 (224-607)	456 (397-643)	0.161
S-ALT, U/l				
Serial median	107 (40-216)	83 (36-141)	169 (94-332)	0.099
Maximum	479 (275-965)	366 (103-1635)	870 (856-1071)	0.059
% proportions of serial ALT measurements (means and ranges)				
≥ 200 U/l	31 (5-57)	13 (0-34)	47 (17-77)	0.038
≥ 300 U/l	12 (0-39)	4 (0-19)	33 (10-59)	0.014
S-bilirubin, µmol/l	10 (6-19)	20 (4-46)	8 (6-11)	0.025
S-γGT, U/l	15 (11-36)	9 (4-18)	19 (18-120)	0.523
P-TTSPA, %	82 (71-143)	69 (50-107)	83 (64-105)	0.147
S-total cholesterol, mmol/l	4.05 (3.60-4.70)	3.75 (2.20-5.50)	5.60 (4.80-5.70)	0.025
S-HDL cholesterol, mmol/l	1.34 (0.92-1.73)	1.00 (0.66-1.97)	0.98 (0.98-1.31)	0.231
S-LDL cholesterol, mmol/l	2.30 (2.10-2.60)	2.30 (1.10-3.50)	3.80 (3.10-4.00)	0.042
S-triglycerides, mmol/l	0.92 (0.64-3.15)	0.77 (0.49-1.70)	1.40 (0.93-1.98)	0.117

Kruskall Wallis one-way ANOVA test, [#] Chi-Square test

4. Liver iron overload and serum iron status in children with ALL (IV)

4.1. *Liver iron overload and serum iron parameters at the end of therapy*

At the time of liver biopsy the median liver TIS was 24 (range 0-37) among all study patients. Eight out of 30 patients (27%) had a TIS value of 0, 3 out of 30 patients (10%) had TIS from 1 to 14, 12 out of 30 patients (40%) from 15 to 29, and 7 out of 30 patients (23%) 30 or more, indicating no, mild, moderate, or severe iron overload histologically respectively. The median serum ferritin was 450 μg/l (range 14-3158 μg/l), iron 19.4 μg/l (range 6.7-38.4 μg/l), transferrin iron saturation 38% (range 11-102%), transferrin 1.9 g/l (range 1.1-3.9 g/l), and sTfR 2.57 mg/l (range 1.30-8.73 mg/l).

Serum ferritin and transferrin iron saturation showed the strongest postive correlation with TIS (Fig. 6). Serum iron also correlated positively with TIS ($r = 0.642$, $p < 0.001$). On the other hand, serum transferrin and sTfR correlated negatively with TIS ($r = -0.800$, $p < 0.001$; $r = -0.400$, $p = 0.035$ respectively). There was also a strong positive correlation between the volume of transfused RBCs and TIS (Fig. 6). In all patients with a TIS value of 0 serum ferritin was within the normal range. Two out of the 3 patients with TIS from 1 to 14, and all patients with TIS over 15 had serum ferritin above the normal upper limit. Serum transferrin iron saturation was within the normal range in the patients with TIS below 15. However, each patient with serum transferrin iron saturation above the normal range had a TIS value of 27 or more (Fig. 6).

The results of the serum iron status and serum/plasma liver tests in patients with varying degrees of liver iron overload are presented in Table 9. Serum ferritin, iron and transferrin iron saturation levels were highest, but transferrin level was lowest in the patients with severe iron overload compared to the patients with less iron overload. The patients with smaller or greater iron overload as measured by liver TIS values did not differ from each other with respect to serum sTfR or any other liver test than alkaline phosphatase, which was highest in the patients with no or mild iron overload (Table 9).

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Table 9. Serum iron parameters, serum/plasma liver tests and the volume of transfused red blood cells (RBCs) in the three liver TIS (total iron score) groups. Medians and ranges.

