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Analysis of 3-OMD levels on DBS in the Ligurian neonatal population: towards AADCd screening

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ABSTRACT

Background: Aromatic L-Amino Acid Decarboxylase deficiency (AADCd) is a rare neurometabolic disorder caused by biallelic pathogenetic variants in the dopa decarboxylase (*DDC*) gene. AADCd appears early in life with a heterogeneous clinical picture including hypotonia, oculogyric crises and other movement disorders, developmental delay, and autonomic symptoms.

We aimed at evaluating the real-life applicability of a new method for AADCd early identification on dried blood spots (DBSs) and, consequently, the 3-O-methyldopa (3-OMD) reference levels in the Ligurian neonatal population in view of a regional AADCd newborn screening program.

Methods: DBSs, collected according to the current newborn screening procedures, were blindly analysed for 3-OMD levels by flow-injection analysis tandem mass spectrometry (FIA-MS/MS) with labelled tyrosine as internal standard. The DBS performed at birth of a currently two years aged affected child was used as an internal disease reference value.

Results: On a total of 9,876 DBSs, collected between the first 0-116 hours of life, the mean 3-OMD concentration was 0.827 µmol/L (SD ± 0.286; range: 0.037-2.271 µmol/L), and the median concentration was 0.808 (IQR, 0.615-1.016) µmol/L. On 9,664 out of 9,876, collected precisely between the first 48-72 hours of life, the mean 3-OMD concentration was 0.830 µmol/L (SD ±0.285; range: 0.037-2.271 µmol/L), and the median concentration was 0.811 (IQR, 0.617-1.018) µmol/L. A 99th percentile value of 1.57 µmol/L was found in both analyses. Considering the approximate 99th percentile (1.6 µmol/L) as the cut-off value, 3-OMD assay would be re-evaluated on 85 (0.86%) samples using a second-tier test.

Conclusions: The method has proven to be valid as a first-tier test for AADCd newborn screening, allowing to move forward to second-tier tests in only 0.86% of the studied population. Early AADCd diagnosis would allow early administration of the recently approved disease-modifying gene therapy.

1. AROMATIC L-AMINO ACID DECARBOXYLASE DEFICIENCY

Aromatic L-Amino Acid Decarboxylase deficiency (AADCd; **OMIM: #608643**) is a rare neurometabolic disorder, with an autosomal recessive inheritance, caused by biallelic pathogenetic variants in the dopa decarboxylase (*DDC*) gene. The *DDC* gene encodes for aromatic L-Amino Acid Decarboxylase (AADC) enzyme, the deficiency of which results in a severe combined deficiency of monoamine neurotransmitters (dopamine, serotonin, norepinephrine, and epinephrine). These neurotransmitters are crucial for motor and autonomic functions, and behaviour.

AADCd appears early (mean age: 2.7 months) with a heterogeneous clinical picture characterised by neurological and non-neurological symptoms. The typical clinical presentation includes hypotonia, oculogyric crises and other movement disorders, developmental delay, and autonomic symptoms. (1) (2)

Despite its early onset, because of the variability in clinical manifestations and non-specific neurological symptoms, AADCd is often undiagnosed, misdiagnosed or diagnosed late (mean age at diagnosis: 3.5 years). (1) (3)

AADCd is significantly more prevalent in the Asian population, especially in the Taiwan population, due to a founder mutation in the dopa decarboxylase (*DDC*) gene.

Through early diagnosis with newborn screening, patients will benefit from the administration of the recently approved gene therapy, eladocagene exuparvovec (Upstaza™).

1.1 Epidemiology

AADC deficiency is an extremely rare neurometabolic disease. Although the worldwide incidence is not precisely reported, over 135 cases have been described. (1) (2)

The predicted birth rates of individuals with AADCd are 1:162,000 in Japan, 1:116,000 in the European Union, and 1:90,000 in the United States. (4)

In Asia the prevalence of AADC deficiency is significantly increased, especially in Taiwan, where newborn screening, using 3-O-methyldopa (3-OMD) as a biomarker, showed a prevalence of 1:32,000. (5)

In at-risk population the prevalence of AADC deficiency is estimated to be approximately 0.112% or roughly 1:900 (6), furthermore in Asian at-risk population the prevalence is 50% greater than in non-Asian population. (7) (8)

The causal and founder *DDC* mutation, c.714 + 4A>T (IVS6 + 4A>T) is a splicing mutation and the most common in the Taiwan population. The c.714 + 4A>T (IVS6 + 4A>T) splicing mutation causes a 37-nucleotide insertion of intron 6 into the *DDC* mRNA. This probably explains the higher prevalence of AADC deficiency in Asia, especially in Taiwan, and the founder effect. (9) (10) (11)

1.2 Pathogenesis and the *DDC* gene

Pathogenetic mutations in the *DDC* gene and the secondary lack of function of the AADC enzyme led to monoamine neurotransmitters deficiency. The deficiency of dopamine, serotonin, and consequently norepinephrine and epinephrine underlie the key clinical symptoms of the disease.

The AADC enzyme (EC 4.1.1.28), encoded by the *DDC* gene, is a lyase enzyme, pyridoxal 5'-phosphate (PLP) dependent, located in mammalian tissues and is essential to synthesize 3,4-dihydroxyphenethylamine (dopamine) and 5-hydroxytryptamine (serotonin). AADC, as a homodimeric enzyme, catalyses decarboxylase reaction of aromatic L-amino acids: Levodopa (L-DOPA) to dopamine, 5-Hydroxytryptophan (5-HTP) to serotonin, L-Phenylalanine to phenethylamine, L-Tyrosine to tyramine, L-Histidine to histamine, L-Tryptophan to tryptamine. (12) (13)

Dopamine, 3,4-dihydroxyphenethylamine, is a catecholamine neurotransmitter synthesized by three main enzymatic reactions, starting from L-Phenylalanine, predominantly in brain tissue and adrenal medullary cells. L-Phenylalanine (Phe) is converted to L-Tyrosine (Tyr) and L-Tyrosine to L-DOPA by Phenylalanine Hydroxylase (PAH) and Tyrosine Hydroxylase (TH), respectively, both of which use tetrahydrobiopterin (BH4) as a cofactor. The last step consists of the decarboxylation reaction: dopamine is obtained from L-DOPA by removal of the carboxyl group (-COOH), catalysed by the enzyme AADC, with PLP as a cofactor. Dopamine beta-hydroxylase (DβH) converts dopamine into norepinephrine and norepinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase (PNMT). (14) (4)

Dopamine, norepinephrine, and epinephrine are inactivated by two enzymes: catechol O-methyl transferase (COMT) or monoamine oxidase (MAO). The end product of Dopamine breakdown is homovanillic acid (HVA) and the metabolites derived from norepinephrine and epinephrine degradation are vanillmandelic acid (VMA) and 3-methoxy-4-hydroxyphenylglycol (MHPG).

L-Phenylalanine ➔ L-Tyrosine ➔ L-DOPA ➔ Dopamine ➔ Norepinephrine ➔ Epinephrine

Serotonin, 5-hydroxytryptamine, is synthesized from L-Tryptophan (Trp) by two enzymatic reactions. L-Tryptophan is converted to 5-hydroxy-L-tryptophan (5-HTP) by Tryptophan hydroxylase (TPH) with tetrahydrobiopterin (BH4) as a cofactor. The enzyme AADC, with PLP, decarboxylates 5-HTP to form serotonin. Monoamine oxidase (MAO) inactivates and converts serotonin to 5-hydroxyindoleacetic acid (5-HIAA), the main end product of serotonin breakdown. (4) (15)

L-Tryptophan ➔ 5-HTP ➔ Serotonin

Deficiency of the AADC enzyme results in the failure to synthesize monoamine neurotransmitters (dopamine, serotonin, norepinephrine end epinephrine), leading to the accumulation of upstream substrates and a lack of downstream products.

The failure of serotonin and dopamine biosynthesis results in increased concentrations of 5-HTP and L-DOPA, respectively. L-DOPA is converted into 3-O-methyldopa (3-OMD) by COMT and vanillactic acid (VLA) is obtained from 3-OMD. This is reflected in an increase in the concentration of the aforementioned.

Deficiency of monoamine neurotransmitters also leads to reduced concentrations of the final breakdown products: 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylglycol (MHPG). (1)

