

Summary and Discussion

Since the first discovery of SLC6A8 deficiency in 2001¹, the knowledge of this disorder has rapidly increased. More than 150 patients harbouring over 80 pathogenic mutations (Figure 1) have been diagnosed up to now. In this thesis, the development of our knowledge base focussing on the DNA analysis of the *SLC6A8* gene in diagnostics and its clinical implications has been outlined. In this final chapter, these progressions are discussed in more depth.

Introduction of amino acid replacements in proteins is a tool to study structure function of proteins. In this study the effect of mutations introduced by nature were studied in our diagnostic model by reintroducing these alleles in primary SLC6A8 deficient fibroblasts. Using these studies, some variants appeared to have residual activity. These data, together with the tertiary structure of the LeuT transporter as well as the alignment within the SLC6 family were used in our studies. For instance, the c.1271G>A; p.(Gly424Asp) variant resides in transmembrane domain 8 (TM). Although this TM is considered to play an important role in the binding of Na⁺ and creatine², the glycine at this position is not conserved at all throughout different species, suggesting no absolute necessity for glycine on this position for correct functioning of SLC6A8. Also, so far no pathogenic missense variants downstream of TM11 and TM12 were found. In addition, compared to SLC6A8 proteins of different species and the superfamily of neurotransmitter transporters, these regions seem to be non-conserved. So it appears that these parts are not crucial for creatine uptake. This hypothesis is strengthened by the residual activity of variants in these regions, mentioned in chapter 4. However, the actual presence of these regions does seem to be necessary, since truncating mutations that predict proteins that lack only the C-terminus end (including aa 424) have been found to diminish creatine transport by SLC6A8. On the other hand we have not studied the presence of these truncated proteins, and thus it also could be that these truncated proteins are not stable. Since the majority of variants resulted in the absence of residual activity it is concluded that all investigated residues are essential for proper protein folding and or function.

