Discussion
Glutamine

In the past decades, the role of the amino acid glutamine in cell function and metabolism has been studied extensively. Key research issues included the role of glutamine in maintaining intestinal integrity and modulation of the immune response. At a basic level, this role primarily comprises providing fuel for rapidly dividing cells and serving as precursor for protein and nucleic acid synthesis. In addition, recent research has focused on glutamine acting as signaling molecule in states of illness and injury. In a landmark study published in 1990, Lacey and Wilmore state that in critically ill patients, endogenous glutamine synthesis cannot meet increased demand and hence glutamine should be considered as a conditionally essential amino acid. Subsequently, numerous randomized controlled trials have evaluated the effect of glutamine supplementation on morbidity and mortality in adult burn, trauma, bone marrow transplantation, and critically ill patients. In a recent meta-analysis, Novak et al. showed that glutamine supplementation decreased infectious morbidity and mortality in burn and trauma patients (update available at www.criticalcarenutrition.com). In a guideline on enteral nutrition in intensive care patients, the European Society for Parenteral and Enteral Nutrition now recommends enteral glutamine supplementation in burn and trauma patients.

Glutamine supplementation in very low birth weight infants

For the fetus, glutamine is an important source of nitrogen. During pregnancy, there is a substantial flux of glutamine from the maternal to the fetal circulation, increased by glutamine synthesized in the placenta. Very low birth weight (VLBW) infants may be susceptible to glutamine depletion, as placental supply ceases at birth, tolerance of enteral nutrition is limited and parenteral nutrition does not contain glutamine for solubility and stability reasons. However, stable isotope studies on glutamine kinetics have shown that VLBW infants have a high rate of de novo glutamine synthesis as compared to full term infants and adults. In addition, in response to intravenous administration of amino acids (without glutamine) VLBW infants are able to increase glutamine synthesis. Nevertheless, it is possible that even increased glutamine synthesis cannot meet the (increased) demand. The question arises whether in the immediate postnatal period, systemically available glutamine meets the demands of organs with a high glutamine consumption, e.g. the gut. In addition, several experimental studies showed that supply of glutamine from the apical side is of critical importance for maintaining intestinal integrity.

In chapter 2 we described a randomized controlled trial of glutamine-enriched enteral nutrition in 102 VLBW infants. In this study, we investigated the effect of enteral glutamine supplementation (in a dose of 0.3 g/kg/d between days 3 and 30 of life) on
feeding tolerance, infectious morbidity and short-term outcome. We found that glutamine-enriched enteral nutrition did not improve feeding tolerance (time to full enteral feeding) nor short-term outcome (e.g. growth, age at discharge from NICU and age at discharge home) in VLBW infants. However, the incidence of serious infections was significantly lower in infants receiving glutamine-enriched enteral nutrition. These results were not influenced by gestational age, birth weight nor by birth weight <10th percentile (Usher et al.62). Other trials of both parenteral39-41 and enteral8,42 glutamine supplementation in VLBW infants have found varying results.

With regard to feeding tolerance, the results of our study are in contrast with the results of other trials of enteral glutamine supplementation in VLBW infants. Vaughn et al.42 and Neu et al.8 found that enteral glutamine supplementation decreased gastrointestinal dysfunction (Bell stage I) and decreased the number of days when feeding was withheld, respectively. In our study, the use of feeding guidelines may have attributed to the lack of difference between the treatment groups with regard to time to reach to full enteral feeding. Furthermore, a comparison between our study and the studies by Vaughn et al.42 and Neu et al.8 is difficult, because those studies did not report data on either time to full enteral feeding or the age when parenteral nutrition was discontinued. Trials of parenteral glutamine supplementation showed varying effects on feeding tolerance.39-41 In general, comparison of all studies may be hampered by the use of different feeding guidelines for both the introduction or withholding of enteral nutrition.