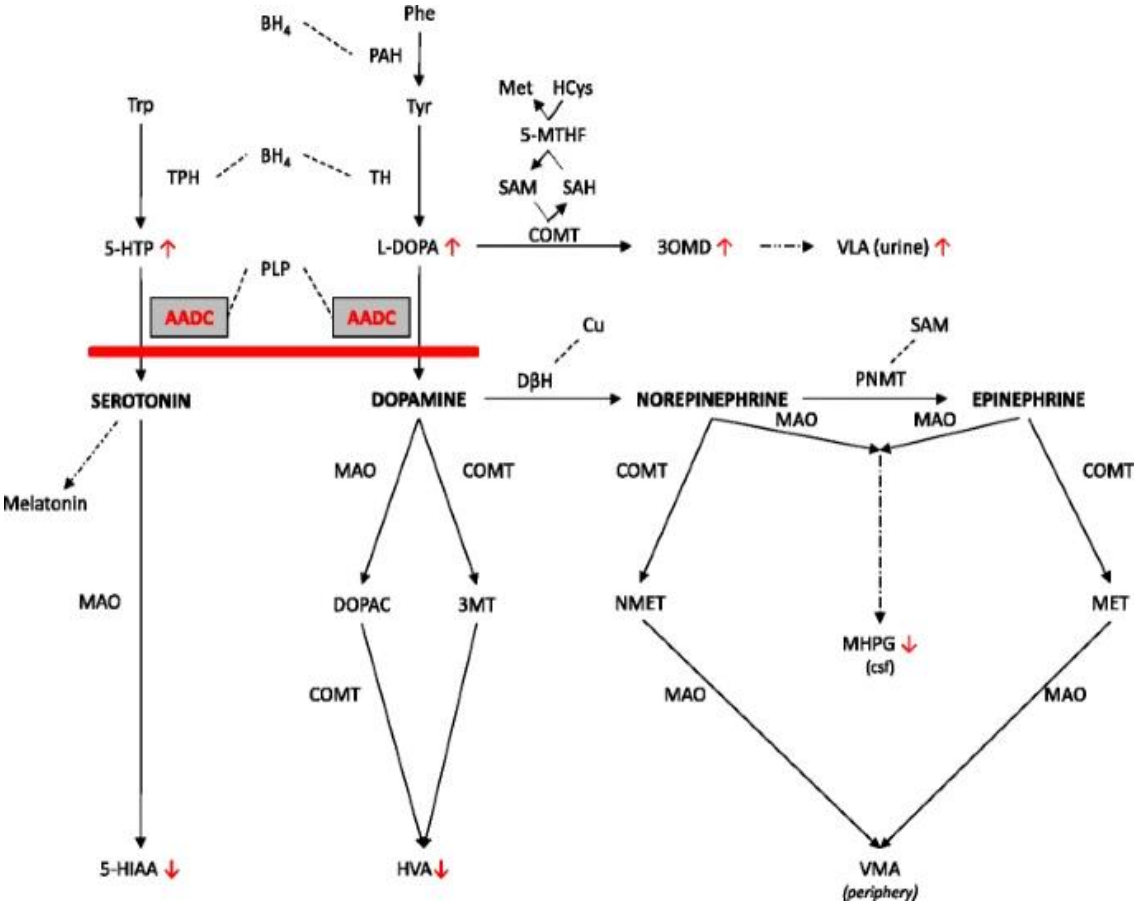

Figure 1. Biosynthesis and breakdown of serotonin and the catecholamines, and the metabolic block in AADC deficiency. Simplified scheme of the biosynthesis and breakdown of serotonin and the catecholamines (dopamine, norepinephrine, and epinephrine), and melatonin synthesis. Cofactors (BH4, PLP, Cu) and methyldonor (SAM) are connected to the respective enzyme with dashed lines. Dashed arrows do not show intermediate steps. The metabolic block caused by AADC deficiency is shown as a red bar. Metabolites above the block are increased, metabolites below the block are decreased, indicated by red arrows. The implication of 5-MTHF in L-dopa to 3-OMD metabolism is shown in a simplified manner. Norepinephrine and epinephrine are broken down to NMET and MET only in the periphery. In CSF, the main metabolite of norepinephrine and epinephrine is MHPG. Abbreviations: AADC: aromatic l-amino acid decarboxylase; BH4: tetrahydrobiopterin; COMT: catechol O-methyl transferase; CSF: cerebrospinal fluid; Cu: cupper; DβH: dopamine beta hydroxylase; DOPAC: dihydroxyphenylacetic acid; HCys: homocysteine; 5-HIAA: 5-

hydroxyindoleacetic acid; 5-HTP: 5-hydroxytryptophan; HVA: homovanillic acid; L-Dopa: 3,4-dihydroxyphenylalanine; MAO: monoamine oxidase; MET: metanephrine; Met: metionine; MHPG: 3-methoxy 4-hydroxyphenylglycol; 3MT: 3-Metyramine; 5-MTHF: methyltetrahydrofolate; NMET: normetanephrine; 3-OMD: 3-O-methyldopa (=3-methoxytyrosine); Phe: phenylalanine; PhH: phenylalanine hydroxylase; PNMT: phenylethanolamine N-methyltransferase; SAH: S-adenosylhomocysteine; SAM: s-adenosylmethionine; TH: tyrosine hydroxylase; TrH: tryptophan hydroxylase; Tryp: tryptophan; Tyr: tyrosine; VLA: vanillactic acid; VMA: vanillmandelic acid: Vit B6 vitamin B6 (pyridoxine). (Wassenberg et al., - 2017). (1)

Dopamine is synthesized in the substantia nigra, ventral tegmentum, and hypothalamus; it regulates voluntary movements, emotions, cognition, and hormonal-related functions. Serotonin deficiency affects sleep, mood, memory and learning, body temperature, appetite, cardiovascular and endocrine functions. Alterations in mood, sleep, attention, and cognition are related to disturbances in norepinephrine and epinephrine synthesis. (16)

DDC gene

The dopa decarboxylase (*DDC*) gene [(OMIM#107930), gene ID 1644], is a protein-coding gene, located on chromosome 7 (7p12.2-p12.1) in a single copy, that encodes for the enzyme aromatic L-amino acid decarboxylase (AADC).

The *DDC* gene is involved in several conditions and it may modulate the age of male schizophrenia onset (17), autism susceptibility (18), and the occurrence of attention-deficit and hyperactivity disorder (ADHD) (19). Polymorphisms in the *DDC* gene are linked to differences in the striatal *DDC* activity and may be associated with neuropsychiatric conditions with ventral striatal involvement. (20)

In addition, it is suggested that *DDC* gene single-nucleotide polymorphisms (SNPs) are associated with susceptibility to nicotine dependence (21) and that the aforementioned gene and migraine susceptibility may also be associated (22).

The *DDC* gene is also implicated in motor response to L-DOPA in patients with Parkinson's disease. Precisely the AADC enzyme, is essential for converting L-DOPA to dopamine. *DDC* promoter polymorphisms underlie the response to L-DOPA, which is variable and unpredictable. *DDC* gene may be a genetic modifier

of the response to L-DOPA in Parkinson's disease. (23) Moreover, patients with Parkinson's disease treated with long-term administration of L-DOPA experience adverse effects (dyskinesia, on-off and wearing off symptoms). Especially 3-OMD, derived from L-DOPA, is believed to be responsible for the side effects of long-term L-DOPA therapy through neurotoxicity and oxidative stress. (24)

1.3 Clinical features

In 1990, AADCd was first described in monozygotic male twins. Onset was at two months of age, and the twins were born to consanguineous parents, specifically first cousins. The main symptoms of clinical presentation were hypotonia, developmental delay, oculogyric crises, and choreoathetoid movements of the extremities. In addition, normal concentrations of biopterin, neopterin, and aromatic amino acids, and low concentrations of HVA and 5-HIAA were demonstrated in CSF. The concentrations of L-DOPA and 5-HTP in CSF and plasma were high. AADC enzyme deficiency was verified by assessment of the enzyme activity in plasma and in the liver. (25)

The clinical onset of the AADC deficiency is early, in most cases within the first 12 months of life, often within the first 6 months of age. (8)

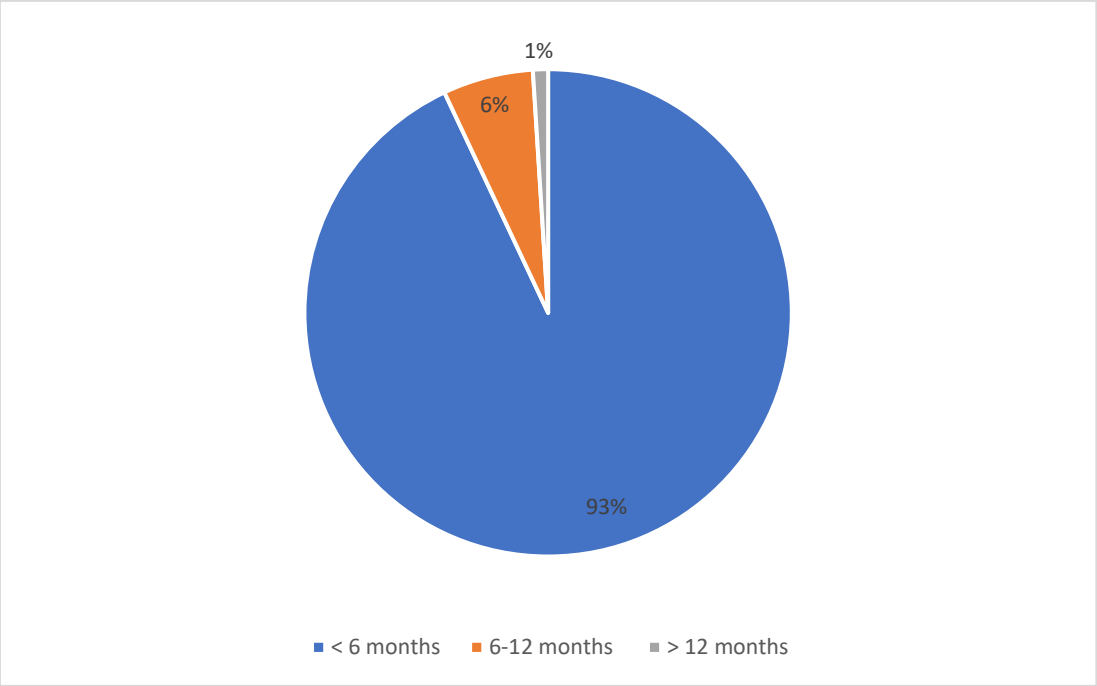

Figure 2. Age at onset. Modified from Rizzi et al., - 2022. (8)

Patients with AADCd manifest neurological and non-neurological symptoms with a heterogeneous clinical picture and a broad phenotypic spectrum. At the onset, the most common symptoms described are hypotonia, developmental delay, oculogyric crises (OGCs), and autonomic symptoms. (8)