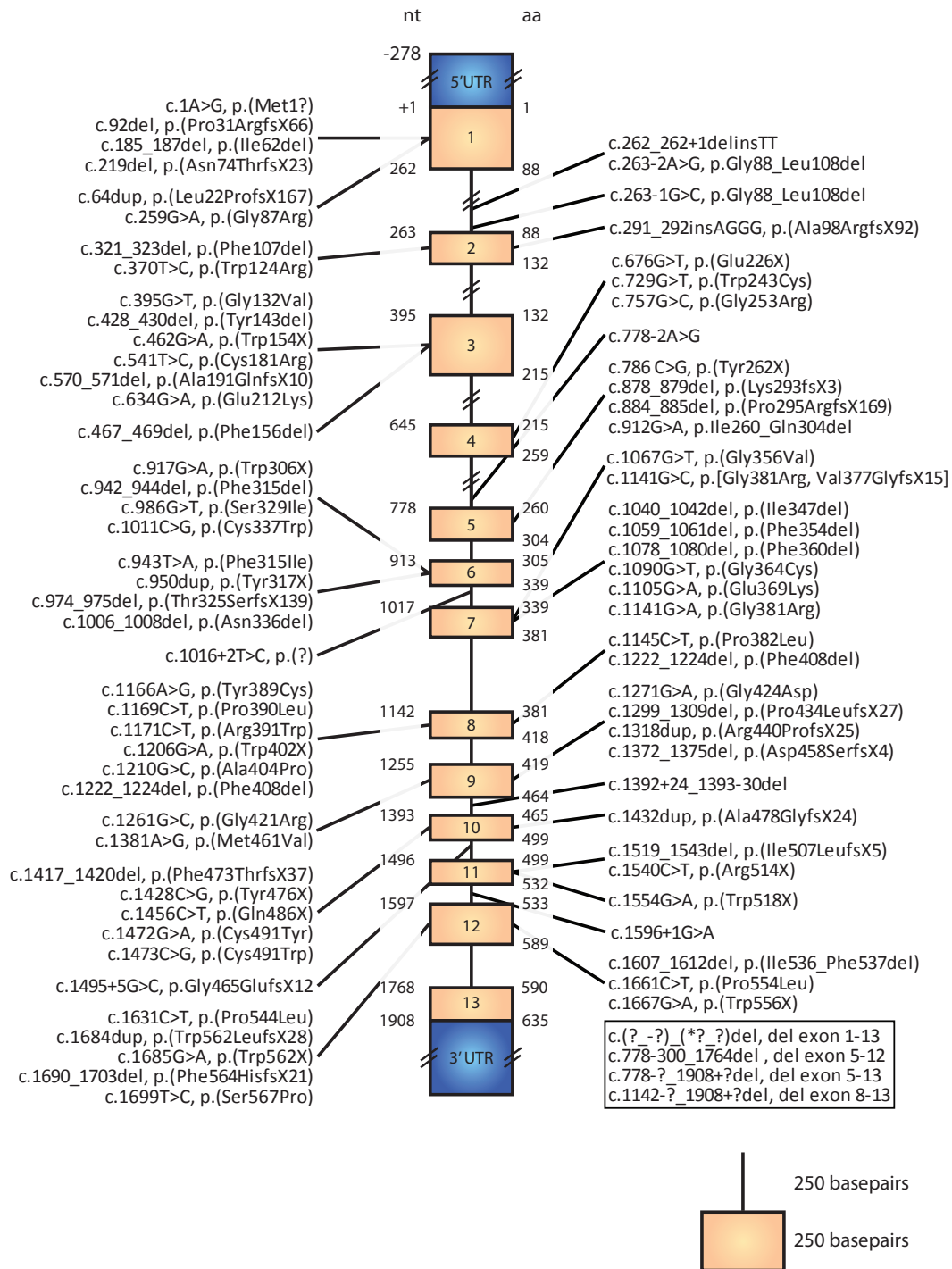


Figure 1. An overview of the pathogenic mutations detected in patients with SLC6A8 deficiency. The location of the mutations is indicated by each line. The bottom right box contains mutations spanning several exons or complete loss of the SLC6A8 gene. nt= nucleotide, aa= amino acid, UTR= Untranslated region

MOLECULAR DIAGNOSIS

Currently, the methods of definitive diagnosis for CCDS include a wide range of biochemical and molecular analyses. Previously, we set up guidelines for the diagnosis of CCDS in males, starting from patients suffering from ID through to the definitive molecular analysis³. As methods evolve, so do the guidelines. In chapter 4, a flowchart is presented to guide physicians towards the correct diagnosis in the case of suspected SLC6A8 deficiency. In this flowchart the molecular characterization of variants detected in DNA of patients with suspected SLC6A8 deficiency is included. Here the nature of the variant should be determined, which can offer quite the challenge (This thesis). If the variant is known, its effect can be confirmed using the LOVD database (www.lovd.nl/slc6a8). This database, which was developed by the Free University Medical Center in cooperation with the Leiden University Medical Center, lists all published variants and pathogenic mutations of SLC6A8. These data are freely accessible and are maintained and updated by the whole community working on SLC6A8. Moreover, this database also offers information that is usually inaccessible (e.g. the molecular proof of pathogenicity).

In the case of a novel unclassified variant, a few elements should be considered. If the variant has an apparent pathogenic effect, (i.e. nonsense, large deletions, frameshift, splice error causing variants), further investigation is usually not essential. Missense, 1-2 amino acid deletions, intronic or neutral variants require further work-up. For correct classification of missense and 1-2 amino acid deletions, creatine uptake studies in patient fibroblasts should be performed. If these are not available, construction of the variant in an expression vector, followed by *in vitro* overexpression in SLC6A8 deficient fibroblasts should give an indication of the pathogenicity of the variant. For intronic or neutral variants, a different approach is necessary. In chapter 2.3, we describe a method where with the use of online splice site analysis tools, these types of variants can be analysed. However, as important as these tools are when no additional patient material is available, they cannot substitute *in vitro* experimentation, either with mRNA analysis or with the use of a minigene. These findings illustrate the importance of collecting and sharing variant data. Especially with next generation sequencing around the corner, which will result in vast amounts of novel variants, waiting to