LIVER TIS GROUPS	GROUP 1 No or mild iron overload TIS 0-14 n = 11	GROUP 2 Moderate iron overload TIS 15-29 n = 12	GROUP 3 Sevre iron overload TIS $\geq$ 30 n = 7	p
ALL group, SR/IR/HR	6/5/0	5/5/2	2/4/1	0.674*
Gender, F/M	9/2	6/6	2/5	0.079*
Age, years	6.0 (3.5-12.5)	6.6 (2.6-15.8)	7.7 (4.3-17.6)	0.501
Ferritin, μg/l	63 (14-502)	516 (153-3158)	1621 (495-3068)	< 0.001
Iron, μmol/l	16.1 (6.7-24.7)	17.7 (7.3-29.0)	31.2 (22.2-38.4)	0.003
Transferrin iron saturation, %	26 (11-49)	39 (13-86)	85 (39-102)	0.002
Transferrin, g/l	2.5 (1.9-3.9)	1.7 (1.1-2.3)	1.7 (1.4-2.3)	< 0.001
Transferrin receptor, mg/l	3.45 (1.81-8.73)	2.15 (1.30-3.12)	2.39 (1.58-4.71)	0.075
ALT, U/l	95 (45-631)	87 (11-280)	144 (27-442)	0.470
Alkaline phosphatase, U/l	563 (315-687)	455 (339-643)	376 (224-541)	0.035
Plasma thromboplastin time, %	77 (56-90)	78 (51-105)	70 (50-107)	0.732
Transfused RBCs, ml/kg	106 (51-139)	163 (110-380)	225 (152-378)	< 0.001
(~ unit/kg, median)	(~1/3 unit/kg)	(~ 1/2 unit/kg)	(~ 3/4 unit/kg)	

Kruskall- Wallis one-way ANOVA test, *Chi-Square test
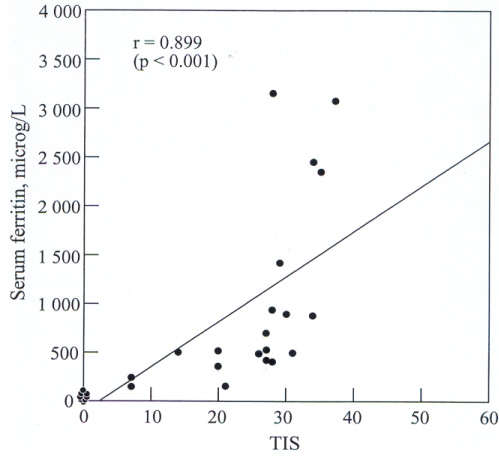
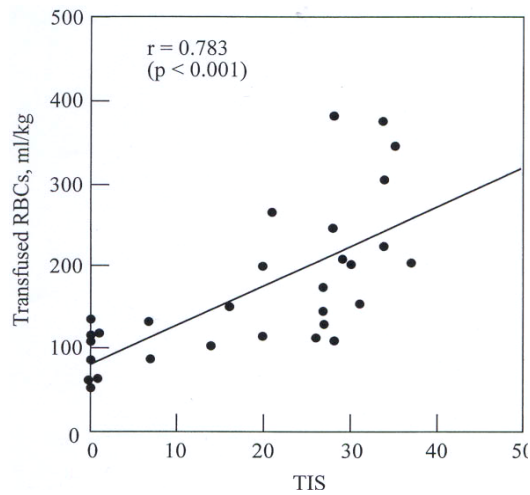(a)   (b)
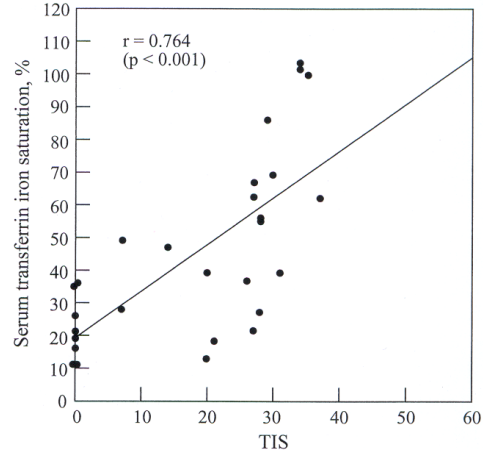
(c)

Figure 6. *Correlation of liver TIS (total iron score) to (a) serum ferritin at the end of therapy, (b) serum transferrin iron saturation at the end of therapy, and (c) volume of transfused RBCs (red blood cells).*