Hypotonia (mainly axial) and delays in motor, cognitive, and language development are key signs (1) (8). Central movement disorders are OGCs, dystonia, and hypokinesia. Autonomic dysfunction includes nasal congestion, abnormal sweating, excessive drooling, temperature instability, and bradycardia. Hypotension and orthostatic hypotension also may occur in adolescence or late childhood. Furthermore, pseudo-myasthenic symptoms, such as ptosis and fatiguability, are described. The clinical picture of AADC deficiency may also manifest with less common symptoms. Notably, other neurological manifestations include behavioural disorders (dysphoria, irritability, excessive crying, and autism-like symptoms), sleep disturbances (insomnia, hypersomnia, and severe sleep apnea), and epileptic seizures, which are rare. Moreover, episodes of intermittent hypoglycaemia and gastrointestinal symptoms, such as

diarrhoea, constipation, gastroesophageal reflux, and feeding difficulties have been reported. (1) (2) (3) (8) (16)

Additional clinical features are growth retardation and prematurity. (11) (26) In the study published *by Kuseyri Hübschmann et al.*, the rate of prematurity in AADCd is higher than the global incidence of prematurity. (26)

Developmental delay

Patients diagnosed with AADC deficiency may present a variable clinical picture. Regarding psychomotor development, the broad phenotypic spectrum can be classified into mild (mild developmental delay, independent walking, mild intellectual disability), moderate (intermediate), and severe (very limited or no attainment of developmental milestones, complete dependence). (1) In particular, *Pearson et al.* analysed the achievement of the three main motor developmental milestones: head control, independent sitting, and walking. They observed that none of the patients younger than 6 years old acquired independent sitting or walking, while a proportion of the patients aged 6 to 12 years old and over 12 years old gained sitting position and walking. The study showed that in the older patients the clinical phenotype was usually mild, with mild motor impairment. Feeding difficulties, such as swallowing difficulties and vomiting, were also common. (2)

Oculogyric crises

OGCs are a clinical sign that manifests as a conjugate, involuntary, and usually upward deviation of the eyes, with a duration ranging from seconds to hours. (27) OGCs are often associated with body movements or dystonia. The phenomenon has been observed in almost all patients of all ages, but the prevalence is highest in the age group of 2 to 12 years and lowest above 18 years. In younger patients (< 6 years), the OGCs are more prolonged (> 4 hours) and more frequent (≥ 3/week). In younger patients, OGCs are also more severe, associated with trunk and/or limb dystonia. (2)

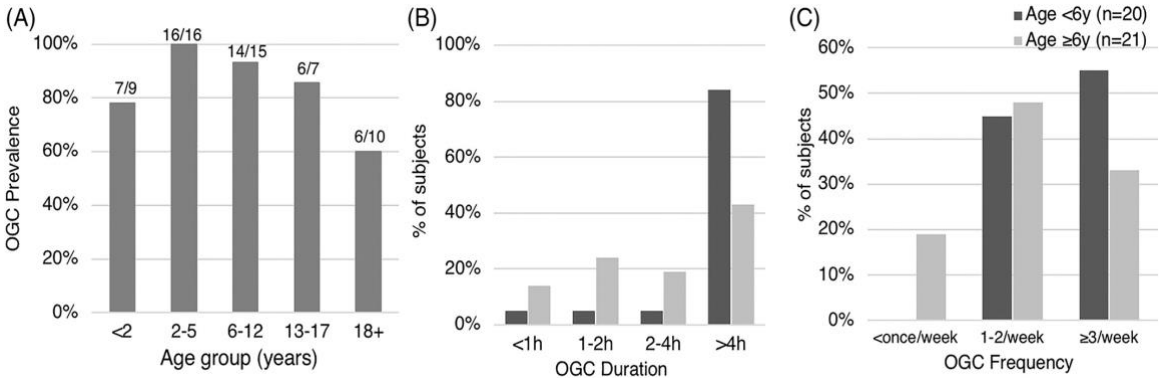

Figure 3. Oculogyric Crises (OGC). A, Reported current prevalence of OGC's across age groups (n = 57): 91% of subjects under 18, 60% of subjects over 18. B and C, OGC duration and frequency, respectively, in younger (age < 6 years, dark gray) vs older (age ≥ 6 years, light grey) subjects. Over 80% of younger subjects experienced prolonged episodes (> 4 hours duration), and the majority experienced episodes at least three times per week. (Pearson et al., - 2020). (2)

Sleep disturbances

Regarding sleep disturbances, *Pearson et al.* observed that between the ages of 2 and 12 years insomnia predominates, while subjects younger than 2 years of age experience excessive sleepiness. (2)

Growth retardation

Hwu et al. showed severe growth retardation in patients with AADC deficiency. The patients had normal body weight in the first few months of life, while at the onset of symptoms their growth started to slow down. Notably, after the age of 1 year, patients stopped gaining weight, and between 1 and 4 years had minimal body weight gain. In patients who survived after age 4 years, body weight improved but was still below the 50th percentile.

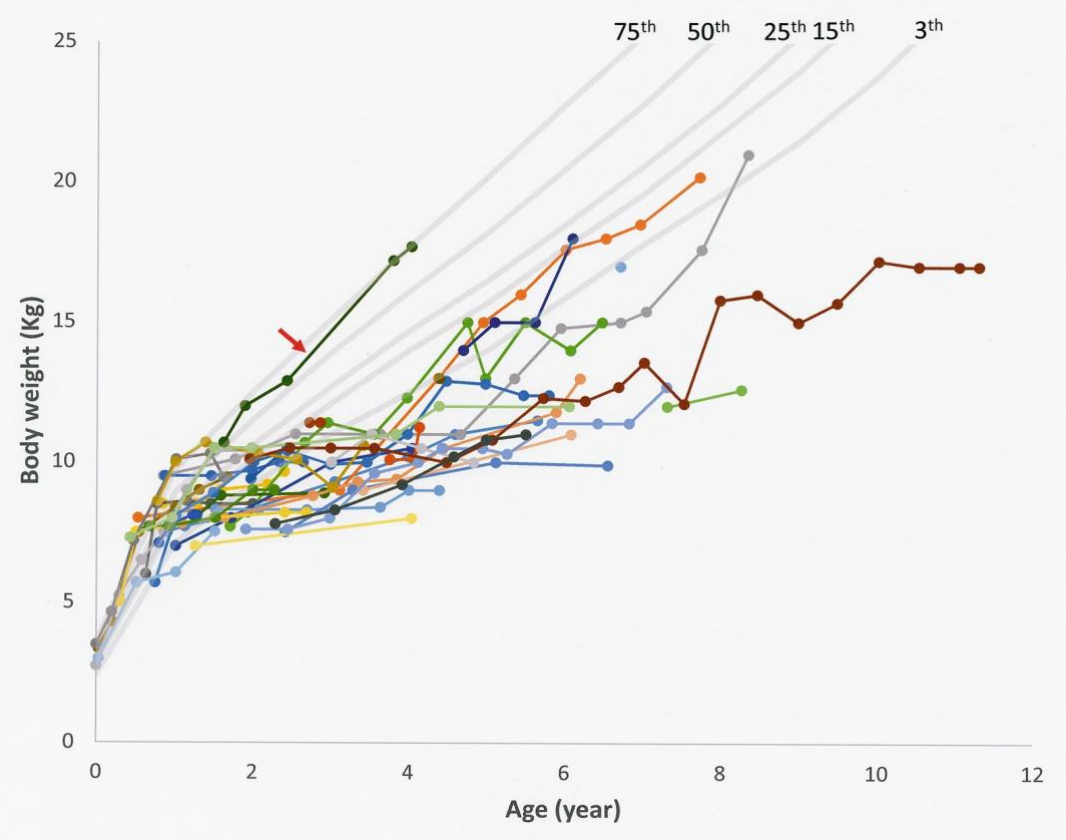

Figure 4. Body weight of 37 patients with AADC deficiency. Body weight at each visit was plotted according to age, in comparison with the body weight distribution of normal Taiwanese female children (Chen and Chang 2010). Each line represents one patient. One outlier (patient No. 37), who had significantly greater growth than other patients, is indicated by the red arrow. (Hwu et al., - 2018). (11)

Phenotypic spectrum and genotype-phenotype correlation

AADC deficiency presents a heterogeneous clinical picture and a broad phenotypic spectrum, ranging from relatively mild to very severe. AADCd typically manifests with a severe phenotype, the onset of the disease is in the neonatal period, and children express all key symptoms and signs with maximal expression. They do not acquire developmental milestones and are completely dependent on caregivers. Patients with severe phenotype sometimes die early; the increased mortality risk can be traced mainly to pneumonia and acute complications occurring during OGCs. (2) (8)

AADCd presents as a continuum of signs and symptoms between the typical and atypical clinical picture. (8) Particularly, *Arnoux et al.* described an AADC deficiency atypical clinical picture in a 5-year-old girl with mild phenotype and normal psychomotor development. The central symptoms were mostly autonomic and included recurrent and severe long-fasting hypoglycaemia and recurrent aqueous diarrhoea. Moreover, chronic nasal obstruction, hypomimia, and mild dyspraxia were reported. (28)

The typical clinical course of AADC deficiency is not progressive, although regression in motor or developmental acquired skills is possible. The main skills lost involve feeding, head control or other motor skills, and language. Regression of acquired skills occurs early at the onset of OGCs and other symptoms. (2) (29)

Considering the clinical picture, we can conclude that AADC deficiency should be considered in patients with unexplained hypotonia, movement disorders, developmental delay, and autonomic symptoms. In addition, the AADCd clinical course is not expected to be deteriorating; thus, in case of a deteriorating clinical course a diagnostic work-up for other diseases should be triggered. (1)