In our study, glutamine-enriched enteral nutrition decreased the risk of serious infections, which is in line with the results of the other single-center trial, as performed by Neu et al.8 However, Vaughn et al.42 and Poindexter et al.40 did not find decreased nosocomial sepsis in recent multi-center trials of enteral and parenteral glutamine supplementation in VLBW infants. Differences in the way the supplementation was administered and adaptation of the supplementation dose during the study, may have contributed to the different results of the studies. In addition, in our study, we found a relatively high infection rate in both the glutamine-supplemented and control groups. This is in agreement with the results of a recent surveillance study of nosocomial infections in our NICU.69 In both studies we used predefined definitions for nosocomial infections in neonates that were adapted from the current definitions from the Centers for Disease Control and Prevention for nosocomial infections in children <1 year. The relatively high infection rate can be partially explained by our definitions for nosocomial infections and the relatively high device-associated utilization ratios. Furthermore, the definition of serious infections in our study not only included culture-proven sepsis, but also culture-proven pneumonia, meningitis, pyelonephritis and arthritis.

With regard to short-term outcome, we did not find a statistically significant effect of glutamine-enriched enteral nutrition on length of ventilator support, growth, age at
discharge from NICU and age at discharge home. This is in line with the other two studies on enteral glutamine supplementation. In the study of Lacey et al., parenteral glutamine supplementation was associated with fewer days on the ventilator (infants with BW <800 g). In our study, the smaller number of days on ventilator support in the glutamine-supplemented group approached statistical significance. An explanation for these findings may be that glutamine supplementation decreased the intestinal inflammatory and thereby attenuated distal organ injury, as further discussed in the ‘cytokine responses’ section.

Safety
To assess safety of glutamine-enriched enteral nutrition in VLBW infants, we determined plasma amino acid concentrations as part of the afore-mentioned randomized controlled trial (chapter 2). We found that enteral glutamine supplementation in a dose of 0.3 g/kg/d between days 3 and 30 of life did not alter plasma amino acid concentrations. In particular, plasma glutamine and glutamate concentrations were not different between the treatment groups. Therefore, adverse effects of glutamine-enriched enteral nutrition, as a consequence of elevated plasma levels of glutamine, glutamine metabolites (e.g. glutamate, which is potentially neurotoxic) or interference with the metabolism of other amino acids (as reflected by changes in the plasma amino acid profile) seem unlikely.

It is striking that in all studies on enteral glutamine supplementation, including our study, plasma glutamine concentrations did not change, whereas in 3 of 4 studies on parenteral supplementation plasma glutamine concentrations were increased in the glutamine-supplemented group. This difference can be attributed to the fact that enterally administered glutamine is almost entirely metabolized by the gut and does not reach the systemic circulation, as shown in stable isotope studies.

The unchanged plasma amino acid concentrations are in line with the finding that no adverse effects of glutamine supplementation were observed in any of the infants. At this point it is important to note that all infants who developed severe periventricular-intraventricular hemorrhage (PIVH) (stage ≥III) were randomly assigned to the glutamine-supplemented group. In all of these infants, PIVH occurred before any supplementation was administered. The higher mortality in the glutamine-supplemented group can be explained because 3 of the 6 infants with severe PIVH in the glutamine-supplemented group died. In the study of Vaughn et al., there was a tendency towards a lower prevalence of grades 3 and 4 intraventricular hemorrhages and/or periventricular leukomalacia in the glutamine-supplemented group. However, it is not clear whether PIVH occurred before or after start of the supplementation.

Another aspect of safety of glutamine-enriched enteral nutrition in VLBW infants includes long-term neurodevelopmental outcome. We assessed the neurodevelopmental outcome at the corrected age of two years in 72/88 (82%) infants
who participated in the initial trial (13 infants had died, 1 infant was excluded because of a severe chromosomal abnormality associated with poor neurodevelopmental outcome). The Bayley Scales of Infant Development-II were performed by a psychologist, who was unaware of the treatment allocation in the neonatal period. Both the mental developmental index (MDI) and the psychomotor developmental index (PDI) were not different in the glutamine-supplemented and control groups (MDI 96 (49-139) vs. 101 (58-136) and PDI 93 (69-119) vs. 101 (49-122) in glutamine-supplemented (n = 40) and control groups (n = 32), respectively). In a secondary analysis of 1018 infants who participated in a multicenter trial of parenteral glutamine supplementation, infants were stratified by early versus late provision of parenteral amino acids. At 18 months’ corrected age both the MDI and the PDI were not different between groups. Unfortunately, the authors did not provide data about the MDI and PDI in the glutamine-supplemented versus the control group.