be classified. Ideally only in cases where the variant is expected to be likely pathogenic, the patients are subjected to further biochemical and clinical workup. There is one specific type of variant which is not easily classified as either pathogenic or non-disease causing, the missense variant with residual transporter activity in transfection studies. In chapter 4, we present nine patients harbouring this type of mutation. In one remarkable case, a missense variant (c.1271G>A;p.Gly424Asp) was detected in a mother and her 3 sons. Creatine uptake studies of SLC6A8 deficient fibroblasts after transient transfection with pEGFP-SLC6A8-Gly424Asp resulted in a relative uptake of around 29% compared to the wildtype transfected fibroblasts. Also, the EGFP-SLC6A8-Gly424Asp fusion protein was detected using an antibody against EGFP. Clinically, these brothers had a very different pattern. Two of them had relatively mild ID, while the third had a more for SLC6A8 deficiency typical phenotype with moderate ID, autistic features, expressive dysphasia and epilepsy. Three other variants, c.1661C>T; p.(Pro544Leu), c.1699T>C; p.(Ser567Pro) and c.1190C>T; p.(Pro397Leu), also showed to have residual transporter activity and resulted in variable clinical phenotypes. The discrepancies between the molecular and clinical findings make this kind of variants difficult to definitively classify, they might however help us understand the *in vivo* functioning of the SLC6A8 protein.

Another interesting finding was the identification of a family with the pathogenic mutation c.1059_1061delCTT; p.Phe354del, which was not previously described (Chapter 3). Additional molecular workup on the mother of the index patient showed that she displayed low-level (6%) somatic mosaicism for this amino acid deletion. This might not seem to be clinically relevant, especially with a *de novo* occurrence rate of 30% in index patients. But with a relatively high percentage (7%) of mothers with somatic mosaicism, we underline the importance of awareness of mosaicism in the counselling of families with a *de novo* mutation in the *SLC6A8* gene and the need for prenatal diagnosis, unrelated from the outcome of DNA sequencing, which does not detect low-level somatic and germline mosaicism.

In conclusion, our diagnostic workup flowchart, in combination with the LOVD database should be considered important tools in aiding and simplifying the process of diagnosing SLC6A8 deficiency.

CLINICAL PRESENTATION

The extensive investigation of SLC6A8 deficiency in males has provided us with a clear overview of the clinical hallmarks.

In males, the most common clinical features are ID with prominent speech delay, behavioural abnormalities and seizures. Most adult male patients had severe ID, while younger patients showed a mild ID.

Most patients were above all delayed in speech development while their motor development was only mildly delayed. Other common symptoms were behavioural problems, mostly autistic features and attention deficit and hyperactivity, and seizures. Hypotonia, mild signs of spasticity and coordination disturbances are also very frequent in males.

In females, heterozygous for pathogenic mutations in *SLC6A8*, similar symptoms have been described, such as mental retardation, learning difficulties and constipation. However, the biochemical profile of females (e.g. urinary creatine to creatinine ratio and/or cerebral creatine) is usually within the range of normal controls.

TREATMENT

Initially, when the first patient was diagnosed, the general hope was that an effective treatment was within reach. Unfortunately until present day this goal has not been reached. With the discovery of SLC6A8 deficiency, several therapeutic trials have been commenced. Initially, patients were treated with high doses of creatine monohydrate. After these did not show any clinical improvements, studies followed with the creatine precursor L-arginine or lipophilic creatine analogs. Regrettably, none of these resulted in the desired clinical effect in males⁴⁻⁹, which keeps the search open for the right treatment of SLC6A8 deficiency. In females with a heterozygous pathogenic mutation in *SLC6A8*, treatment was proven to be mildly effective in several cases. One example is a female with a heterozygous c.1067G>T, p.Gly356Val mutation, that showed complete loss of her intractable epilepsy after treatment with creatine monohydrate and L-arginine and L-glycine, but no significant increase in her intellectual development¹⁰.