Furthermore, the spectrum of the identified mutations in the *DDC* gene is constantly evolving. Missense, nonsense, deletion, and splice-point variants have been identified. The splice site and founder variant c.714 + 4A>T (IVS6 + 4A>T), in homozygous or in compound heterozygous, is the most common one, followed by c.1234C>7 (p.R412W) and c.1297dupA (p.I433Nfs*60), all associated with severe phenotypes. (2) (4) (11) (30)

A clear correlation between genotype and phenotype has not been established, except for the homozygous c.714 + 4A>T (IVS6 + 4A>T) splice variant, which always underlies a severe phenotype. (1)

1.4 Diagnosis

To date, the diagnosis of AADC deficiency has been based on three main diagnostic tests:

- Cerebrospinal fluid (CSF) analysis by lumbar puncture
- Measurement of AADC enzyme activity in plasma by enzyme assay
- *DDC* genetic test

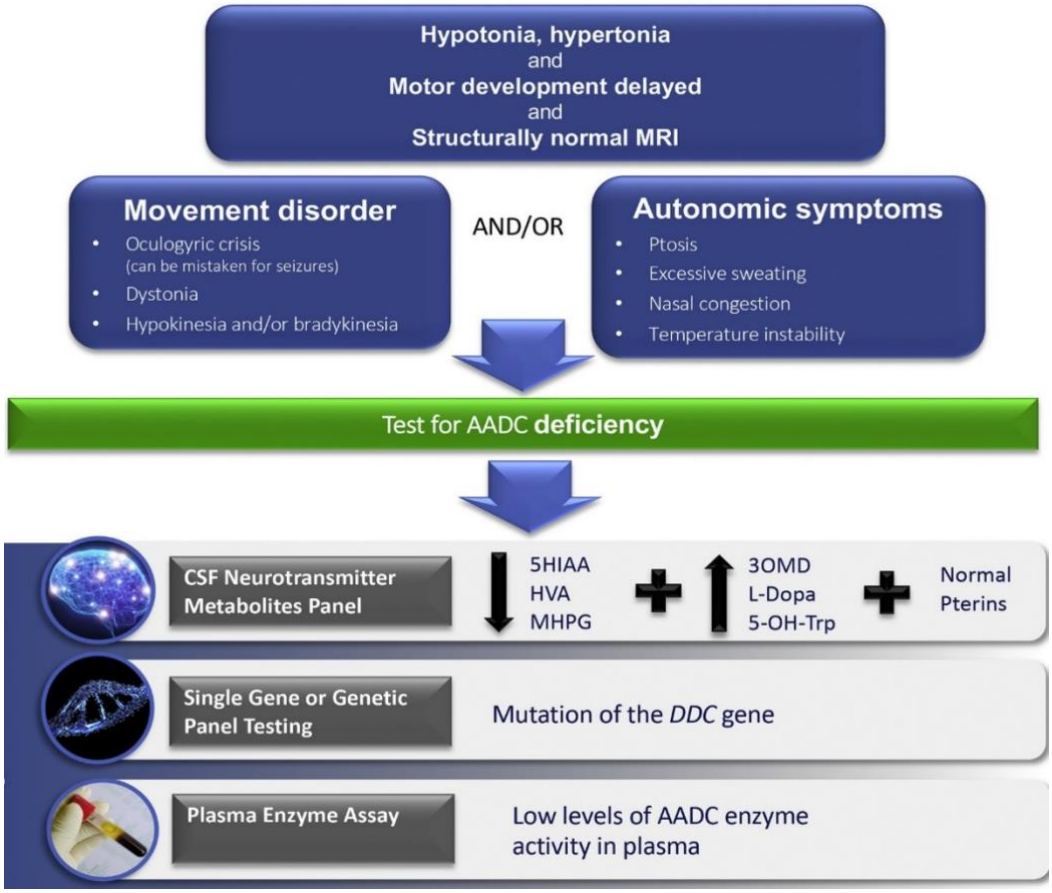

Figure 5. Modified from Himmelreich et al., - 2019. (4)

To diagnose AADC deficiency, at least two of the three key diagnostic tests should be positive and the genetic test should be performed; however, if local resources allow, it is recommended that all three core diagnostic tests be performed. (1)

Lumbar puncture and CSF analysis

Pathogenic mutations in the *DDC* gene and the resulting lack of function of the AADC enzyme result in a deficit in the synthesis of monoamine neurotransmitters (serotonin, dopamine, norepinephrine end epinephrine), leading to increased concentrations of upstream substrates and deficiency of downstream products.

Blockade of neurotransmitter synthesis is demonstrated by the typical CSF profile:

- Low concentrations of 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylglycol (MHPG)
- Normal concentrations of pterins (neopterin and biopterin)
- High concentrations of 3-O-methyldopa (3-OMD), L-DOPA, and 5-OH tryptophan (5-HTP) (1) (8) (16)

The CSF pattern allows the differential diagnosis between AADC deficiency and tetrahydrobiopterin (BH4) deficiencies. Disturbance of tetrahydrobiopterin synthesis or regeneration underlies the six neurometabolic disorders, which are characterized by severe depletion of monoamine neurotransmitters. The clinical picture includes hypotonia often associated with poor head control and hypertonia of the extremities, movement disorders (dystonia and OGCs), developmental delay, and impaired cognitive and speech development. Early-onset parkinsonism or hypokinetic rigid syndrome, autonomic dysfunction (e.g., temperature instability), excessive salivation, feeding and swallowing difficulties, behavioural and psychiatric problems, and sleep disorders are also described. The CSF profile in BH4 deficiencies usually shows low levels of 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and altered concentrations of pterin (neopterin and biopterin), and 5-methyltetrahydrofolate (5-MTHF) are observed. Given the similarity between the typical clinical manifestations of AADCd and BH4 disorders, the concentration of pterins in CSF is crucial to discern the two aforementioned diseases. (1) (31)

The AADC enzyme is also involved in pyridox(am)ine 5’-phosphate oxidase (PNPO) deficiency, a severe neonatal epileptic encephalopathy. Specifically, mutations in the *PNPO* gene, a protein-coding gene, lead to pyridoxal 5’-phosphate (PLP) deficiency, which is a cofactor of AADC. In PNPO deficiency, PLP deficiency and subsequent secondary failure of AADC result in a CSF pattern similar to that of AADCd, with the exception of reduced PLP and increased glycine and threonine concentrations. (1) (32)

Therefore HVA, 5-HIAA, 3-OMD, L-dopa, 5-HTP, and pterins levels in CSF should be measured and, if available, the measurement of PLP and 5-MTHF should also be included. (1)

AADC activity in plasma

The enzymatic activity of AADC in plasma is measured by enzymatic assays using L-DOPA and 5-HTP as substrates, but because of its higher analytical yield, L-DOPA is considered the standard method. Enzyme assays have shown that in AADC-deficient patients plasma AADC activity is strongly decreased, and in heterozygous carriers plasma AADC activity is moderately reduced (35-40% of normal). (33)

According to *Fusco et al.* to diagnose AADC deficiency, enzyme assay measuring the AADC activity in plasma should be considered if CSF analysis shows non-significant neurotransmitter abnormalities. (3)

Molecular diagnosis

If the genetic diagnosis is performed first, it should be confirmed by measurement of neurotransmitter metabolites in CSF and/or the AADC enzyme activity in plasma. Genetic confirmation should not be waited for initiation of therapy, results of plasma AADC activity and CSF analysis are usually available sooner. (1)

According to *Rizzi et al.*, genetic testing detected homozygous and compound heterozygous pathogenic variants in the *DDC* gene, most of which were compound heterozygous, while due to the founder effect, homozygous variants were prevalent in the Asian population. (8) (9)

According to *Fusco et al.* in patients with adult onset and mild phenotype, it is useful to consider whole-exome sequencing in the diagnostic work-up. (3)

Neurotransmitter metabolites in urine and blood

Measurement of urinary metabolites of neurotransmitters demonstrates normal, increased, or decreased levels of dopamine, VMA, and HVA; on the other hand, increased concentrations of VLA in urine are observed. Therefore, AADCd should

be considered if the urinary concentration of VLA is increased, but it is not excluded in case of normal levels of VLA in urine. (1) (16)

However, in clinical practice, the measurement of catecholamine metabolites in blood and urine is not helpful, because the results do not allow the diagnosis or exclusion of AADCd. (1)

Magnetic Resonance Imaging (MRI) of the brain and Electroencephalography (EEG)

In patients with AADC deficiency, a specific MRI pattern is not described. Although neuroimaging is not useful and necessary to diagnose AADC deficiency, it should be considered in the work-up of patients with neurodevelopmental delay and in patients with AADCd with unexpected clinical course deviation. (1)

EEG is not necessary to diagnose AADC deficiency, however, it is useful in the work-up of AADCd to differentiate epileptic seizures from OGCs and in the work-up of neurodevelopmental delay. (1)

1.5 Misdiagnosis

The typical clinical picture of AADC deficiency includes nonspecific neurological and non-neurological signs and symptoms leading to misdiagnosis or delayed diagnosis. The mean age of onset is 2.7 months, while the mean age of diagnosis is 3.5 years. (1)

Because of the broad spectrum and heterogeneity of clinical presentation and nonspecific signs and symptoms, misdiagnosis with other neurological diseases (e.g., cerebral palsy, epilepsy, myasthenia, and autism) is common.