4.2. Serum iron parameters 1 to 3 years after cessation of therapy

The medians of serum iron parameters from 22 re-studied patients at the end of therapy and 1 to 3 years after cessation of therapy are presented in Table 10. Results of serum sTfR measurements were only available for 14 patients. Serum ferritin, iron, transferrin iron saturation and sTfR levels were significantly lower and transferrin level significantly higher 1 to 3 years after therapy than at the end of therapy in all re-studied patients (Table 10). Three out of 22 patients (14%) had serum ferritin level over 1000 µg/l 1 to 2 years after therapy (Fig. 7). All

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three were boys and had been treated according to the IR ALL protocol. At the time of cessation of therapy the three patients were older than the others (median 17.3 years, range 13.7-17.6 years, vs median 5.8 years, range 2.6 –15.8 years, p = 0.003), and their TIS values were also higher (median 35, range 34-37, vs median 20, range 0-34, p = 0.001).

Of the serum iron parameters measured 1 to 3 years after cessation of therapy ferritin level was positively (r = 0.908, p < 0.001), and transferrin level negatively (r = -0.656, p = 0.001) correlated with TIS taken at the end of therapy. However, serum iron, transferrin iron saturation or sTfR levels taken 1 to 3 years after cessation of therapy did not correlate with TIS.

Table 10. Serum iron parameters at the end of therapy for ALL, and 1 to 3 years after cessation of therapy in 22 children. Medians and ranges.

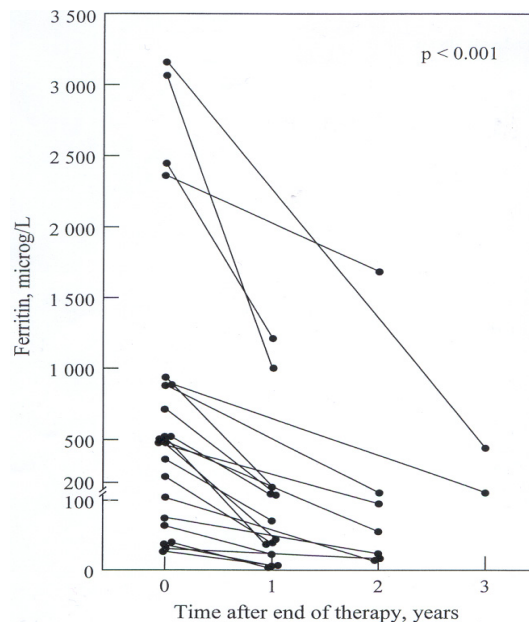|	At the end of therapy n = 22	1 to 3 years after therapy n = 22	P
Ferritin, μg/l	494 (28-3158)	63 (5-1693)	< 0.001
Iron, μmol/l	19.4 (6.7-38.4)	14.0 (3.8-26.1)	0.010
Transferrin iron saturation, %	38 (11-102)	22 (7-64)	0.001
Transferrin, g/l	1.9 (1.4-3.9)	2.5 (1.7-3.3)	0.001
Transferrin, receptor, mg/l	2.96 (1.30-4.76) (n = 14)	2.00 (1.06-2.81) (n = 14)	0.013

Wilcoxon Signed Ranks Test

Figure 7. Serum ferritin levels at the end of therapy and 1 to 3 years after cessation of therapy in 22 patients treated for ALL.

DISCUSSION

1. Fasting hypoglycaemia and related metabolic changes in children receiving maintenance therapy for ALL

The study showed that fasting hypoglycaemia is a potential, yet reversible, adverse effect of MT in children being treated according to the Nordic regimens for SR or IR ALL. Children under six years of age are especially prone to fasting hypoglycaemia; in our study 74% of the children with hypoglycaemia were younger than 6 years of age. Moreover, only 26% of the children under the age of 6 years remained normoglycaemic during fasting. Over half of all study patients reached hypoglycaemia within 11 to 16 hours of fasting. This is a pathological finding, since the normal lower limit for a blood glucose level (2.5th percentile) in healthy children aged 3 to 15 years after a 14 –hour fast is reported to be 3.7 mmol/L (Lamers et al. 1985). In almost a fifth of all patients (6/35) hypoglycaemia was detected after 11 to 12.5 hours of fasting. This indicates that in the course of overnight sleep, children with ALL are at risk of becoming hypoglycaemic, with or without symptoms. No differences in sex, height (SD score), BMI, or ALL risk group between the patients with hypoglycaemia and those with normoglycaemia were detected, and did not explain the tendency to hypoglycaemia.