**Mechanisms of glutamine action in very low birth weight infants**

In VLBW infants, the mechanisms of glutamine action have hardly been investigated. As part of the randomized controlled trial of glutamine-enriched enteral nutrition in VLBW infants (chapter 2), we performed several studies to elucidate the role of glutamine in postnatal adaptation of the gut (intestinal permeability, intestinal microflora) and modulation of the immune response (cytokine responses).

**a. Intestinal permeability**

It has been shown that depletion of dietary or endogenously synthesized glutamine has a negative effect on the integrity of intestinal epithelium. Furthermore, in animal and experimental studies, glutamine supplementation reduces intestinal atrophy and decreases intestinal permeability following parenteral nutrition, abdominal radiation and pharmacological agents. Animal studies have shown that improved intestinal integrity leads to decreased bacterial translocation, decreased systemic spread of bacteria and consequently to decreased infectious morbidity. In human studies, glutamine-enriched enteral nutrition resulted in a lower systemic inflammatory response in multiple trauma patients and changes in immune cell subtype distribution in VLBW infants, reflecting decreased bacterial challenge in these patients. One study in adult burn patients found that increased intestinal permeability was associated with increased infectious morbidity. In another study, burn patients who developed clinical infections had a greater increase in intestinal permeability than patients who did not develop infections. In our study, the lower infection rate in infants receiving glutamine-enriched enteral nutrition was not associated with decreased intestinal permeability, as measured by sugar absorption.
test (chapter 4). This is the first study into the effect of glutamine-enriched enteral nutrition on intestinal permeability in an unselected group of very low birth weight infants throughout a 4-week study period. Studies of glutamine supplementation in adults have found both no\textsuperscript{17,18} and beneficial\textsuperscript{19-22} effects on intestinal permeability.

Several factors may explain why we did not find a relation between intestinal permeability and decreased infectious morbidity in VLBW infants receiving glutamine-enriched enteral nutrition. In an experimental study of intestinal glutamine deprivation in rats, De Marco et al. showed that only complete glutamine depletion (both endogenous and nutritional) resulted in a decrease of intestinal barrier function.\textsuperscript{91} It is unlikely that the infants in our study had complete glutamine depletion in the first weeks after birth as minimal enteral feeding was started immediately after birth and enteral nutrition on average after 3.5 days. Under these circumstances, glutamine-enriched enteral nutrition may have no additional effect on the postnatal decrease in intestinal permeability. Furthermore, studies of Van der Hulst et al.\textsuperscript{22} and Hulsewé et al.\textsuperscript{18,92} suggest that glutamine supplementation particularly improves intestinal integrity in patients with both inflammation and nutritional depletion. Inflammation plays a significant role in the morbidity of VLBW infants (e.g. chronic lung disease, brain injury).\textsuperscript{93,94} The high intestinal permeability directly after birth and the rapid postnatal decrease may reflect (attenuation of) a neonatal inflammatory response. We could not find an additional effect of glutamine-enriched enteral nutrition on this process. This finding may be explained by the fact that the largest decrease in intestinal permeability occurred before day 7, whereas the supplementation dose was maximal at approximately day 10 after birth. Finally, the passage of saccharides may not adequately reflect the passage of bacteria across the intestinal barrier.\textsuperscript{96} However, in a recent review on intestinal permeability and systemic infections in critically ill adults, de Souza et al. mentioned several well designed clinical studies showing an association between increased permeability and systemic infections.\textsuperscript{97} One of these studies reported that the higher the increase in intestinal permeability, the higher the risk of infection.\textsuperscript{98} These studies at least suggest some causal relationship between increased intestinal permeability and systemic infections.

