Tyrosinemia type 1

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DOI:
10.33612/diss.137426908

CHAPTER 6

General discussion

This thesis comprises studies that highlight several challenges in the treatment of TT1, while focussing on the following subjects: (1) monitoring the risk of HCC, (2) the cognitive-behavioral phenotype of TT1 patients and the associated pathophysiology, and (3) monitoring metabolic control. For each subject, one or two hypotheses have been formulated. These hypotheses will be addressed by discussing the main findings of the different research projects in relation to the existing literature. Possible implications for current treatment recommendations will be highlighted and suggestions and opportunities for future research will be presented.

How to monitor the risk of HCC development?

HCC screening in TT1 patients

TT1 was previously especially characterized by severe liver failure and HCC development[1]. This changed after introduction of NTBC in 1992. However, patients are still considered to have an increased risk for HCC development, especially when NTBC treatment is initiated late due to delayed diagnosis or unavailability of NTBC, when a slow decrease of AFP is seen or when AFP concentrations remain just above the normal range of 0 to 10 μg/L[2-5]. Due to this increased risk for HCC, screening for liver cancer is recommended. Most clinical centers adopted their own approach, but the common advice is to screen all TT1 patients at regular intervals using AFP and imaging such as ultrasound and/or magnetic resonance imaging (MRI)[6,7]. However, based on available literature, it may be assumed that patients can be divided into different groups based on their risk to develop HCC and that the screening approach should be adjusted accordingly. We therefore hypothesized that in low risk patients, routine liver ultrasound and MRI are no longer necessary and screening can be done merely using regular AFP measurements.

In chorionic villus material of TT1 affected foetuses, lower FAH activity and increased SA concentrations can already be found in the first trimester of gestation[8-10]. Next to this, AFP concentrations are already increased at birth in TT1 affected neonates[11]. The combination of both, increased SA concentrations in utero and increased AFP concentrations at birth, make a prenatal start of liver disease likely. Despite this, after almost 30 years of NTBC treatment, no

liver cancer has been reported in patients who are pre-clinically diagnosed in the first week of life and treated with NTBC immediately afterwards[5]. This is in contrast to other TT1 patients who started with NTBC later in life and patients whose AFP concentrations failed to normalize within 1-2 years. These patients, including the patient we presented in **Chapter 2**, have reportedly shown to be susceptible for HCC[2-5]. Next to this, patients are thought to have an increased risk for HCC when hepatic nodules are already present at diagnosis, or when new hepatic lesions develop during NTBC treatment, which is highly suspicious for HCC. It is thought that these hepatic lesions are more likely to develop when NTBC treatment is started late, or when adherence to NTBC treatment is non-optimal.

Based on the information above, a distinction can be made between TT1 patients who have a "low" risk and patients who have a "higher" risk for HCC development. Low risk patients could be defined as patients who are diagnosed and treated with NTBC pre-clinically in the first month of life, have AFP concentrations within the normal range (within 2 years after treatment), have no hepatic nodules on the first ultrasound and have a good adherence to NTBC treatment. In these patients, our hypothesis is that AFP measurements would be sufficient for HCC screening during follow-up.

Despite a low sensitivity for detecting HCC in other diseases, such as hepatitis B and C, AFP is always considered to be sensitive marker for HCC in TT1. In TT1 patients, AFP concentrations are already increased at birth[11], can be used to identify patients at risk for HCC[3,4] and an increase in AFP is considered to be pathognomonic for HCC[4]. So far, literature provides only one case report of a TT1 patient with HCC without a substantial rise in AFP (**Chapter 2**). However, considering the pathological classification of early-stage HCC in this patient, we do not know whether AFP concentrations would eventually have started to rise. Furthermore, this patient was diagnosed and treated late, the AFP concentrations never fully normalised and nodules were found at liver imaging. All these factors are indicative for the very high risk of HCC development in this patient as explained above.

Considering all information mentioned above, including that no HCC has been reported in pre-clinically diagnosed patients since introduction of

NTBC, routine liver imaging never showed any abnormalities in these patients and AFP has been considered a sensitive marker in TT1, we believe that the identified low risk patients could be screened for HCC using regular AFP measurements only. In these patients, ultrasound, CT and/or MRI of the liver would only be indicated when risk factors become present, for example a non-optimal adherence to NTBC treatment or AFP concentrations that fail to normalize or even increase.