Hypoglycaemia was clearly ketotic and free fatty acids were increased in the patients with hypoglycaemia, pointing respectively to intact fatty acid oxidation and lipid mobilization. There was no evidence of hyperinsulinism or hypo-cortisolism. Deficiency of growth hormone was not suspected in anyone, even though the fasting and hypoglycaemia did not stimulate growth hormone secretion. Hyperlactic acidaemia, an indicator of defective gluconeogenesis (Lee and Leonard 1995), was not detected. Serum levels of free carnitine were low, but levels of total carnitine within the normal range, suggesting high acylcarnitine formation in the liver rather than deficiency of carnitine because of low biosynthesis or intake in these patients. High urinary excretion of dicarboxylic acids in the hypoglycaemic patients was thought to be in accordance with abundant oxidation of fatty acids to ketoacids.

Reduced plasma levels of gluconeogenic amino acids alanine and glutamine were found to be associated with hypoglycaemia. Plasma alanine level was at or below the normal lower limit in 67% (12/18) of the hypoglycaemic children. This would suggest that an insufficient supply of gluconeogenic substrates is at least partly responsible for hypoglycaemia. An impaired response to a glucagon

stimulation test was detected in four out of 11 patients with hypoglycaemia after 4 to 15 hours of fasting, but in only one out of 8 patients with normal blood glucose levels and in the latter not until after 16 hours of fasting, pointing to either lowered hepatic glycogen stores or defective glycogenolysis in the patients with hypoglycaemia.

Most biochemical changes in children with ALL and with a tendency to fasting hypoglycaemia are similar to findings in idiopathic ketotic hypoglycaemia of childhood. Characteristics include raised serum levels of free fatty acids and ketone bodies (Chaussain 1973), appropriate endocrine responses, and normal plasma levels of lactate but low levels of alanine (Pagliara et al. 1972, Haymond et al. 1974, Lee and Leonard 1995). In children with idiopathic ketotic hypoglycaemia the glycaemic response to glucagon has also been reported to be less after fasting, suggesting that hepatic glycogen is more rapidly exhausted in them (Chaussain et al. 1974).

The impaired fasting tolerance was obviously associated with the MT, particularly with the 6MP and MTX. In all re-fasted patients with hypoglycaemia, fasting tolerance had improved by the time of the control fasts performed 3 to 4 months after cessation of ALL therapy. There were no significant differences between the hypoglycaemic and normoglycaemic children with ALL in relation to the dose levels of 6MP and MTX, the erythrocyte concentrations of metabolites of 6MP and MTX or the stage of MT at which fasting tolerance was studied. This suggests that concentrations or cumulative doses of 6MP and MTX do not need to reach particular levels for hypoglycaemia to occur.

The liver test results were more abnormal in the patients with hypoglycaemia than in those with normal blood glucose levels, suggesting that the tendency to fasting hypoglycaemia may be associated with liver dysfunction in children with ALL. It is widely known that liver damage, abnormal liver tests, and abnormal liver biopsy results are among findings during oral 6MP and MTX therapy (Einhorn and Davidsohn 1964, Nesbit et al. 1976, Berkovitch et al. 1996). Both 6MP and MTX are anti-metabolites. 6MP is a purine analogue and MTX a folic acid analogue. They affect the synthesis of RNA and DNA precursors and the functioning of normal cells (Peters et al. 1993), also liver cells. However, only few studies on the effects of 6MP and MTX on glucose metabolism in the liver are available. MTX has been shown to be capable of increasing hepatic glycogenolysis in an isolated perfused rat liver (de Oliveira et al. 1986). It has also been reported to accelerate gluconeogenesis in cells in vitro (Hudakova et al. 1992). We have found no studies concerning the effects of 6MP and its metabolites on hepatic glycogenolysis and gluconeogenesis.