b. Intestinal microflora

In VLBW infants, the intestinal colonization by health promoting bacteria (e.g. \textit{Bifidobacterium} and \textit{Lactobacillus} species\textsuperscript{24,25}) is delayed, as compared to healthy breast-fed infants (chapter 5-I). The mucus layer is an important site for bacterial colonization\textsuperscript{27} and its composition may modulate bacterial adherence.\textsuperscript{28} A study in rats suggested that parenteral glutamine supplementation improved thickness and optical density of the mucus gel.\textsuperscript{4} These glutamine-mediated changes of the mucus layer in turn may lead to altered bacterial adherence and colonization of the gut. Preliminary data indicate that supplementation of probiotics is associated with decreased infectious morbidity, probably by stimulating colonization with health promoting
bacteria. However, we found that glutamine-enriched enteral nutrition did not affect the prevalence of bifidobacteria, lactobacilli, *E. coli*, streptococci and clostridia in the intestinal microflora of VLBW infants (chapter 5-II). Thus, decreased infectious morbidity in infants that received glutamine-enriched enteral nutrition was not associated with higher numbers of health promoting species, such as *Bifidobacterium*, *Lactobacillus* and *Enterococcus*.

In our study, the development of the intestinal microflora in the first 30 days of life was characterized by large inter-individual differences both in terms of composition and counts. In general, the number of fecal bacterial groups was low at birth, whereas the number of species increased significantly in the first 30 days of life. This is in line with previous studies, using both conventional culture and molecular techniques. Furthermore, colonization with bifidobacteria was delayed, whereas numbers of *E. coli* and streptococci rapidly increased over time. These findings are consistent with other studies in VLBW infants.

Host-, treatment-, and nutrition-related factors may play a role in the development of the intestinal microflora in VLBW infants. In our study, treatment with systemic antibiotics substantially decreased the prevalence of all bacterial groups, which is in line with previous studies. Moreover, we found that stay at a general hospital ward (versus stay at a neonatal intensive care unit) increased the prevalence of all bacterial groups. In term infants, delivery by cesarean section (CS) is associated with decreased colonization with bifidobacteria. However, in our study in VLBW infants, the prevalence of bifidobacteria was higher in infants born by cesarean section as compared to infants born by vaginal delivery. Possibly, this finding is explained by the smaller percentage of infants in the CS group that was treated with systemic antibiotics directly after birth. Finally, in contrast to findings in term infants, we found that exclusive breast milk feeding was not associated with increased prevalence of bifidobacteria. Other studies in preterm infants have shown varying effects of breast milk feeding on the development of the intestinal microflora. We hypothesize that in VLBW infants, treatment with systemic antibiotics has an important negative effect on the development of the intestinal microflora in the first weeks of life. Therefore, the effect of mode of delivery, breast milk feeding or glutamine supplementation may be negligible.

c. Cytokine responses

Experimental studies have indicated that the presence of glutamine in vitro increases the cytokine production by T-lymphocytes following stimulation with various mitogens. A subset of T-helper cells denoted as T helper type 1 (Th1) cells, is implicated in the cell-mediated resistance to infections, whereas Th2 cells are involved in the regulation of antibody responses. The Th subsets can be differentiated based on the production of a specific panel of cytokines: IFN-γ, TNF-α and interleukin (IL) 2 are representative for the Th1 response, whereas IL-4, IL-5 and IL-10 are
representative for the Th2 response. It has been shown that glutamine supplementation increases the Th1 response in septic mice\textsuperscript{32} and in adult trauma patients.\textsuperscript{33} In our study on cytokine responses in VLBW infants, glutamine-enriched enteral nutrition did not specifically enhance Th1 cytokine responses, as determined following in vitro stimulation of whole blood cells with $\alpha$-CD\textsubscript{3}/$\alpha$-CD\textsubscript{28} or LPS (chapter 6). Therefore, the beneficial effect of glutamine-enriched enteral nutrition on the incidence of serious neonatal infections, does not result from major changes in innate or adaptive immune responses.