Epilepsy

Epilepsy can occur in patients diagnosed with AADC deficiency, although this is a rare occurrence. Epileptic seizures have been observed in a small percentage

of patients with AADCd; in some cases the seizures were symptomatic, secondary to hypoglycaemia and hydroelectrolytic imbalance during diarrhoea. (8) In patients with AADCd paroxysmal events such as OGCs, tonic or dystonic limb postures, myoclonus, and chorea are frequently described and are misinterpreted as epileptic seizures. Clinical and ictal EEG recording are helpful in making a differential diagnosis between epileptic seizures and involuntary non-epileptic movements, ensuring proper treatment. Thus, epilepsy can occur in patients with AADCd and sometimes requires treatment with antiseizure medications (ASMs). (34) (35)

On the other hand, patients may be misdiagnosed as epileptic and/or treatment treated with ASMs before confirmation of AADCd diagnosis. (2)

Myasthenia

Developmental delay, hypotonia/hypokinesia, ptosis, and fatigue can be misinterpreted as features of neuromuscular origin, resulting in misdiagnosis of myasthenia. (3) (36)

Cerebral palsy

Cerebral palsy (CP) comprises a heterogeneous group of permanent neurodevelopmental disorders with an early onset, usually within the first 2 years of life, characterized by movement and posture disorders. CP consists of motor and non-motor neurological signs and symptoms, usually non-progressive. The main motor features are hypotonia, spasticity, dystonia, chorea, and often developmental delay. Non-motor neurological features include intellectual disability, behavioural symptoms, and seizures. AADC deficiency may be included among CP mimics and thus may be misdiagnosed. (37)

Autism

Autism is a set of heterogeneous neurodevelopmental conditions. A high percentage of individuals with autism have co-occurring conditions, such as intellectual disability, language disorders, gastrointestinal problems (e.g., constipation, chronic diarrhoea, and gastroesophageal reflux), insomnia, and aggressive behaviours. (38) The dopaminergic and serotoninergic systems and

common variants of modest effect in the *DDC* gene may be implicated in autism susceptibility. (18) Neurological manifestations of AADC deficiency include behavioural disorders (e.g., dysphoria, irritability, excessive crying) and delays in cognitive and language development, which could lead to the misdiagnosis of autism.

2. NEWBORN SCREENING

2.1 Generalities on newborn screening

The newborn screening program is a secondary prevention medical activity with the aim of enabling early detection of congenital diseases in the pre-symptomatic phase. The earlier the diagnosis, the earlier the specific treatment can be administrated. Specific therapeutic interventions can change the natural history of congenital diseases, leading to improved prognosis and quality of life for patients and their families, and can often prevent death. (39)

Newborn screening is based on non-invasive tests, a blood sample is taken at the birth Centre between the first 48-72 hours of life from the newborn's heel, is applied on the Guthrie Card (Dried Blood Spot, DBS) and the concentration of specific metabolites is evaluated. Alterations in the screening test do not imply a diagnosis of the disease, but further investigations should be conducted to confirm or exclude the diagnosis. In the event of diagnosis, the referring clinician will take charge of the patients, and provide treatment and follow-up. (40)

In Italy, since 1992 (L. 104/1992), newborn screening for phenylketonuria, congenital hypothyroidism, and cystic fibrosis is mandatory and free for all newborns. It was possible to expand newborn screening for specific metabolic inherited diseases, which were included in expanded newborn screening. It should be noted that with the approval of Law 167 in 2016 (L.167/2016) and with the DPCM of January 12, 2017, expanded newborn screening was included in the LEAs, so it is mandatory to be offered to all newborns. Subsequently, it was further expanded to include genetic neuromuscular diseases, lysosomal storage

diseases, and severe congenital immunodeficiencies. This was enabled by the simultaneous measurement of disease biomarkers using a multiplex platform and the approval of specific and innovative therapies for congenital diseases. (41)

2.2 Newborn screening for Aromatic L-Amino Acid Decarboxylase deficiency

AADC deficiency is a debilitating inherited neurometabolic disorder; lack of function of AADC enzyme results in deficiency of serotonin, dopamine, and subsequently norepinephrine and epinephrine. On the other hand, the concentration of metabolites upstream of AADC blockade is increased, especially the blood concentration of 3-O-methyldopa (3-OMD), derived from L-dopa catabolism, is high. The increased blood concentration of 3-OMD and its possible detection in patients with AADCd, as well as the recent approval of a specific, safe, and efficacy gene therapy, provide the rationale for newborn screening of AADCd. (42) (43)

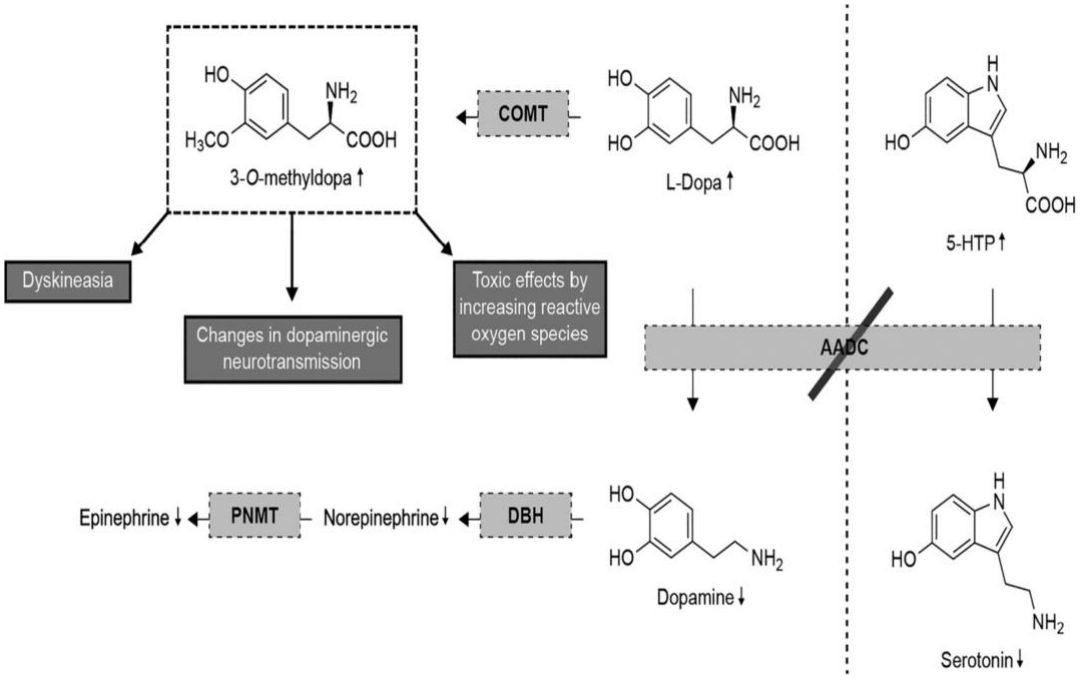

Figure 6. Metabolic pathway of dopamine and serotonin synthesis. L-Dopa is decarboxylated to generate dopamine by aromatic l-amino acid decarboxylase. In AADC deficiency, l-Dopa concentrations are increased leading to

substantial methylation of l-Dopa by catechol-O-methyltransferase (COMT) to 3-O-methyldopa (3-OMD). AADC, aromatic l-amino acid decarboxylase; COMT, catechol-O-methyltransferase; DBH, dopamine beta-hydroxylase; PNMT, phenylethanolamine- N-methyltransferase; 3-OMD, 3-O-methyldopa; 5-HTP, 5-hydroxytryptophan. (Brennenstuhl et al., - 2020). (44)

Due to the similarities and overlapping signs and symptoms between AADC deficiency and other diseases, misdiagnosis and delayed diagnosis are common. Although the onset of AADCd occurs within the first few months of life (mean age: 2.7 months), diagnosis is often delayed (mean age: 3.5 years). (1)

In 2014, *Chen et. al* developed a method to detect 3-OMD concentrations in dried blood spots (DBS) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and demonstrated that, given the stability of 3-OMD in DBS, this method can be used for both high risk and newborn screening of AADCd. The method is quantitative and has high sensitivity and reliability. They analysed 3-OMD concentrations in normal newborns and children and observed a decrease in 3-OMD concentrations with increasing age. In patients with AADC deficiency 3-OMD concentrations were significantly increased (mean 1,113 ng/ml; range 530-2,430 ng/ml), furthermore in older patients the 3-OMD levels were lower than in younger patients. (43)

Chien et al. conducted a newborn screening (NBS) program for AADC deficiency during the period from September 2013 to December 2015. In Taiwan, 127,987 newborns were screened for AADC deficiency using the methods proposed previously by *Chen et al.* The concentrations of 3-OMD in DBS were detected by LC-MS/MS. The mean 3-OMD concentration detected was 88.08 ng/mL, and four subjects had 3-OMD concentrations above 500 ng/mL. The four screening-positive patients had confirmatory testing: medical history, neurological and physical examinations, and *DDC* gene sequencing. Two pathogenic mutations in the *DDC* gene were detected in all four patients, leading to the diagnosis of AADC deficiency. Three of the four patients had the homozygous IVS6 + 4A> T mutations and manifested a severe phenotype, while one had the heterozygous compound mutation. The diagnosis of AADC deficiency was confirmed in all four

subjects. The incidence of AADC deficiency in Taiwan was 1:32,000. Noteworthy, the newborn screening method for AADC deficiency is rapid, non-invasive, and simple and 3-OMD in DBS is a convenient biomarker. (5)