The catabolism of 6MP includes pathways of oxidation to thiouric acid, methylation to 6-methyl mercaptopurine, and desulphuration (Van Scoik et al. 1985). To be cytotoxic 6MP must undergo intracellular conversion to 6-thio-guanine nucleotides (TGNs), which are incorporated into DNA (Tidd and Paterson 1974a, Tidd and Paterson 1974b, Zimmerman et al. 1974). A major metabolic pathway of 6MP is methylation to 6-methyl mercaptopurine catalysed

by the thiopurine-methyltransferase (TMPT) (Sarcione and Stutzman 1959, Weinshilboum et al. 1978, Weinshilboum 1989, Aarbakke 1995). There are large individual variations in TPMT activity regulated by a genetic polymorphism at a single locus on chromosome 6 with two co-dominant alleles, including a low acitivity gene with a population frequency of approximately 6% (Szumlanski et al. 1996). Patients homozygous for a low acitivity TPMT gene will have increased conversion of 6MP to 6TGN, and are at increased risk for severe toxicity when treated with thiopurines (Evans et al. 1991, Lennard et al. 1993). Thus, it is possible that patients with low activity of TPMT could also be more prone to hepatotoxicity and impaired fasting tolerance. No measurements of concentrations of methylated 6MP metabolites or TPMT were performed in the present study. However, the data on TPMT concentrations were available for 14 children with hypoglycaemia and 6 children with normoglycaemia from a study by Kjeld Schmiegelow et al. (personal communication). According to the data TMPT concentrations did not differ in the hypoglycaemic and normoglycaemic children (p = 0.458), suggesting that low activity of TPMT is not associated with fasting hypoglycaemia during ALL therapy.

An interesting question which also remained unanswered in our study is whether repeated high-dose MTX courses included in the Nordic therapy regimens for SR and IR MT of ALL are associated with the tendency to hypoglycaemia. No reports concerning the issue are available from countries with different therapy regimens.

In a recent retrospective Italian study Ziino et al. (2002) described symptomatic hypoglycaemia during the early morning hours in 6 out of 86 children (6.9%) receiving oral purine analogues for treatment of childhood ALL. Symptomatic hypoglycaemia episodes occurred not only during maintenance but also during consolidation and reinduction phases of ALL therapy. The researchers suggested that the administration of purine analogues appears to be associated with hypoglycaemia more than the combination of 6MP and MTX.

The occurrence of therapy-related hypoglycaemia during overnight fasting is often insidious, and potentially hazardous. However, it can be prevented. We recommend that children under the age of 6 years should be awakened after 8 to 9 hours of sleep to drink juice or milk. In the evening it is important to eat meals high in proteins and complex carbohydrates. Obviously, concurrent illnesses with vomiting and/or diarrhea as well as prolonged day-time fasting are also risk factors for hypoglycaemia during therapy for ALL. To prevent hypoglycaemia in these situations easily absorbed carbohydrates should be provided.

In conclusion, the present study proved that overnight fasting hypoglycaemia is an adverse effect of MT for ALL. However, no single exact mechanism of hypoglycaemia was revealed in the study. Liver dysfunction and low plasma levels of gluconeogenic amino acids, especially alanine, are associated with hypoglycaemia. Reduced hepatic glycogen stores or impaired hepatic glycogenolysis may partly explain hypoglycaemia. The effects of 6MP and MTX on hepatic glycogenolysis and gluconeogenesis need further investigation.

2. Liver disease in children after modern ALL therapy

The main histological manifestations of liver disease in our patients after modern therapy for childhood SR and IR ALL were microvesicular fatty change and siderosis, mostly grade II/IV or III/IV. Liver fatty change was detected in 93% and siderosis in 70% of all study patients. Half had both fatty change and siderosis. Only 11% of the patients had fibrosis and it was mild. Each patient with liver fibrosis also had grade II/IV or III/IV siderosis and microvesicular fatty change. None of the patients had hepatitis or cirrhosis histologically. There were no differences in liver histology findings between the SR and IR ALL treatment groups.