Comparison of the studies on cytokine responses and glutamine supplementation is hampered by differences in study populations and methods to determine cytokine responses. In our study, supraphysiological stimulation with $\alpha$-CD\textsubscript{3}/$\alpha$-CD\textsubscript{28} or LPS resulted in strong cytokine responses with large interindividual differences, which may explain why we did not find a stimulating effect of glutamine-enriched enteral nutrition on Th1 cytokine responses. This may indicate that immune response patterns are determined by individual (genetic) predisposition and are less affected by exogenous factors.\textsuperscript{146} Another explanation may be that enteral glutamine supplementation does not reach the systemic circulation and mainly affects the intestinal immune system. This hypothesis is supported by the finding that enterally administered glutamine is almost entirely metabolized by the gut and does not reach the systemic circulation.\textsuperscript{46,79} Several experimental studies showed that glutamine reduced the synthesis of pro-inflammatory cytokines in the gut (IL-6 and IL-8)\textsuperscript{38,74,147-150} and stimulated the synthesis of anti-inflammatory cytokines (IL-10).\textsuperscript{149} This in turn may attenuate the gut-mediated systemic inflammatory response and may prevent distant organ injury.\textsuperscript{73,74,151} This hypothesis is supported by the finding of Lacey et al. that parenteral glutamine supplementation was associated with fewer days on the ventilator (infants with BW <800 g).\textsuperscript{39} In addition, in our study, the smaller number of days on ventilator support in the glutamine-supplemented group approached statistical significance (chapter 2). Although glutamine-mediated attenuation of the intestinal inflammatory response reveals an important effect of glutamine on the immune system, it does not explain why glutamine supplementation leads to decreased infectious morbidity. Neu et al. found that decreased infectious morbidity in VLBW infants who received enteral glutamine supplementation was associated with decreased HLA-DR expression on lymphocytes, which may reflect decreased bacterial challenge.\textsuperscript{8} Decreased bacterial challenge in the glutamine-supplemented group may represent improved intestinal integrity and decreased bacterial translocation, although only animal studies have provided direct evidence for this hypothesis.\textsuperscript{70-72} In adult trauma patients, enteral glutamine supplementation was associated with decreased infectious morbidity in combination with increased HLADR expression on monocytes, which may have improved cell-mediated resistance to infections.\textsuperscript{152} Thus, studies into the mechanism of decreased infectious morbidity in patients receiving

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glutamine supplementation show varying results and until now no convincing explanation has been found.

Follow-up

Little is known about the long-term effects of glutamine supplementation in VLBW infants. In a follow-up study in 77 of 90 (86%) surviving infants, who participated in the initial trial (chapter 2), we assessed the incidence of allergic and infectious diseases during the first year of life by means of validated questionnaires (chapter 7). The results of this study indicate that glutamine-enriched enteral nutrition in VLBW infants between days 3 and 30 of life decreased the incidence of atopic dermatitis during the first year of life. The lower incidence of bronchial hyperreactivity in the glutamine-supplemented group approached statistical significance.

We hypothesized that glutamine-enriched enteral nutrition in the neonatal period may enhance the maturation of the immune response and may lead to decreased allergic disease later in life. Th2 cytokine responses dominate the neonatal immune response, as during pregnancy the maternal immune response is skewed towards Th2 immunity. After birth, microbial exposure is the most important stimulus for the Th1 cytokine responses and the deviation of the neonatal immune response towards balanced Th1/Th2 cytokine responses. Delayed transition from fetal Th2-polarized cytokine responses to adult Th1-polarized cytokine responses may lead to long-term dysregulation of Th2 responses and allergic disease. The shift from Th2 towards Th1 cytokine responses can be manipulated in early infancy. Glutamine supplementation increased the Th1 response in septic mice and in adult trauma patients. However, in our study in VLBW infants we did not find an effect of glutamine-enriched enteral nutrition on neonatal cytokine responses (chapter 6). Thus, in our study, decreased allergic diseases during the first year of life cannot be explained by glutamine-mediated enhancement of Th1 cytokine responses in the neonatal period. As part of the follow-up study at the corrected age of one year, we also determined cytokine responses. These data may provide more evidence for our hypothesis, but have not yet been analyzed.