Prevention of HCC

As explained above, apart from HCC screening, treatment monitoring is essential, since sub-optimal NTBC treatment is considered to be one of the main risk factors for HCC development. After NTBC treatment is initiated, the main treatment goal is too maximally suppress FAA formation[12]. We hypothesized that treatment monitoring can therefore be done by exclusively measuring blood SA concentrations, as an indirect marker of FAA.

The current general recommendations in Europe, the US and Canada are to treat patients with a daily dose of 1mg/kg bodyweight[6,7], taken as a single dose due to the long half time of NTBC[13,14]. However, chronic treatment in TT1 patients is sometimes carried out with much lower dosages around 0.4-0.6 mg/kg bodyweight/day[15,16]. Most laboratories, evaluate the efficacy of this treatment by measuring both blood NTBC and SA concentrations. However, NTBC measurements could be difficult to interpret as target NTBC concentrations are not clearly established. Furthermore, recommendations for target NTBC concentrations are hampered by large inter- and intra-individual variability and lack of standardization of NTBC assays. As a consequence, target NTBC concentrations vary between 50-150 umol/L[12,17]. Despite the inter- and intra-variability of blood NTBC concentrations, we have shown that blood spot NTBC and blood spot SA concentrations are clearly correlated (**Chapter 2** and **Chapter 5**). This led to our conclusion in **Chapter 2** that NTBC concentrations measured in blood spot should be above 45 µmol/L to fully suppress SA formation.

SA concentrations can be measured in blood and urine. As the majority of SA is bound to albumin, the half-time of blood SA is very long[18], which makes

blood SA measurements suitable for treatment monitoring. However, due to sensitivity problems, urinary SA measurements have long been considered the best method. Urinary SA is now being replaced by SA measurements in dried blood spots (DBS) as new techniques for blood SA measurement have led to a clear improvement in sensitivity[19]. Even during the research presented in this thesis, a continuous improvement in sensitivity of the method was seen, which is also reflected by the different limits of quantification in the studies presented in this thesis (**Chapter 2** versus **Chapter 5**). The improvement in sensitivity allowed some laboratories to detect SA in healthy individuals as well. Since then, it has been claimed that SA concentrations should be targeted to the reference range[12], although the reference range still needs to be established.

With the current treatment regimen, increased SA concentrations (i.e. > 0.1 μmol/L) are frequently found, which is also highlighted in **Chapter 2** and **Chapter 5**. As increased SA concentrations are an indirect marker for increased concentrations of hepatotoxic FAA, increased SA concentrations should, independent of NTBC concentrations, lead to adjustment of therapy. Adjustment of therapy can be done by increasing the NTBC dose or alternatively change to a twice daily dosing regimen as was shown in **Chapter 2**.

To return to our hypothesis, we reason that monitoring can be done by exclusively measuring SA concentrations, if the laboratory can measure SA concentrations sensitively enough. This hypothesis is based on the reasoning that the main treatment goal is to maximally supress FAA concentrations, which is reflected by SA concentrations within the normal range. However, due to the correlation between NTBC and SA, blood NTBC concentrations can still be helpful when the laboratory is not able to measure SA sensitively enough or when questions arise about adherence to NTBC treatment.

Cognitive – behavioral phenotype of TT1 patients

The neuropsychological and behavioral outcome was never much of a concern in TT1 patients. However, this gradually changed after Masurel-Paulet et al. retrospectively showed that 35% of French TT1 patients had experienced school problems[20]. Subsequent research mainly focused on IQ, and showed

a lower-than-average IQ[21-24] and even regression of IQ over time[25,26]. Next to these neurocognitive problems, structural changes in the brain were found in some TT1 patients. MRI of two TT1 patients showed white matter problems and myelination deficits[27,28]. Based on these findings, our hypothesis was that cognitive-behavioral phenotype is a true concern in TT1 patients and that the impaired neuropsychological outcome is not only restricted to IQ.

Chapter 3 showed the results of an international study in which the cognitive-behavioral outcome of a maximum of 42 TT1 patients was investigated. In the absolute sense, this is a small sample size, but considering the prevalence of TT1 this study could be considered as relatively large. However, it should be acknowledged that the patient group was very heterogeneous, for example regarding age at diagnosis, clinical presentation, and treatment. Observed differences in the cognitive-behavioral outcome may thus be partly explained (or hidden) by the variability within the patient population.