We detected liver fatty change in practically all study patients. In comparison, previous studies on liver histology in children treated for ALL have reported fatty change to be present in approximately 10 to 50% of the patients depending on the study (McIntosh et al. 1977, Topley et al. 1979, Parker et al. 1980, Harb et al. 1983). Moreover, in our study fatty change was grade 3+ in as many as in half of the patients. A new finding was that liver fatty change is mainly microvesicular. One explanation for this may be that in the earlier studies frozen sections of the liver were not evaluated with a fatty stain as in the present study. However, increased intensity and thereby increased toxicity of ALL therapy are the most probable explanations for the phenomenon, since in our experience microvesicular fatty change is primarily considered to be a toxic change.

Microvesicular steatosis is generally a more severe finding than macrovesicular lesion (Burt et al.1998). It occurs in conditions characterized by damage of the mitochondrial beta-oxidation process (Fromenty and Pessayre 1997, Fromenty et al 1997). Drugs that impair mitochondrial DNA transcription or DNA replication like dideoxynucleosides (Fromenty and Pessayre 1997), and also dexamethasone (Letteron et al. 1996), have been reported to be responsible for microvesicular steatosis. Milder forms of microvesicular steatosis usually have a good short-term prognosis (Fromenty and Pessayre 1997). In the long-term, however, chronic steatosis whether macrovesicular or microvesicular, can lead to the progressive development of steatohepatitis lesions in some patients. These lesions include steatosis, necrosis, Mallory bodies, a mixed inflammatory infiltrate and fibrosis or cirrhosis. Steatosis itself is sufficient to cause lipid peroxidation, which might explain these lesions. Steatohepatitis has been reported to occur rarely in patients with obesity or diabetes and more frequently in patients receiving amphiphilic cationic drugs or abusing alcohol. In the two latter conditions, not only is fat for substrate peroxidation present in the liver, but the formation of reactive oxygen species is also increased, resulting in more severe peroxidation and more frequent steatohepatitis (Fromenty et al. 1997). It has also been detected that in pure alcoholic fatty liver the presence of a mixed

macrovesicular and microvesicular pattern of steatosis is associated with a greater risk of the development of fibrosis and cirrhosis (Teli et al. 1995a).

Liver fibrosis was found in only 3 out of 27 patients (11%) in our study, and it was mild in each patient. No histological hepatitis changes were detected. Our findings are in contrast to the results of the most recent study on liver histopathology after therapy for childhood leukaemia by Guido et al. (1991), who reported liver fibrosis in as many as 93% (67/72) and hepatitis in 36% (26/72) of the study patients. The most probable explanation for this is that none of our patients was infected by hepatotropic viruses, which were the major cause of chronic liver disease and histological picture of hepatitis in the study by Guido et al. (1991). The absence of hepatitis in our study may be the result of the effective virus screening by the Finnish Red Cross Blood Transfusion Service. It has screened for HBV since 1972 and for HCV since 1990. We also emphasize that in our study liver fibrosis was more rare than in several small studies in the 1970´s and 1980´s which reported hepatic fibrosis in about 18% to 63% of the study patients (McIntosh et al. 1977, Topley et al. 1979, Harb et al. 1983).

Liver fibrosis is shown to be associated with hepatic iron overload (Jean et al. 1984, Bassett et al. 1986, MacDonald et al. 2001), lipid peroxidation and steatosis (Fromenty et al. 1997, MacDonald et al. 2001). Of our 3 patients with fibrosis two had grade II/IV and one grade III/IV liver siderosis. Their degree of steatosis varied from 1+ to 3+. It is worth mentioning that there were 5 patients with grade III/IV liver siderosis without any fibrosis in our study.