Another explanation for the decreased incidence of allergic diseases in the first year of life may be that glutamine-enriched enteral nutrition leads to altered microbial colonization of the gut. As previously mentioned, microbial colonization of the gut is considered the most important stimulus for the development of Th1 cytokine responses. Sudo et al. showed that germ-free mice had prolonged Th2 cytokine responses, whereas Th1 cytokine responses rapidly developed after introduction of commensal intestinal microflora. In a study of probiotic supplementation (Lactobacillus GG) in term infants at high risk for developing atopy, the frequency of atopic dermatitis in the probiotic group at the age of two years was half that of the
control group. Unfortunately, the authors did not provide data on the composition of the intestinal microflora in these infants. In our study, we did not find increased numbers of lactobacilli or bifidobacteria in the intestinal microflora in the first four weeks of life (chapter 5-II). As part of the follow-up study at the corrected age of one year, we have determined the composition of the intestinal microflora, but these data have not yet been analyzed. These data will provide interesting information about the relation between allergic diseases and intestinal microflora.

Another question with regard to the long-term effect of glutamine-enriched enteral nutrition in VLBW infants was whether the decreased incidence of serious infections in the neonatal period (chapter 2), would have consequences for the long-term resistance against infectious diseases. The results of the follow-up study indicate that glutamine-enriched enteral nutrition in VLBW infants does not affect the incidence of infectious diseases during the first year of life. This is in accordance with the finding that glutamine-enriched enteral nutrition in VLBW infants is not associated with major changes in innate or adaptive immune responses (chapter 6).

The corrected age of one year may be too early to investigate the incidence of allergies and infections, as these diseases can manifest themselves at an older age. Long-term follow-up of this well-defined cohort VLBW infants may reveal further differences between glutamine-supplemented and control group and may contribute to better understanding of the maturation of the immune response and the role of glutamine supplementation in this process.

Methodological considerations

The varying results of single- and multi-center trials of parenteral and enteral glutamine supplementation in VLBW infants, as summarized in the Cochrane review of Tubman et al., have raised several questions regarding the design of these studies. Perhaps the most intriguing question is whether glutamine should be added to enteral or to parenteral nutrition. A meta-analysis of clinical trials of glutamine supplementation in adults showed a trend towards a larger effect when glutamine was supplemented via the parenteral route. In VLBW infants however, it appears that the most important clinical effects were found in trials of enteral glutamine supplementation: Neu et al. and our group (chapter 2) showed decreased infectious morbidity, whereas Vaughn et al. found a decrease in gastro-intestinal dysfunction. Nevertheless, experimental studies using Caco-2 cells as model of human enterocytes do not fully answer the question whether glutamine should be added to enteral or to parenteral nutrition. Panigrahi et al. found that supply of glutamine from the apical side is of critical importance for maintaining intestinal integrity. However, Le Bacquer showed that following luminal fasting, supplementation with free glutamine, regardless of its route of delivery (basolateral or
apical), restores intestinal cell protein synthesis and decreases intestinal permeability.  

In all studies of enteral glutamine supplementation, including our study, glutamine was provided in a dose of approximately 0.3 g/kg/d. A stable-isotope study on protein kinetics in 18 VLBW infants showed that enteral glutamine supplementation in a dose of 0.6 g/kg/d was entirely metabolized by the gut and had no significant effect on whole-body protein and nitrogen kinetics. These data suggest that it is safe to increase the supplementation dose in future studies on enteral glutamine supplementation in VLBW infants.

No data are available with regard to the optimal duration of the supplementation period. Probably, infants on full enteral feeding (breast milk or formula) receive sufficient glutamine via the protein fraction of their feeding. In our study, we chose a supplementation period of 30 days, to guarantee that the majority of the infants reached full enteral feeding during this period. Based on these assumptions, we hypothesize that glutamine supplementation should be started as early as possible and should last until full enteral feeding has been achieved.