In this study, different neuropsychological instruments have been used, including computerized tests, paper and pencil tasks, and questionnaires. In this way, various cognitive and behavioral domains have been assessed, including executive functioning and social cognition, internalizing and externalizing behaviour, and quality of life. As noted in **Chapter 3**, executive functions are a set of inter-related high level skills across several cognitive domains[29,30]. Inhibition, working memory and cognitive flexibility are usually considered the "core" executive functions, which were all measured using the Amsterdam Neuropsychological Tasks (ANT)[31,32]. Social cognition mostly refers to the mental operations underlying social interactions such as the perception, interpretation and generation of responses to the intentions, dispositions and behaviors of others[33].

The results in **Chapter 3** showed that the neuropsychological dysfunction of TT1 patients is not restricted to a lower-than-average IQ, but TT1 patients exhibit problems in a broad range of cognitive and behavioral domains. Executive functioning has been one of the main focus points during these studies and was shown to be clearly impaired, in both computerized tests and questionnaires.

Interestingly, effect sizes were larger when day-to-day executive functioning

was analysed with the Behavior Rating Inventory of Executive Functioning (BRIEF), compared to effect sizes when specific "core" executive functions were measured with the ANT. These type of differences between measurements have been shown before[34,35]. They have been attributed to the fact that most operations in day-to-day life require multiple executive functions simultaneously and/or the incorporation of executive functioning with other cognitive abilities, which is especially reflected in the BRIEF[36]. In agreement with this concept, it is interesting to notice that, while using the ANT, the biggest differences between TT1 patients and healthy controls were found in tasks that require a dual action of both executive functioning and social cognition as well.

Regarding behavior, both internalizing and externalizing behavior problems have been observed, such as thought/mood and social problems and problems related to aggressive behavior. However, most striking are the attention problems that have been found frequently, with about half of the TT1 patients scoring in the clinical range. This seems to be in line with other studies that showed attention problems as well[37-39]. Interestingly, in our study, children seemed to be more severely affected than adults, although these findings might be biased due to the small sample size of adults with TT1. So far, no specific group of TT1 patients at risk for neurocognitive deficiencies could be identified, as non-optimal neurocognitive outcomes have been observed in pre-clinically diagnosed, clinically diagnosed (**Chapter 3**) and transplanted patients[38].

The cognitive-behavioral phenotype of TT1 patients seems to resemble the cognitive-behavioral phenotype of patients with phenylketonuria (PKU), which is an associated disorder of amino acid metabolism. PKU is characterized by a deficiency of the enzyme phenylalanine hydroxylase that normally converts phenylalanine into tyrosine. This enzymatic deficiency consequently leads to high phenylalanine and low-normal tyrosine concentrations (the opposite from what has been observed in TT1). If left untreated, PKU is characterized by severe mental retardation and seizures. Dietary restriction of phenylalanine can lower (but not normalise) phenylalanine concentrations and subsequently improve the clinical outcome. However, even in early treated PKU patients, deficiencies in several cognitive domains have been reported, with executive

function deficits being the most consistent[40]. Compared to TT1 patients, much more research into the cognitive-behavioral phenotype of PKU patients has been carried out. Based on the neurocognitive and behavioral problems that were found, standard neurocognitive follow-up of PKU patients has been recommended[41-43]. However, when comparing both patient groups in **Chapter 3**, TT1 patients show more problems than early treated PKU patients, with regard to executive functioning and mental health.

Before this study started, not all clinicians agreed that the cognitive, behavioral and social functioning of TT1 patients were a true concern. However, we think that the results presented in this thesis (**Chapter 3**) support the idea that more attention should be paid to neuropsychological and psychosocial functioning of TT1 patients. Additionally, research should focus on the pathophysiology underlying the cognitive, behavioral and social problems observed in TT1-patients. This in turn will lead to new clinical challenges such as how to monitor the neurocognitive-behavioral outcome adequately efficiently.