Serum ALT levels reached high levels in many patients during therapy. This is in accordance with the recent study by Farrow et al. (1997). Previous studies have reported that the extent of aminotransferase elevation is not predictive of abnormal liver histology (McIntosh et al. 1977, Topley et al. 1979, Parker et al. 1980, Harb et al. 1983). We likewise found no association between single high ALT levels and liver histology. However, the patients with fibrosis reached ALT levels of 300 U/L or more significantly more often in the serial measurements during total therapy than the patients with fatty change or siderosis. Of other laboratory parameters in our study serum total cholesterol and LDL-cholesterol concentrations were highest in the patients with fibrosis at the end of therapy. Serum HDL-cholesterol levels tended to be lowest and triglyceride levels tended to be highest in the patients with fibrosis. Since no long-term data on serum cholesterol levels for the study patients were available, the association between disturbed serum lipid profile and liver fibrosis plus impaired liver metabolism remains unclear. Serum γGT, which is a sensitive method for measuring toxic damage of hepatocytes, was elevated at the end of therapy in only one patient who had hepatic fibrosis grade II. Regarding the measurements of liver dysfunction there are several shortcomings in the present study. No other liver function test than plasma thromboplastin time was used. We did not measure plasma coagulation factors V and VII, or antithrombin III. Nor did we measure serum bile acids or prealbumin.

Taken together, the more intensified IR ALL therapy did not seem to predispose to more severe liver histopathology than the SR ALL therapy.

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Hepatic fibrosis occurred more rarely than expected, suggesting that modern intensified ALL therapy is not associated with more extensive liver fibrosis than the former therapy. Fibrosis is probably a permanent hepatic lesion, while fatty change instead is generally thought to be reversible (Teli et al. 1995b). However, further studies will reveal whether the microvesicular fatty change and siderosis are of long-term importance by being associated with sustaining liver histopathology like steatohepatitis and fibrosis, especially in cases of childhood obesity or abundant alcohol consumption in adolescence and adulthood.

3. Transfusional iron overload and its course in children treated for ALL

Our study revealed that altogether 22 out of 30 patients with ALL (73%) had iron overload in the liver by histological evaluation with total iron score (TIS) at the end or near the end of therapy. Moreover, in 19 out of 30 (63%) patients liver siderosis was graded as II/IV (TIS > 15) or more. There was a definite positive correlation between the volume of transfused RBCs and liver TIS. Of serum iron markers serum ferritin and transferrin iron saturation correlated most significantly and positively with liver TIS. Two out of 3 patients with TIS from 1 to 14 and all patients with TIS 15 or more had increased serum ferritin levels. Moreover, the correlation of serum ferritin with liver TIS remained significant during the follow-up of 1 to 3 years, indicating that serum ferritin may be the most useful serum parameter for long-term monitoring of the resolution of iron overload in this patient group. In other diseases with transfusional iron overload, as in thalassaemias and sickle cell anaemias, some previous studies have not been able to detect a linear relationship between serum ferritin and hepatic iron concentration (Brittenham et al. 1993, Mazza et al. 1995, Harmatz et al. 2000). In the present study no viral hepatitis, severe fibrosis or cirrhosis were found. Thus, there were no confounding factors which would have raised serum ferritin levels and diminished the value of ferritin as an indicator of iron overload.

Extensive iron overload is potentially associated with endocrine, cardiac and hepatic complications (Schafer et al. 1981, Bassett et al. 1986, Barton 1989). Therefore recognizing it is also important in survivors of childhood ALL after current intensified therapy including increasing amounts of RBCs. Previous studies on iron overload are available only for adults, suggesting that 15 to 20% of adults with acute leukaemia in long-term remission after routine chemotherapy develop iron overload, often with hepatic abnormalities (Harrison et al. 1996, McKay et al. 1996, Barton and Bertoli 2000). In our study serum iron parameters including serum ferritin normalized in most patients during the follow-up. This implies that the transfusion-related iron overload decreases in the course of time, and during growth, and is largely reversible in children treated conventionally for ALL. Physical growth is known for its ability to consume iron stores. However, serum ferritin values in excess of 1000 µg/l persisted in 3 out of

22 patients (14%) 1 to 2 years after cessation of therapy. The three patients with sustained iron overload were older than the rest of the study patients, indicating that the remaining growth potential at the time of cessation of therapy may be directly related to the reversibility of iron overload. No difference in age was detected at the end of therapy between the patients with more extensive liver iron overload and those with no or mild iron overload. None of the three patients with sustained iron overload had liver fibrosis at the end of therapy or manifested endocrine or cardiac abnormalities during the follow-up.