Patients harbouring mutations known to have residual activity *in vitro* may benefit more from treatment with creatine and/or creatine precursors. Currently, this

hypothesis was not in line with the treatment of 3 patients with the c.1631C>T; p.Pro544Leu mutation (residual activity of 38% of wildtype transfected cells), whom were treated with either a combination of L-arginine and creatine or just with L-arginine. All three showed no significant clinical improvement^{11,12}. Possibly, treatment of patients harbouring the other two published variants with residual activity, c.1271G>A; p.(Gly424Asp) and c.1699T>C; p.(Ser567Pro), will show a different course of improvement.

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Chapter 6

Future Perspectives



Future Perspectives

Currently molecular analysis of *SLC6A8* is performed by direct DNA sequencing. Although this method is very robust and trustworthy, new techniques will emerge which will replace this current gold standard, with next generation sequencing being the most probable candidate. With this technique, not only will larger groups of patients be sequenced simultaneously, but the spectrum of disorders sequenced will also be widened. At present patients are being selected based upon biochemical findings. After suspicion of ID, firstly the urine is analysed biochemically, followed by enzymatic analysis. If the pattern is consistent with *SLC6A8* deficiency, the patients' DNA is sequenced. In the future this order will be reversed. One (will) start(s) with whole exome or even whole genome sequencing, of for instance patients suffering from epilepsy, followed by biochemical analysis in case of unclassified variants in *SLC6A8*. This approach has two collateral effects. First, awareness of *SLC6A8* deficiency will increase, as the number of correctly diagnosed patients will rise. Second, new variants, both inside and outside of the ORF as well as intronic, will be detected. This will result in better understanding of the correct functioning of *SLC6A8* as a protein, the function of each transmembrane domain, the intronic regions, the regulatory regions and the promoter. In its turn, this will result in faster and more accurate diagnosis of this disorder.

With this in mind, the introduction of *SLC6A8* sequencing in newborns is also within reach. Although this disorder does not meet the criterion of being treatable, its detection does have significant consequences for genetic counselling. Also, this might have an even bigger impact once a treatment becomes available in the future.

The proper strategy of treating *SLC6A8* deficiency still has to be elucidated. Initially patients were treated with creatine monohydrate and creatine precursors in order to restore cerebral creatine levels, which did not yield the desired results. Lipophilic creatine analogs have now moved to the spotlight. Although creatine-benzyl-ester, phosphocreatine-Mg-complex acetate, creatine-ethyl-ester and dodecyl-creatine-ester did not have any effect in clinical pilots¹⁻⁶, cyclocreatine did show it could be transported to the brain in brain-specific *Slc6a8* knockout mice⁷. This even led to improved cognitive functioning. It remains the question if a


similar effect will be perceived in a model more comparable to human SLC6A8 deficient patients, the ubiquitous Slc6a8 knockout mouse model.

Recently, the function of the monocarboxylate transporter 12 (MCT12), encoded by the *SLC16A12* gene was elucidated. Apparently this protein, which is highly expressed in human kidney, retina, lung and testis, also transports creatine⁸. Unlike SLC6A8, which transports creatine in a Na⁺ and Cl⁻ dependent manner, MCT12 performs facilitated transport of creatine. Additional research might reveal the function and role of MCT12 and especially how its cooperation with SLC6A8 in the transport of creatine.

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Nederlandse samenvatting 

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Vanaf de eerste beschrijving in 2001¹ van *SLC6A8* deficiëntie, is de kennis over deze aandoening in snelle mate toegenomen. Op dit moment zijn er meer dan 150 patiënten gediagnosticeerd. In totaal zijn er meer dan 80 pathogene mutaties (Hoofdstuk 5, Figuur 1) beschreven. In dit proefschrift wordt beschreven wat de ontwikkeling binnen de DNA analyse van het *SLC6A8* gen teweeg heeft gebracht, voor zowel de diagnostiek alsmede de kliniek.