Pathophysiological mechanisms underlying neuropsychological dysfunction

Several hypotheses have been proposed to explain the cognitive and behavioral issues in TT1 patients. Firstly, despite the fact that cognitive and social problems were not reported extensively before introduction of NTBC, the disease itself, with (hepato) toxic metabolites and associated liver failure might be related to the problems. Early liver disease of any kind has been associated with cognitive deficits, learning disabilities and behavioral problems[44]. In addition to this, research in TT1 mice also showed behavioral problems that were possibly associated to the (hepato)toxic metabolites and disease itself[45,46]. However, pre-clinically treated patients, who have not been suffering from severe liver failure, also show clear deficits in neurocognitive outcome (**Chapter 3**).

Secondly, it can be hypothesized that NTBC, which was considered to be a herbicide with clear potential[47], may have a direct negative effect on the cognitive-behavioral outcome. In the early days of treatment with NTBC, the physiological distribution of NTBC was analysed in rats. It was indicated

that NTBC accumulated in the liver and eye, but that a small fraction of it was found in the brain as well[47]. Despite these findings, evidence for a direct negative effect of NTBC on the brain is limited. In mice, no structural negative effect of NTBC on the brain has been found and behavioral abnormalities were not associated with NTBC[45,46]. Although a regression of IQ has been found in TT1 patients[25,26] and the duration of NTBC treatment has been associated with lower IQ scores in our study, no other associations between NTBC and neurocognitive outcome were found (**Chapter 3a**).

Thirdly, it is thought that NTBC (and dietary) treatment are only indirectly associated with the neuropsychological and behavioral outcome by inducing alterations in blood amino acid concentrations. By creating a new block upstream from the primary enzymatic defect, NTBC causes tyrosine concentrations to rise. As increased tyrosine concentrations are associated with eye problems in both TT1 and tyrosinemia type 2, and possible neurocognitive problems in tyrosinemia type [2][18,48,49], NTBC treatment has always been combined with a tyrosine and (precursor) phenylalanine restricted diet. However, even with dietary treatment, blood tyrosine concentrations are still clearly increased. At the same time it turns out to be difficult to prevent low phenylalanine concentrations[50,51]. Both, high tyrosine and low phenylalanine concentrations have been associated with a non-optimal neurocognitive outcome and developmental delay in infancy[52]. It is thought that changes in blood phenylalanine and tyrosine concentrations do not only affect brain concentrations of these amino acids, but affect brain concentrations of other amino acids as well. All large neutral amino acids (LNAA) including phenylalanine and tyrosine are primarily transported across the blood brain barrier (BBB) by the so called L-type amino acid transporter 1 (LAT-1)[53]. Under normal physiological circumstances, the LAT-1 transporter is saturated for more than 95%[54]. Therefore, high plasma concentrations of a particular LNAA would not only increase the brain influx of that specific LNAA, but also outcompete the uptake of other LNAA. This mechanism has already been shown in other disorders of LNAA metabolism such as PKU and maple syrup urine disease[55,56]. When taking PKU as an example again, high plasma phenylalanine concentrations have been shown to be associated with: (1) high brain phenylalanine concentrations that are

considered to be neurotoxic, (2) low brain concentrations of other LNAA that might be related to an impaired cerebral protein synthesis and consequently (3) changes in brain neurotransmitter synthesis, as the LNAA tyrosine and tryptophan are the precursors for dopamine and serotonin respectively[56-59]. Due to the alterations in blood phenylalanine and tyrosine concentrations, all these pathophysiological mechanisms can play a role in TT1 as well. Therefore, considering all information mentioned above, we hypothesized that NTBC and dietary treatment are only indirectly associated with the neuropsychological and behavioral outcome, by inducing changes in blood amino acid concentrations.

To investigate these different mechanisms, brain LNAA concentrations have been estimated using a theoretical model and measured in TT1 mice in **Chapter 4**. In general, the theoretical calculations and research in TT1 mice showed clear similaritties in brain biochemistry. The most important findings were:(1) increased brain tyrosine influx and subsequent brain tyrosine concentrations, even when treated with a diet, (2) decreased brain phenylalanine influx and subsequent brain phenylalanine concentrations, whereas other brain LNAA concentrations were normal or only slightly lower than normal, and (3) unaffected brain catacholaminergic neurotransmitter concentrations, whereas brain serotonin concentrations tended to be lower in TT1 mice.