Brennenstuhl et al. published a new method based on tandem mass spectrometry with an electrospray source (ESI-MS/MS) for the analysis of 3-OMD concentrations on DBS. In July and August 2019, after obtaining parental consent, 38,888 newborns were screened for AADCd. The novel method was also used to measure 3-OMD concentrations in the DBS filter cards of 7 patients with a confirmed diagnosis of AADCd; a retrospective analysis was performed on the original NBS filter card of one patient with AADC deficiency. Measurement of 3-OMD concentrations was also performed in 1,079 non-AADCd control subjects, and in fourteen asymptomatic heterozygous carriers of *DDC* variants. A mean 3-OMD concentration of 1.16 µmol/L was found in DBS samples from newborns screened for AADCd. Retrospective analysis of the original filter card of the patient diagnosed with AADCd, after repeated measurements, revealed a mean concentration of 3-OMD of 35.95 µmol/L; this showed that in AADCd patients the concentration of 3-OMD was highly increased and that 3-OMD was a valid and reliable biomarker for early diagnosis of AADC deficiency. The mean concentration of 3-OMD in heterozygous carriers was 0.69 µmol/L, indicating that heterozygous carriers were not detected by the screening test. The analysis of the samples from 1,079 healthy subjects, showed a mean 3-OMD concentration of 0.78 µmol/L and found that 3-OMD concentrations decreased with age and that 3-OMD levels were not gender dependent. The study also revealed that the mother's intake of a medical therapy that acts on the dopaminergic system (e.g., L-Dopa/carbidopa) could influence the concentration of 3-OMD in the newborn DBS samples, leading to false-positive screening results. (44)

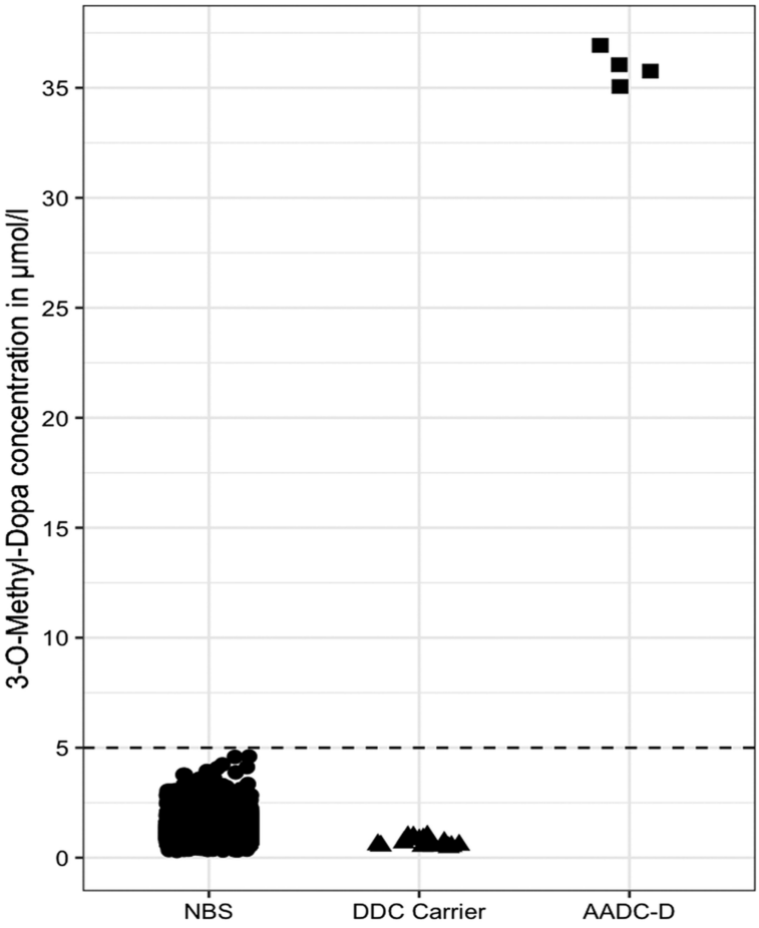

Figure 7. 3-OMD concentration in DBS samples of 38 888 newborns, DBS samples of 14 adult carriers and one NBS sample of an AADC deficiency patient. Measurement of 3-OMD in non-AADC deficiency newborns, carriers of monoallelic DDC variants, and one NBS DBS sample of a patient with AADC deficiency. The patient revealed a mean 3-OMD concentration of 35.95 µmol/L, displayed are four independent measurements of the same DBS filter card. 3-OMD, 3-O-methyldopa; AADC, aromatic l-amino-acid decarboxylase; DBS, dried blood spots; NBS, newborn screening. (Brennenstuhl et al., - 2020). (44)

This is the first described method for high-throughput screening of AADC deficiency; the method is valid, simple, rapid, and non-invasive. Compared with the LC-MS/MS method proposed previously by *Chen et al.*, the new ESI-MS/MS method reduces the measurement time per sample. The novel ESI-MS/MS method can be included in existing workflows for high-throughput newborn screening, leading to the early detection of AADC deficiency by measuring the concentration of 3-OMD in DBSs. In case of increased 3-OMD concentration,

confirmatory diagnostic tests (enzymatic assay or genetic testing) should be performed. (43) (44)

The methods developed by *Chen et al.* and by *Brennenstuhl et al.* are complicated by the use of deuterated 3-OMD-d3 as the internal standard. Burlina and colleagues, in 2021, described a method, flow-injection analysis tandem mass spectrometry (FIA-MS/MS), using the labelled tyrosine (^{13}C6-Tyr) as an internal standard, which is already part of the reagents routinely used in expanded newborn screening. They proposed a rapid, specific, and quantitative method to measure 3-OMD concentrations in newborn DBS samples, on the same cards routinely used in newborn screening. Samples from 1,000 healthy newborns were analysed and the 3-OMD mean concentration was 1.33 µmol/L. The method was also used for retrospective measurement of 3-OMD concentration in a patient with confirmed AADCd and was correctly identified (3-OMD concentration 10.51 µmol/L). In non-AADCd controls, the mean 3-OMD concentration was 1.19 µmol/L. The pilot project was conducted from April 2020 to November 2020 at the University Hospital of Padua, and measurement of 3-OMD concentration was performed in 21,867 DBSs. They did not identify patients with AADC deficiency, but the sample of a screened newborn revealed a high concentration of 3-OMD correlated with the mother's intake of L-Dopa. Notwithstanding, *Burlina et al.* demonstrated a novel method based on the combination of flow-injection analysis tandem mass spectrometry and NeoBase™ 2 kit reagents. ^{13}C6-tyrosine, already used for routine expanded newborn screening, was used as an internal standard. In case of positive screening results, LC-MS/MS with 3-OMD-d3 was performed as a second-tier test. The use of ^{13}C6-tyrosine as an internal standard, instead of 3-OMD-d3, makes this new method easily implementable in current NBS workflows. The study showed that due to the advantages of the newly published method, the measurement of 3-OMD in DBS samples could be implemented in expanded newborn screening, allowing early diagnosis of AADC deficiency and the subsequent administration of timely treatment. (45)

3. TREATMENT

3.1 Medical treatment

To date, first-line therapy includes dopamine agonists, MAO inhibitors, and vitamin B6. Anticholinergic drugs, melatonin, and benzodiazepines, on the other hand, are used as symptomatic therapy. (1) (3)

FIRST LINE TREATMENT

Dopamine agonists

Dopamine agonists act by directly activating postsynaptic dopamine receptors and include ergot-derived and non-ergot derived dopamine agonists:

- Ergot-derived dopamine agonists with strong serotoninergic (5HT2b) agonist action (pergolide and cabergoline)
- Ergot-derived dopamine agonists without 5HT2b agonist action (bromocriptine)
- Non-ergot derived dopamine agonists (pramipexole, ropinirole, and rotigotine)

Cabergoline and pergolide should not be used for the treatment of AADC deficiency; in fact, they are associated with high-risk fibrotic complications (fibrotic cardiac valvulopathy, pleuropulmonary and retroperitoneal fibrosis). Fibrotic changes in cardiac valve leaflets result in incomplete leaflet coaptation and clinically significant regurgitation. This is potentially explained by the high affinity of cabergoline and pergolide for 5-HT2b serotonin receptors on cardiac valvular fibroblasts, which mediates fibroblast proliferation. (1) (46)

Bromocriptine has a lower risk, but dose-dependent retroperitoneal, pulmonary, and (peri)cardiac fibrosis have been reported. (47)

Dopamine agonists should be tried for the treatment of AADCd, particularly non-ergot derived dopamine agonists (pramipexole, ropinirole, and rotigotine) are

preferred, because of their probably very low risk of fibrotic complications.(48) Treatment benefits outweigh side effects; in particular, improvements in head control, hypotonia, OGCs, voluntary movements, and autonomic symptoms were observed for bromocriptine, pramipexole, rotigotine, and pergolide. On the other hand, the main side effects observed include irritability, progressive weight loss, worsening of failure to thrive, vomiting, and dyskinesia. (1) (49)

MAO inhibitors

MAO inhibitors increase the availability of monoamines by preventing their breakdown. Improvement in at least one clinical endpoint (e.g., hypotonia) was observed with no effect on the others; all patients described were taking co-treatment with dopamine agonists and/or pyridoxine. Although the evidence of clinical benefit is low, from a biochemical perspective the recommendation to administer MAO inhibitors to patients with AADCd is strong. (1) (10)

Pyridoxine/pyridoxal phosphate

Vitamin B6 is available in the form of pyridoxine and pyridoxal phosphate (PLP); PLP is the active form of pyridoxine and the cofactor of the AADC enzyme, these drugs might increase and optimize the residual activity of AADC. Pyridoxine is the most frequently administrated drug because it is cheaper and more readily available. Favourable or unclear responses have been observed. Side effects such as gastrointestinal and sleeping disturbances and extreme motor restlessness have been described in patients taking very high doses of pyridoxine and co-treating with L-DOPA. Pyridoxine and PLP administrated in high doses and for a long time can cause reversible polyneuropathy. Dose limits should be respected, and if pyridoxine is not tolerated, PLP can be tried. (1) (28)

ADDITIONAL SYMPTOMATIC TREATMENT

Additional symptomatic therapy mainly includes anticholinergic agents, melatonin, and benzodiazepines. Specifically, anticholinergic agents are used for autonomic symptoms and movement disorders, melatonin is used for sleep disturbances, and benzodiazepines for movement disorders. (1)