Our study also showed that serum sTfR is not a useful marker for iron overload in children with ALL. Even though serum sTfR concentration correlated negatively with TIS at the end of ALL therapy, it consistently decreased during the follow-up of 1 to 3 years. If it had been a marker for lowering iron stores, it would have increased instead. There may be several explanations for the finding that sTfR was higher at the time of cessation of therapy than after the follow-up of 1 to 3 years. First, elevated serum TfR could signal functional iron deficiency at the end of ALL therapy, i.e. iron-deficient erythropoiesis in the presence of adequate or increased iron stores (Suominen et al. 2000), a condition which is usually induced by a sustained acute-phase reaction and a deranged cytokine balance. Second, at the end of therapy erythropoiesis may have been compensatorily accelerated in some patients resulting in elevated sTfR concentrations. Third, serum TfR concentrations are shown to decrease with age until the age of 16 years (Suominen et al. 2001).

In conclusion, transfusional iron overload is common in children and adolescents after modern conventional ALL therapy. Even though it is reversible in most cases, it may not resolve as readily in patients who have already reached their adult stature by the end of therapy. Our study patients were mostly those treated with SR and IR protocols. Only three patients were treated according to an HR protocol, all under 5 years of age. If there had been more older patients with HR ALL receiving intensive therapy and also more RBC transfusions, long-term iron overload would probably have been detected more often. Ferritin was found to be a good but sTfR inappropriate serum marker for iron overload in this patient group. We recommend that serum iron status is measured at the end of ALL therapy, and followed up when necessary. The volume of transfused RBCs during therapy should be calculated for each patient. A liver biopsy should be considered in cases of sustained iron overload. After intensive therapy phases (induction, consolidation, reinduction) a haemoglobin level for RBC transfusions could be lower than 90 g/l. Therapy for iron overload such as venesections may also be needed, especially in children with very high-risk ALL who have been treated according to intensive therapy regimens with allogeneic bone marrow transplantation accompanied by numerous RBC transfusions.

SUMMARY AND CONCLUSIONS

The aims of the present study were to investigate glucose homeostasis and fasting tolerance during therapy, and to evaluate the severity of liver disease and iron overload after therapy for childhood ALL.

To study fasting tolerance an overnight fasting test lasting for up to 16 hours was performed on 35 children and adolescents receiving MT for SR and IR ALL. The MT consisted of daily oral 6MP and weekly oral MTX. Nineteen out of 35 patients reached ketotic hypoglycaemia during 11 to 16 hours of fasting. Children under the age of 6 years were especially prone to hypoglycaemia. Reduced plasma levels of alanine and abnormal liver tests were associated with hypoglycaemia. No exact single etiological mechanism was revealed. Three to 4 months after cessation of chemotherapy the tendency for fasting hypoglycaemia was improved. In conclusion, hypoglycaemia after prolonged overnight fasting is a potential and insidious adverse effect during MT for ALL. However, it is reversible, and can be prevented.

Liver disease after modern ALL therapy was studied by obtaining a liver biopsy and by measuring serum liver tests at the end of therapy in 27 children and adolescents with SR and IR ALL. The main liver histological findings were microvesicular fatty change and siderosis, which occurred in 93% and 70% of the patients respectively. Liver siderosis was most often grade II/IV or III/IV. Liver fibrosis was detected less frequently than expected, in only 11% of the patients. Serum ALT levels reached temporarily high levels in most patients during therapy. The long-term sequelae of liver microvesicular fatty change and siderosis, which could be associated with more severe liver histopathology like steatohepatitis and fibrosis, were not ascertained in the present study.

Iron overload was evaluated histologically from liver biopsy specimens taken from 30 patients at the end of ALL therapy, and by measuring serum iron status at the end of therapy, and 1 to 3 years after cessation of therapy. At the end of therapy mild, moderate and severe liver iron overload were detected in 10%, 40% and 23% of the patients respectively. In most cases iron overload seemed to be reversible. However, it was long-term in 14% of the re-studied patients. Serum ferritin was a good serum marker for iron overload, whereas serum TfR was not useful in detecting it. We recommend that serum iron status is measured at the end of ALL therapy, and followed up whenever iron overload is detected. In cases of severe iron overload treatment should be considered.

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Päivi Halonen

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