Out of all non-tyrosine brain LNAA, the results of **Chapter 4** showed that brain phenylalanine concentrations tend to be most severely affected. This could be explained by two different mechanisms. Firstly, plasma phenylalanine concentrations are often low in both TT1 patients and TT1 mice. Secondly, phenylalanine has a strong affinity to the LAT-1 transporter. Therefore, small changes in blood phenylalanine concentrations can lead to large changes in brain phenylalanine influx and consequent brain concentrations. This mechanism also explains that in PKU mice, which show very high plasma phenylalanine concentrations, all non-phenylalanine LNAA are outcompeted at the BBB and brain LNAA concentrations are therefore more affected[60].

With regard to neurotransmitter concentrations, despite increased brain tyrosine concentrations, brain dopamine and noradrenalin concentrations

are usually normal or even slightly below normal in TT1 mice. This is most likely caused by highly regulated substrate and product inhibition on tyrosine hydroxylase. This is the rate limiting step in dopamine synthesis[39,61,62], which aims to keep dopamine concentrations within range. On the other hand, in our study, very high brain tyrosine concentrations were associated with lower brain serotonin concentrations. This is in agreement with some preliminary data on cerebral spinal fluid (CSF) concentrations in TT1 patients, that showed increased CSF tyrosine concentrations and low CSF 5-HIAA (a degradation metabolite of serotonin) concentrations[63]. In the discussion of **Chapter 4b**, we hypothesized that low brain serotonin concentrations could be caused by an inhibitory effect of brain tyrosine on tryptophan hydroxylase. This hypothesis has recently been confirmed in another study[39].

In contrast to our expectations, none of these above mentioned biochemical changes were associated with behavioral changes in the mice from our study. In fact, all TT1 mice showed normal learning behaviour and did not show major behavioral problems. Although not part of this thesis, these normal behavioral outcomes in TT1 mice have been confirmed in a follow-up study that has been performed with TT1 mice with the same mutation and background in Portland (OR, USA). These TT1 mice also showed normal memory retention, a normal learning curve and normal hippocampus-dependent and –independent fear learning (unpublished data).

In other studies, high brain tyrosine concentrations have been thought to be neurotoxic to rat brains mainly by creating oxidative stress[64,65]. In tyrosinemia type 2, which is characterized by tyrosine concentrations which are even higher than in TT1, high tyrosine concentrations are associated with a variable degree of developmental delay and cognitive dysfunction in patients[49]. In agreement with these findings, our study on the neuropsychological outcome (**Chapter 3**) shows some correlations between recent high tyrosine concentrations and behavior and quality of life as well, possibly indicating a toxic effect of tyrosine. This has recently been shown again in a smaller sample of TT1 patients as well[39]. Alternatively to the high tyrosine concentrations, low plasma and consequent brain phenylalanine concentrations might be associated with impaired brain protein synthesis and thus be related to the observed cognitive-behavioral phenotype. The clinical relevance of low phenylalanine concentrations is

shown in a case report of an infant in which low phenylalanine concentrations were among others associated with growth problems and developmental delay[52]. Our study on the cognitive-behavioral phenotype of TT1 patients also showed that low phenylalanine concentrations during infancy were associated with more behavioral problems and lower quality of life later in life (**Chapter 3**).

To conclude, we cannot confidently confirm our hypothesis that changes in plasma amino acid concentrations lead to cognitive and behavioral dysfunction. However, based on our findings, it seems that low blood phenylalanine concentrations might be harmful during infancy (the period in which the need for phenylalanine is high to promote growth), whereas sustained high blood tyrosine concentrations are harmful later in life. Reliable monitoring of dietary treatment and resulting phenylalanine and tyrosine concentrations is therefore clearly indicated, and rapid adjustment needs to be possible. However, finding a balance in natural protein restriction that leads to tyrosine concentrations within target range, without resulting in low phenylalanine concentrations, may be a challenging objective[50,51,66].

Metabolic control: amino acid concentrations in TT1

How and when to measure amino acid concentrations?