Anticholinergic drugs

Anticholinergic drugs can be considered for additional symptomatic treatment of autonomic symptoms, dystonia, and OGCs. The precise mechanism of action is not known, but they are believed to act on the relative imbalance between dopaminergic and cholinergic pathways. All patients treated with anticholinergic drugs received concurrent medications and, in most cases, improvement in at least one clinical endpoint (e.g., hypotonia, excessive sweating, dystonia) was reported. (1) (3)

Melatonin

Evidence on melatonin administration for the treatment of sleep disorders in patients with AADC deficiency is limited. Many patients with AADCd experience sleep disturbances. Melatonin may be decreased, considering that it is derived from serotonin; therefore, from a pathophysiological point of view, melatonin supplementation is reasonable. Transient episodes of night terrors have been observed but have not been published. (49)

Melatonin should be considered in patients with AADC deficiency for the treatment of sleep disturbances. (1)

Benzodiazepines

Evidence on the use of benzodiazepines in patients with AADC deficiency is very limited. Intermittent use of benzodiazepines can be considered in sustained oculogyric or dystonic crises. (1) (3)

Others

For the treatment of nasal congestion, the value of alpha-adrenoreceptor nose drops, which result in local vasoconstriction, is evident in clinical practice. In addition, clonidine can be used to treat irritability and sleep disturbances. (1)

To summarize, first-line treatment includes selective dopamine agonists, MAO inhibitors, and pyridoxine, while anticholinergic agents, melatonin, and

benzodiazepines are used as additional symptomatic therapy. Polytherapy is usually required, and doses should be titrated individually and sequentially. (1) (8)

DRUGS TO AVOID

Centrally acting dopamine antagonists, which have antiemetic and antipsychotic properties, should be avoided in patients with AADC deficiency because of the potential worsening of dopamine deficiency symptoms. For the treatment of nausea and vomiting in patients with AADCd, supportive care to avoid dehydration and hypoglycaemia should be optimal, while low-dose domperidone can be considered if medical therapy is needed. (1)

3.2 Complications and follow-up

Cardiac complications

Although, from a pathophysiological point of view, structural cardiac abnormalities are not expected in patients with AADCd, catecholamine deficiency, and autonomic dysfunction may lead to cardiac complications, which may be more evident during illness or stress (e.g., infections, surgical interventions). (1)

One patient was found to have bradycardia on ECG (29), and one patient, at age 9, had an unexplained witnessed cardiac arrest that led to permanent neurological damage (50). One AADCd patient developed serious heart rhythm disturbances during intravenous administration of dopamine and norepinephrine, subsequently spontaneous atrial fibrillation without intravenous catecholamines administration was reported in the same patient, thus patients with AADC deficiency could potentially develop serious spontaneous or stimulus-induced heart rhythm disturbances (51).

Because cardiac complications are possible in potentially stressful situations, cardiac monitoring is recommended in these cases and cardiac screening (clinical evaluation, ECG, and echocardiogram) is recommended before any anaesthesia or intervention, while regular follow-up cardiac screening is not necessary. Furthermore, because of the potential risk of cardiac fibrosis, cardiac screening is indicated before and during bromocriptine treatment. (1)

Orthopaedic complications

Orthopaedic monitoring (e.g., hip and spine X-rays) and a multidisciplinary approach for follow-up and treatment are recommended in AADCd patients, considering that orthopaedic complications, due to severe motor impairment, have been described. (1)

Infections

Impaired feeding and swallowing, reduced mobility, and frequent hospitalization increase the risk of infection in patients with AADC deficiency; in addiction, AADCd patients have a reduced response to the stress of infections, which can be fatal. Therefore, monitoring during infections and vaccinations following local vaccination programs are recommended. (1)

Dystonic crisis

Dystonic crisis, a rare and potentially life-treating complication, consists of severe and sustained muscle contractions, which can lead to airway compromise and rhabdomyolysis, resulting in acute renal failure. Infections and medication adjustments are the main triggers. Because this is a potentially life-treating complication it should be treated promptly; management of a dystonic crisis involves prompt admission to an intensive care unit, fluid and nutrition supplementation, sedation, and possibly respiratory support. (1) (52)

Follow-up visits by the child neurologist should be at least annually, ideally in a multidisciplinary setting. (1) Experts agreed on the need to use standardized and age-appropriate scales to evaluate cognitive and neuropsychological functions,

for the characterization of the movement disorder phenotype, in follow-up to evaluate response to treatment, and cognitive and motor outcomes. All also agreed on the need for a multidisciplinary approach in follow-up. (3)

3.3 Gene therapy

According to *Rizzi et al.*, dopamine agonists and MAO inhibitors provided benefits in slightly more than half of patients, while vitamin B6 provided benefits in only 38% of patients. Pharmacological treatment is unsatisfactory, the success of medical therapy (usually based on combinations of vitamin B6, dopamine agonists, and MAO inhibitors) is very limited, response to therapy is better in the mild and moderate phenotype than in the severe phenotype, and adverse effects, especially for dopamine agonists, often lead to dose limitation and discontinuation of treatment. (2) (8)

From Parkinson's disease to AADC deficiency

AADC deficiency and Parkinson's disease (PD) are two debilitating neurological disorders, in which dopamine deficiency causes motor disturbances. Since dysfunction of the dopaminergic system is involved in both PD and AADCd, and the problem is relatively confined to specific brain areas, intraparenchymal infusion of gene therapy into precise target regions by stereotactic surgery, can act directly on the underlying causes of the diseases and represent a real cure. The putamen constitutes a prime target for gene therapy, as it is directly influenced by the loss of dopamine biosynthesis in the striatum, it is also a large structure in the forebrain and therefore more easily accessible through surgery. (53)

The basal ganglia consist of the dorsal striatum (caudate nucleus, putamen), ventral striatum (nucleus accumbens and olfactory tubercle), globus pallidus, ventral pallidum, substantia nigra (SN), and subthalamic nucleus. The basal ganglia do not have an exclusively motor function, but their role is more complex: they are involved in the full range of goal-directed behaviours through movement,

and in the process that guides action, including emotions, cognition, and motivation. The putamen receives input from the premotor and motor cortices of the frontal lobe, primary and secondary somatic sensory cortices of the parietal lobe, the secondary visual cortices of the occipital and temporal lobes, and the auditory association areas of the temporal lobe. The putamen also receives dopaminergic inputs from substantia nigra pars compacta (SNpc). The γ-aminobutyric acid (GABA)-ergic medium spiny neurons (MSNs) are the main source of the basal ganglia output and form the connection between the putamen and cerebral cortex via the substantia nigra pars reticulata (SNpr), globus pallidus, and thalamus. Dopamine modulates the output of MSNs through the activation of D1 or D2 receptors. Therefore, dopamine has a neuromodulatory effect on motor, cognitive, and limbic functions. The circuit starts from multiple cortical areas and ends in the motor areas of the frontal lobe. (53) (54)

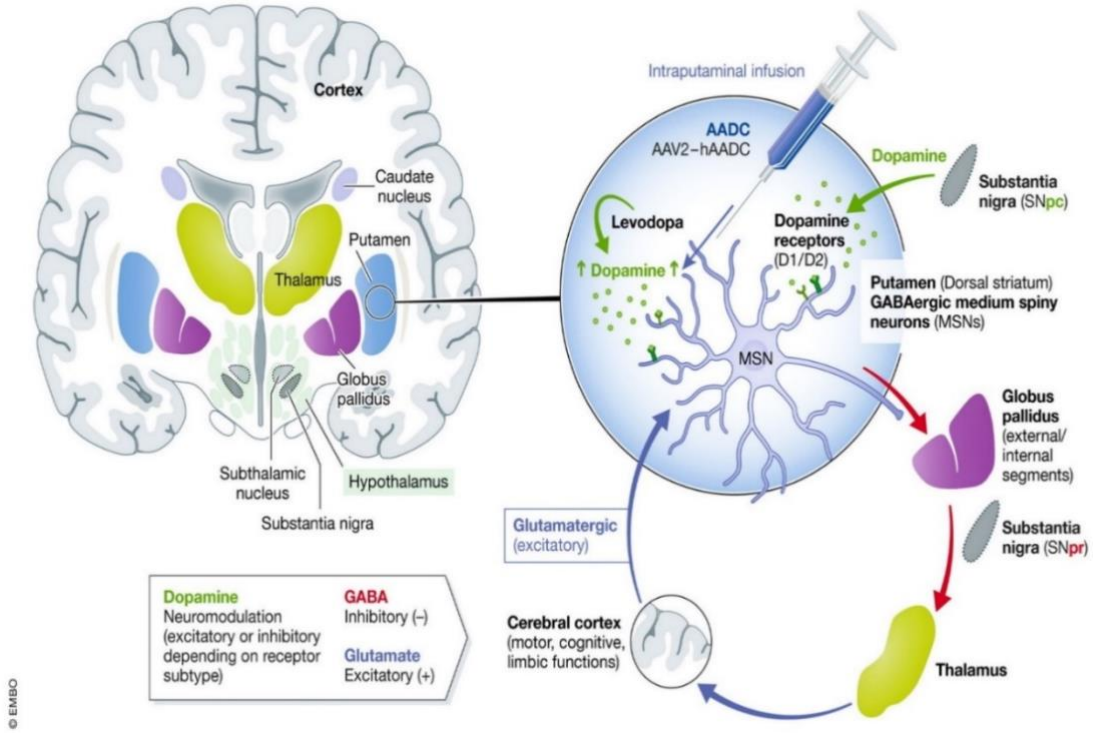

Figure 8 Simplified circuit diagram illustrating the key afferent and efferent connections of the dorsal striatum (putamen) and their role in control of motor, cognitive, and limbic functions. Dopaminergic inputs to the putamen originate from the SNpc. The dopaminergic terminals release dopamine, which modulates the output of the postsynaptic MSNs in the putamen via D1 or D2 receptor activation. MSNs connect with different parts of the cerebral cortex indirectly via their connections with other basal ganglia nuclei (globus pallidus and SNpr) and thalamus. By exerting their inhibitory effects via these indirect connections (cortical and subcortical loops), the MSNs of the putamen

control various functions (motor, cognitive, and limbic). Hence, dopamine, by modulating MSN function, exerts an important neuromodulatory effect on motor, cognitive, and limbic functions. The seat of this neuromodulation is in the striatum (caudate nucleus and putamen). MSN, medium spiny neuron; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata. (Hwu et al. – 2021). (53)