In hoofdstukken 2.1 en 2.2 worden nieuw ontwikkelde methodes beschreven waarmee de pathogeniciteit van nieuw gevonden mutaties met een onbekend effect kunnen worden bepaald. Deze methodes kunnen onder andere goed worden ingezet wanneer er mutaties in grote patiënten cohorten zonder overduidelijk effect (intronische varianten, missense varianten of 1-2 aminozuren deleties) worden gevonden. Het is niet altijd mogelijk om aanvullend materiaal te verkrijgen om de diagnose te bevestigen via bijvoorbeeld een mRNA analyse, creatine/creatinine ratio in urine, of met behulp van een MRS van de hersenen. Om de pathogeniciteit te kunnen testen hebben wij eerst een methode ontwikkeld waarmee creatine opname kon worden bepaald in creatine transporter deficiënte fibroblasten. Hierop voortbordurend kon, met behulp van overexpressie-studies, het effect van varianten in *SLC6A8* op de creatine opname worden bepaald. Om dit te bereiken, werden varianten geïntroduceerd in *SLC6A8* door middel van site-directed mutagenesis. Na overexpressie in *SLC6A8* deficiënte primaire fibroblasten en incubatie met fysiologische en suprafysiologische creatine concentraties, geeft het niveau van creatine opname ten opzichte van normale fibroblasten de pathogeniciteit van de variant weer.

Synonieme varianten of varianten gelegen in het intron, zijn met de hierboven beschreven methodes niet te analyseren. In hoofdstuk 2.3 passen we een *in silico* methode toe om dergelijke varianten te analyseren. Na een eerste schifting kunnen zo kandidaat varianten worden geselecteerd om vervolg onderzoek mee te verrichten, bijvoorbeeld door mRNA of minigene analyse.

Een interessante diagnostische uitdaging wordt beschreven in hoofdstuk 3. Bij het analyseren van de sequentie data, verkregen uit het bestuderen van een groot Europese cohort, bleek het *SLC6A8* gen van één mannelijke patiënt een pathogene mutatie te bevatten. Deze mutatie, c.1059_1061delCTT; p.Phe354del was, zoals

verwacht, ook aanwezig bij zijn broer met een vergelijkbaar ziektebeeld. Geheel onverwacht bleek dit echter niet te detecteren te zijn in het DNA dat geïsoleerd was uit het bloed van zijn moeder. Om deze alsnog te kunnen opsporen is een methode ontwikkeld waarmee het mogelijk is om somatische mosaïcisme van slechts een paar procent te detecteren. Deze techniek is vooral belangrijk bij genetische consultatie voor families waarbij een mutatie *de novo* lijkt te zijn ontstaan.

Met behulp van de verscheidenheid aan beschikbare middelen om *SLC6A8* te bestuderen is in de loop van de jaren een groot aantal mutaties gevonden. Om een beter overzicht en inzicht te verkrijgen hebben we de data van alle patiënten waarbij middels DNA onderzoek in het VU Medisch Centrum de diagnose *SLC6A8* deficiëntie kon worden bevestigd geanalyseerd. Door middel van een uitgebreide vragenlijst, die werd ingevuld door artsen verspreid over vele centra, konden we ook klinische data van patiënten meenemen van de hele wereld includeren. Ook hebben we in de analyse, waar mogelijk, gepubliceerde data meegenomen. Deze studie, bestaande uit 101 mannelijke *SLC6A8* deficiëntie patiënten, leverde de eerste uitgebreide beschrijving op van de fenotype, genotype en biochemische indices van deze aandoening. Deze DNA data zijn ook opgenomen en beschikbaar gemaakt in een online LOVD database, die hierdoor wereldwijd toegankelijk zijn. Zo kan bij het vermoeden op *SLC6A8* deficiëntie het diagnostische proces sneller en handzamer worden en wordt er tegelijkertijd ook meer bewustzijn gecreëerd voor deze toch nog relatief onbekende aandoening.

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Curriculum vitae

Ofir Tsvi Betsalel was born on May 25th 1983 in Rechovot, Israel. After graduating highschool in 2001, he finished his chemistry “propedeuse” at the University of Amsterdam. He continued his bachelors degree at the Hogeschool in Leiden, which led him to the Metabolic Laboratory for his final internship. After completion, he started his PhD research there and in parallel his graduate degree at the Graduate School Neurosciences Amsterdam. He currently manages his own scientific marketing agency in Amsterdam.