Considering that both high and low tyrosine and phenylalanine concentrations might be associated with long-term cognitive and behavioral dysfunction in PKU and TT1, adequate monitoring of amino acid concentrations is necessary. The effect of dietary treatment and possible need for adjustment was always analysed by measuring blood amino acid concentrations at the outpatient clinic in the hospital. After the introduction of DBS analyses for phenylalanine and tyrosine, home monitoring became possible. DBS have the advantage that monitoring can be done in a less invasive way (finger prick instead of venous blood sampling) and more frequently. Although amino acid analysis with plasma obtained at the outpatient clinic is still the golden standard, DBS are used more and more frequently. However, the validity of DBS is of ongoing debate. Several studies showed a large variation in

DBS amino acid concentrations depending on the analytical methods used. Besides, differences between laboratories and differences between plasma and DBS amino acid concentrations are found as well[67-70] (and Coene et al. personal communication). However, we hypothesized that DBS are adequate for assessing metabolic control. We consider the time of blood sampling to be more important, especially when low phenylalanine concentrations are the main focus.

To investigate whether DBS are an adequate way for asssessing metabolic control, the agreement between phenylalanine and tyrosine concentrations measured by different blood sampling methods in both PKU and TT1 patients is investigated in **Chapter 5**. The results showed that when a lab specific correction factor is taken into account, DBS and plasma amino acid concentrations can be made comparable. However, the study was done in a standardized way to prevent pre-analytical factors from influencing the results. At home, most of these pre-analytical factors, such as the applied blood volume on the DBS card, haematocrit and differences in transport to the hospital, are difficult to control for. Another disadvantage of DBS home monitoring is the relatively long time period from blood sampling to results.

The second part of the hypothesis concerns the timing of blood sampling. Most blood samples taken for treatment monitoring are routinely taken after overnight fasting, before breakfast. In the past, pre-breakfast samples were mainly taken for diagnostic purposes. Nowadays, during routine follow-up, pre-breakfast samples are mainly taken for reasons of convenience and because previous research (again mainly in PKU) showed that phenylalanine concentrations remained stable during the day and timing of blood sampling was therefore not really important[71]. Regarding TT1, similar results have been found. Tyrosine concentrations do not show a clear diurnal variation51 and even when phenylalanine supplementation is prescribed, tyrosine concentrations tend to increase only slightly during the course of the day (**Chapter 4**). In both diseases, the timing of blood sampling is not so relevant if you are only interested in concentrations of the particular toxic amino acid. However, to study a possible deficiency of an (associated) essential amino acid, a different approach probably needs to be used. Previous research showed that a deficiency of an essential amino acid is characterized by blood

concentrations that decrease during the course of the day, instead of the slight increase which is normally seen[72-74]. In dietary treated TT1 patients, blood phenylalanine concentrations show such a "deficiency" pattern. Blood phenylalanine concentrations are within normal range before breakfast and clearly decrease during the day with the lowest concentrations before lunch[51]. Based on this, van Dam et al. advised to measure phenylalanine concentrations in the morning and take a decrease in phenylalanine concentrations into account[51]. However, in **Chapter 5** of this thesis, it is shown that pre-breakfast phenylalanine concentrations did not reflect phenylalanine concentrations before lunch, as phenylalanine supplementation increases phenylalanine concentrations during the day, without changing pre-breakfast phenylalanine concentrations. Therefore, we hypothesize that normal pre-breakfast phenylalanine concentrations are most likely caused by net protein catabolism during the night. After breakfast, patients enter an anabolic status in which the available phenylalanine is needed for protein synthesis. A deficiency will only come to light at this moment. Consequently, in C**hapter 5** we concluded that blood samples should be taken before lunch, in order to truly study phenylalanine status in TT1, which could be combined with an overnight fasted bloodspot when in doubt.

How to improve metabolic control?

Although not part of our hypothesis, **Chapter 5** gives insight in how to prevent low phenylalanine concentrations in TT1 patients as well. In the past, amino acid monitoring mainly focused on high tyrosine concentrations, as they were associated with eye problems[2,18] and neurocognitive problems[48]. Low phenylalanine concentrations have been present since the start of NTBC treatment, but these low concentrations were not considered to be significant[75]. However, more recently, low phenylalanine concentrations have been associated with growth problems, skin problems and developmental delay in infancy[52] and maybe also to neuropsychological issues later in life (**Chapter 3**). To prevent these low phenylalanine concentrations, different approaches exist. The natural protein restriction can be made less strict, although this would increase the tyrosine intake as well. Therefore, some clinicians chose to prescribe phenylalanine supplementation instead[52,66],