Efforts to explain the mechanism of action of glutamine have focused on the metabolic pathways of its breakdown products. First, glutamine is converted into glutamate by the release of the amide nitrogen. Further breakdown of the glutamate molecule yields an amino group that will serve as a substrate for amino acid or glutathione synthesis. The remaining carbon skeleton can be oxidized or reused in gluconeogenesis. For obvious reasons, the specific functions of glutamine have been ascribed to the amide nitrogen that glutamate lacks. The amide nitrogen is used in the synthesis of molecules that are important for maintaining intestinal integrity, such as nucleic acids and amino sugars. Nevertheless, recent studies have found that glutamate was as effective as glutamine in maintaining intestinal integrity. Although few data are available, these findings have challenged the metabolic role of the glutamine amide nitrogen. In addition, recent research has focused on the regulatory role of glutamine in cell metabolism and proliferation and the molecular mechanisms by which glutamine may improve outcome in critical illness.

A general point of discussion in studies on glutamine supplementation, either enteral or parenteral, is the choice of a control supplementation. In our study, we compared the effects of supplementing normal nutrition with either glutamine or isonitrogenous alanine. We recognize that a control group without glutamine or alanine supplementation would have been more representative of daily practice. However, a comparison of glutamine supplementation with no amino acid supplementation limits the possibility of drawing conclusions about the effect of glutamine, because the results may reflect the effect of amino acid supplementation per se. As discussed by Neu, the choice of a control supplementation remains controversial and limits the comparability of the various studies.

Discussion
Besides the general methodological dilemmas inherent to trials of glutamine supplementation in VLBW infants, some specific remarks may be formulated with regard to the design of the studies in this thesis. First, the sample size calculation was based on the primary outcome of the main trial (feeding tolerance). As a consequence, the sample size of the studies into the mechanisms of glutamine action (chapter 4, 5 and 6) and the follow-up study (chapter 7) may have been too small to detect differences between the glutamine-supplemented and control groups. In the first studies (chapter 4, 5 and 6) the differences between the treatment groups were small. We believe that a larger sample size would not have lead to significant differences between the glutamine-supplemented and control groups. However, the conclusions of the follow-up study are susceptible to a type II error (chapter 7). In this study, the difference in incidence of bronchial hyperreactivity did not reach statistical significance, though 14% of the infants in the glutamine-supplemented group had bronchial hyperreactivity in the first year of life, compared to 33% of the infants in the control group. In our opinion, this is a clinically relevant difference. To achieve a statistically significant difference with regard to the incidence of bronchial hyperreactivity approximately 80 infants should have been included in both treatment groups.

Second, the infants in the control group may have received considerable amounts of glutamine through breast milk. In a study on free amino acids in human milk, Agostoni et al. calculated that a healthy breast-fed, one month old, 4-kg infant receiving 600 mL human milk per day, ingested approximately 0.120 g of free glutamine and glutamic acid per day (0.03 g/kg/d). The supplementation dose in our study was 0.3 g/kg/d. Consequently, it is unlikely that the amount of glutamine ingested by the control group may have approximated that of the glutamine-supplemented group.

Third, in the study on clinical outcome (chapter 2), infectious morbidity was defined as ≥1 serious infection during the complete study period. In the studies into intestinal permeability and cytokine responses, measurements were performed at 4 and 3 time points respectively during the first 30 days of life. In the statistical analysis of the latter studies, only serious infections within 48 hours preceding the sampling time were taken into account. In addition, results of a small number of infants with a serious infection at a sampling day, were excluded from analysis because of insufficient urine or blood collection. These factors may provide an explanation for the finding that decreased infectious morbidity in VLBW infants, who received glutamine-enriched enteral nutrition, was not associated with changes in intestinal permeability nor with altered cytokine responses.