without comprehensive consideration about how to dose this phenylalanine supplementation. Our research in **Chapter 5** investigated the effect of different amounts of phenylalanine supplementation and showed that it remains a challenge to find a balanced amount of phenylalanine that prevents low phenylalanine concentrations during the day without leading to a large increase in tyrosine concentrations. Generally speaking, 20mg/kg/day phenylalanine supplementation could be a good starting point. However, clear individual (and maybe also age related) differences exist and therefore an individually tailored approach is probably necessary. These individual differences complicate the question whether phenylalanine should be added to the amino acid mixtures and if so, how much phenylalanine should be added. The preferred treatment strategy is mainly dependent on which of the two is most detrimental for long term growth and (cognitive) development, high tyrosine or low phenylalanine concentrations, which clearly requires further investigation.

Implications, future considerations and overall conclusion

The research presented in this thesis provides insight into various challenges TT1 patients and their clinicians nowadays face. It focused on the remaining risk for HCC development, the cognitive-behavioural phenotype of TT1 patients and the pathophysiological mechanisms that might be involved, and adequate ways to assess metabolic control.

The remaining risk for HCC

Chapter 2 showed that the "old" challenges such as HCC development shouldn't be forgotten and can still occur. However, the risk for HCC development clearly decreased after introduction of NTBC, especially in the before mentioned "low risk" TT1 patients. In these patients, HCC has not been diagnosed in almost 30 years of treatment, despite the extensive screening that has been performed. Therefore, although clear attention should be paid to the screening for HCC, the screening protocol can be adjusted, which makes follow-up easier and more convenient.

In order to change the screening protocol, treatment with NTBC should be

optimized as it is considered to be one of the most important risk factors. Although blood SA is only an indirect marker of the hepatotoxic metabolite FAA, its close association with maleylacetoacetate and FAA and the long half-time of make it an adequate marker for sub-optimal NTBC treatment, provided that laboratories can measure SA sensitively enough. In that case, it is not necessary to analyse other indirect markers such as blood NTBC concentrations anymore and the NTBC dose can be titrated down to the lowest necessary dose. However, so far, only few laboratories can detect SA sensitive enough to detect it in healthy participants. Until then, undetectable SA does not necessarily reflect optimal treatment. Therefore, future research should focus on ways to further improve the sensitivity of SA determination.

Cognitive – behavioural profile and associated patho-physiological mechanisms

In **Chapter 3**, it has been shown that various domains of cognitive function and behaviour do not evelop in an optimal way. After comparing TT1 patients to individually matched healthy volunteers, population based norm scores and PKU patients, we concluded that the neuropsychological and behavioural outcome is a true concern in TT1 patients that needs attention during follow-up and intervention when necessary. Unfortunately, we were not able to identify specific patients at risk for these cognitive-behavioural impairments. The sample size was often too small to perform reliable comparisons of different groups of TT1 patients. Consequently, in our study, paradoxical results were found with children being more severely affected than adults, whereas literature describes a decline in IQ over time[25,26]. Future studies therefore require a larger sample size not only to study specific groups of patients at risk for a non-optimal outcome, but also to study the association between the neuropsychological and behavioural outcome measures and metabolic control both of phenylalanine and tyrosine from infancy onwards.

In this thesis, pathophysiological mechanisms of non-optimal brain development have been analysed in several ways. Firstly, correlational analyses between cognitive and behavioral outcome measures and blood phenylalanine and tyrosine concentrations have been performed. Secondly, a theoretical model has been used to calculate brain LNAA concentrations

based on plasma LNAA concentrations. Thirdly, blood and brain biochemistry were studied in TT1 mice. These different analyses showed that the current treatment could result in: (1) high brain tyrosine concentrations despite dietary treatment, (2) low-to-normal brain concentrations of other LNAA, with brain phenylalanine being the most evident example and (3) low brain serotonin concentrations. In TT1 patients, there were some signs that both low phenylalanine concentrations in early childhood and high tyrosine concentrations throughout life were associated with a non-optimal cognitive-behavioral outcome. However, despite these biochemical changes, a specific pathophysiological mechanism could not be identified in TT1 mice yet, since their memory and learning behaviour was not affected. Consequently, it could be argued that TT1 mice do not resemble TT1 patients adequately and are therefore not a suitable model for studying behaviour. Alternatively, it might be the case that a specific condition that causes the neuropsychological impairments have not been identified yet. For example, plasma phenylalanine (and corresponding brain phenylalanine) concentrations were lower than normal in dietary treated TT1 mice, but not as low as concentrations seen in TT1 patients. It might be possible that lowering plasma phenylalanine concentrations even further, would reveal behavioural problems in these mice. This is one of the options that can be analysed further in future studies.

Monitoring metabolic control

Due to the eye problems associated with high tyrosine concentrations and possible associations of the cognitive-behavioral outcome and high tyrosine and/or low phenylalanine concentrations, reliable monitoring of metabolic control and possibility for adjustment of therapy is required. The use of DBS is a great advantage for both patients and clinicians, but this thesis showed that persistent attention should be paid to the reliability of the DBS. Patient education is necessary to prevent pre-analytical factors to influence the results. In addition, laboratories should calculate a proper correction factor to make DBS truly comparable to plasma amino acid concentrations. On the other hand, this thesis showed that clinicians should be aware that an overnight fasted bloodspot alone is not sufficient to study phenylalanine concentrations in TT1 patients, whereas a pre-lunch bloodspot can both highlight a possible

phenylalanine deficiency and study tyrosine concentrations adequately. The timing of blood sampling is therefore truly important, and should be given proper attention.

Although metabolic control can be monitored more frequently with DBS, the timeframe between blood sampling and obtaining results is still a couple of days. This is mainly caused by the time DBS need to dry, transportation to the hospital and time needed for analyses. The time lag between sampling and result, complicates feedback on treatment strategy. Ideally, blood amino acid concentrations can be measured instantly at home in a similar way as diabetes patients measure their glucose concentrations with point-of-care-testing. Thus, both, pre-analytical factors can be prevented and results are immediately available. This is not only convenient, but could even cause an increase in compliance as has been suggested in diabetes[76]. This would be of great value, not only for TT1 patients, but for other patients with amino acid disorders treated with a diet as well, such as PKU patients.

Future perspectives

Since the first description of TT1 around 1956[77,78], treatment has considerably changed over

the years. A phenylalanine and tyrosine restricted diet was introduced as the first available treatment in 1964[79]. However, as dietary treatment could not prevent the occurrence of liver problems, OLT long seemed to be the only option to prevent the metabolic and oncological problems associated with TT1[80]. Introduction of NTBC, about 30 years ago, revolutionised the treatment and outcome, especially when combined with neonatal screening[5]. With recent medical innovations and new treatment techniques, I expect that the treatment of TT1 will further change in the future.

Already some years ago, hepatocyte transplantation showed promising results in TT1 mice[81]. Alternatively, enzyme replacement therapy has shown to be an encouraging treatment strategy in a number of lysosomal storage disorders[82], and recently in PKU as well[83,84]. With the identification of tyrosine ammonia lyase, a similar approach with enzyme substitution could - at least theoretically - be applied in TT1 as well[85]. However, the non-mammalian nature of these

enzymes may cause detrimental immune responses. Instead of enzyme substitution, mRNA or gene therapy might be the solution to TT1. These methods have shown promising results in other metabolic disorders as well. However, as TT1 mice still develop HCC even if 90-95% of the liver cells are corrected, long-term efficacy of cell and gene therapy was thought to require all cells to be targeted to prevent HCC formation in original FAH deficient cells[86,87]. Nonetheless, liver-directed gene therapy in a TT1 pig has been shown to be effective without signs of liver fibrosis or HCC development[88].

Most of the above mentioned treatment options still require extensive investigation and will not be available in the near future. The aim of this thesis was not to focus on possible new treatment options, but rather on several challenges in the current treatment for TT1. It included a broad range of studies that analysed different outcome measures, and should therefore be considered as a starting point for more (in-depth) research. Most likely, this requires extensive international collaboration due to the rarity of the disease. Only in this way it is possible to increase the sample size to truly study specific patient groups or target concentrations of various metabolites such as tyrosine, phenylalanine, NTBC and SA. This knowledge is definitely required in order to make clear guidelines for the treatment of TT1 patients, which should be a focus point in the near future.

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