Other aspects of our study design support a general application to daily practice. First, the study population was composed of a randomly selected group of VLBW infants that had no severe congenital malformations. Infants with both a high probability of rapid transfer to another hospital and a high probability of short-term death were
included. Second, both breast-fed and formula-fed infants were included. Finally, the analysis on an intention-to-treat basis supports a general application to daily practice. In view of the results of our study, in particular the decreased infectious morbidity after glutamine-enriched enteral nutrition and absence of adverse effects, we suggest adding glutamine supplementation to the enteral nutrition of VLBW infants. We propose to start as soon as possible after birth and to continue supplementation until full enteral feeding has been achieved. Whether supplementation to parental nutrition has an additional effect remains to be determined.

**Conclusions**

Glutamine-enriched enteral nutrition did not improve feeding tolerance or short-term outcome in VLBW infants. However, infectious morbidity was significantly lowered in infants who received glutamine-enriched enteral nutrition. In addition, enteral glutamine supplementation in a dose of 0.3 g/kg/d seemed safe. Decreased infectious morbidity in the glutamine-supplemented group was not associated with changes in intestinal permeability or microflora nor with altered cytokine responses. Although in recent years numerous mechanisms were proposed by which glutamine may improve outcome, the exact mechanism of decreased infectious morbidity in patients receiving glutamine supplementation is not fully understood.

Glutamine-enriched enteral nutrition in VLBW infants decreased the incidence of atopic dermatitis during the first year of life, but had no effect on the incidence of bronchial hyperreactivity and the incidence of infectious diseases during the first year of life. Long-term follow-up of this well-defined cohort VLBW infants may reveal further differences between glutamine-supplemented and control groups and may contribute to better understanding of the maturation of the immune response and the role of glutamine supplementation in this process.

**Implications for future research**

In our study on clinical outcome, we found that glutamine-enriched enteral nutrition in VLBW infants reduced the risk of serious infections. However, in our studies into the mechanisms of glutamine action we found that decreased infectious morbidity was not associated with changes in intestinal permeability and microflora nor with altered cytokine responses. To date, the data on mechanisms of glutamine action in VLBW infants are limited. Besides our studies, only Neu et al. have investigated the effect of enteral glutamine supplementation in VLBW infants on immune cell type distribution. To further elucidate the potential beneficial effect of glutamine supplementation in
VLBW infants, future research should focus on the mechanism by which glutamine might improve intestinal integrity and might modulate the (intestinal) immune response. For this purpose, it will be essential to develop non-invasive methods to assess gut function, suitable for application in large groups of VLBW infants. Furthermore, future studies on glutamine action in VLBW infants should take into account the proposed mechanisms by which glutamine may influence outcome in critically ill adults. Actual research in critically ill adults focuses on the role of glutamine in tissue protection, immune regulation, preservation of tissue metabolic function and anti-oxidant expression.

In future studies of glutamine supplementation in VLBW infants, glutamine may be supplemented in a higher dose. Stable isotope studies on glutamine metabolism have shown that a dosage of 0.6 g/kg/d is safe. These studies should evaluate whether subgroups of VLBW infants may especially profit from glutamine supplementation. These subgroups may include infants that will need a long period to achieve full enteral feeding or infants prone to inflammation-mediated diseases of prematurity, such as infants born after chorioamnionitis.

Little is known about the long-term effects of glutamine supplementation in VLBW infants. In our follow-up study at the corrected age of one year, we found that glutamine-enriched enteral nutrition decreased the incidence of atopic dermatitis, but did not significantly decrease the incidence of bronchial hyperreactivity or infectious diseases during the first year of life. Long-term follow-up will be required to reveal further differences between glutamine-supplemented and control groups. Furthermore, data on cytokine responses and intestinal microflora, determined as part of the follow-up study at the corrected age of one year, will provide information with regard to pathophysiological processes that may underlie the effect of glutamine supplementation early in life on allergic diseases later in life. Neurodevelopmental outcome is another important aspect of long-term follow-up in VLBW infants. Nutrition early in life may have permanent effects on cognitive function. In our study, neurodevelopmental outcome at the corrected age of two years was not different in glutamine-supplemented and control groups. However, assessment of neurodevelopmental outcome at an older age will have more value and will provide important additional information.