Because of the similar aspects of PD and AADCd pathophysiology, studies conducted on intraputaminal infusion of gene therapy in PD patients laid the principle for the development of gene therapy of AADCd. Gene therapy is based on intraputaminal infusion of a viral vector, adeno-associated virus type 2 (AAV2), which provides a functional copy of the human *DDC* gene (hAADC), encoding for the AADC enzyme. The rationale for gene therapy is to increase the conversion of L-DOPA to dopamine by delivering a copy of the human *DDC* gene directly to the striatal regions involved in the disease. PD patients are treated with L-DOPA. In PD, due to disease progression, the gradual and progressive degradation of dopaminergic neurons in the SNpc leads to a progressive decline in AADC enzyme levels, resulting in the administration of higher doses of L-DOPA to maintain the desired clinical benefits of therapy. Whereas in AADCd, the lack of function of the AADC enzyme, due to inherited mutations in the *DDC* gene, leads to deficiencies of dopamine, serotonin, norepinephrine, and epinephrine. (53)

Gene therapy was originally and first developed for Parkinson's disease patients, who received bilateral intraputaminal infusion of viral vector (adeno-associated virus type 2) containing the human AADC gene. In PD, degeneration of nigrostriatal neurons leads to declining levels of the AADC enzyme, which converts L-DOPA to dopamine. The progressive loss of AADC may explain why many patients with Parkinson's disease, after an initial improvement in the early and moderate stages of PD with L-DOPA administration, experience a reduction in benefit and require higher doses of L-DOPA to maintain the desired clinical response. Studies demonstrated the long-term safety and efficacy of the gene therapy, the surgical procedure was well tolerated, AADC enzyme activity increased and there was an improvement in the mean scores on the Unified Parkinson's Disease Rating Scale. (55) (56) (57) (58)

PD treatment is based on the administration of exogenous L-DOPA, whereas patients with AADCd, due to the lack of the AADC enzyme, do not convert L-DOPA to dopamine, resulting in high concentrations of endogenous L-DOPA. In AADC deficiency, gene therapy, based on intraputaminal infusion of the human AADC gene, aims to convert the endogenous L-DOPA to dopamine. (53)

Three different clinical trials examined the efficacy and safety of intraputaminal bilateral infusion of AAV2 gene therapy in patients with AADC deficiency. (59) (60) (61)

The viral vector (adeno-associated virus type 2) carrying the human AADC gene was injected bilaterally, via stereotactic surgery, into the putamen of four patients (aged 4 to 6 years) with AADC deficiency for the first time, in 2012, in a phase 1 clinical trial conducted by Hwu and colleagues. Before receiving gene therapy, all four patients had not achieved head control, were bedridden, unable to speak, and had frequent OGCs. Improvement in motor function was observed in all patients, evidenced by increases in raw scores on the Peabody Developmental Motor Scale (PDMS-2) and the Alberta Infant Motor Scale (AIMS). One patient achieved standing position 16 months after gene therapy, while the other 3 patients were able to achieve supported sitting position 6 to 15 months after receiving gene therapy. The Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) scores increased after gene therapy, indicating motor and cognitive improvement. A reduction in OGCs severity was observed, as well as improvements in behavioural stability, sweating, and hyperthermia. Concentrations of Homovanillic acid (HVA) in CSF increased. In positron emission tomography (PET) scans performed 6 months after gene therapy, the putaminal uptake of 6-[^{18}F]-fluoro-L-DOPA, tracer for AADC, increased from baseline. The most common adverse event was transient choreic dyskinesia, which resolved after several months. The study showed that gene therapy is well tolerated and results in improvements especially in motor functions. (59)

After the first compassionate use of AAV2 gene therapy in four AADCd patients in 2012, *Chien et al.* learned how to control the adverse events of gene therapy by medication and perform stereotactic brain surgery in young patients, and in

2017 published an open-label phase 1/2 trial. They treated ten patients (aged 1.7 to 8.4 years) with a diagnosis of AADCd and symptoms of the disease, enrolled from October 2014 to December 2015, with bilateral intraputaminal injections of AAV2-hAADC via stereotactic brain surgery. None of the ten patients achieved full head control before enrolment and, in all patients, very low concentrations of homovanillic acid (HVA) and 5-hydroxyindoleacetic (5-HIAA) were detected in CSF, as well as increased DBS 3-OMD concentrations. Prior to treatment, all patients had very low raw scores on Peabody Developmental Motor Scale (PDMS-2) and Alberta Infant Motor Scale (AIMS). The purpose of the study was to establish the efficacy and safety of intraputaminal gene therapy (AAV2-hAADC). The primary efficacy outcomes were an increase in PDMS-2 score greater than 10 points, and an increase in HVA or 5-HIAA concentrations in CSF, 12 months after gene therapy. All patients achieved the primary efficacy endpoint; 12 months after gene therapy, PDMS-2 scores increased, and HVA concentrations in CSF increased, while no significant change in 5-HIAA concentrations was found. In addition, AIMS scores significantly increased 12 months after the gene therapy, as well as cognitive and language subscales of Bayley-III improved. Before gene therapy, PET scans with 6-[^{18}F]-fluoro-L-DOPA (FDOPA) of all patients showed barely visible or completely absent uptake in the putamen. In the nine patients who completed PET scans 12 months after gene therapy, the uptake in the putamen was clearly visible. Considering that FDOPA is the tracer for AADC, the specific FDOPA uptake demonstrates the activity of the AADC enzyme and thus the conversion of L-DOPA to dopamine. The most common adverse events observed were pyrexia and orofacial dyskinesia. All patients experienced transient dyskinesia after gene therapy, which resolved with risperidone. Only one treatment-related adverse event was of severe intensity, but none resulted in hospitalization or death. Therefore, the study, conducted in a larger and younger population, demonstrated the efficacy and safety of intraputaminal gene therapy (AAV2-hAADC) for the treatment of patients with AADC deficiency. Post-hoc analysis revealed that after gene therapy, HVA concentrations in CSF were higher in younger patients (< 3 years) and the

functional recovery was better than in older patients. Early administration of the gene therapy might lead to better benefits. (60)

In 2019, *Kojima et al.* published an additional study on gene therapy in patients with AADCd; they enrolled six patients (aged 4-19 years) from May 2015 to July 2017 and conducted an open-label phase 1/2 trial. Compared with the Taiwanese studies conducted by *Hwu et. al* and *Chien et. al*, the six patients enrolled had a variable genetic background, one had moderate phenotype, and four patients were older; Kojima and colleagues also used a similar AAV type 2 vector carrying hAADC (AAV-hAADC-2). The primary objective was to verify the safety of the gene therapy, while the secondary objective was to evaluate the clinical response. The five patients with severe phenotype were bedridden and unable to make voluntary movements, had OGCs and generalized dystonic attacks, were unable to speak but understood simple words, and mental status was relatively preserved, while the only one with a moderate phenotype walked with support. Bilateral intraputaminal injection of the viral vector was performed by stereotactic surgery. After gene transfer, improvement in motor function was observed in all patients. Patients with severe phenotype achieved head control and were able to make voluntary movements, two patients achieved standing position with support, and one patient walked with a walker. A marked improvement was observed in the patient with moderate phenotype, being able to walk independently and ride a bicycle. A gradual increase in AIMS scores was shown in all patients. In addition, dystonia attacks disappeared and OGCs decreased in all patients. The patient with moderate phenotype also became able to speak fluently and perform simple arithmetic, showing improvement in cognitive and verbal functions. The study revealed a superior improvement in younger patients compared with older patients with the same or similar mutation, in patients with missense mutation the improvement was earlier than in patients with frameshift IVS6+4A>T mutation, and in patient with moderate phenotype an improvement in cognitive and verbal functions was observed. PET scans with 6-[^{18}F]-fluoro-L-m-tyrosine (FMT, specific tracer for AADC) were performed and showed a persistent and bilateral increase in FMT uptake in the putamen, maintained at 2 years after treatment. All patients manifested transient choreic dyskinesia, which

resolved within a few months. In one patient asymptomatic subdural haemorrhage was detected on brain CT scan. The study provided independent confirmation of the safety, tolerability, and potential efficacy of gene therapy in patients with AADC deficiency. (61)

Summary: In all these studies, bilateral intraputaminal infusion of low doses of AAV2-hAADC (1.8×10^{11} vg or 2×10^{11} vg) was performed in patients of various ages with AADC deficiency. After receiving gene therapy, improvements in motor development, OGCs, and emotional stability were observed in all patients, demonstrating sustained improvements in motor and non-motor symptoms. PET scans showed increased bilateral putaminal uptake of AADC tracers, as well as increased concentrations of dopamine and serotonin metabolites in CSF, demonstrating restoration of dopamine synthesis in the putamen. Regarding adverse events, transient dyskinesia and pyrexia were common; all patients experienced transient dyskinesia, which resolved after several months. One patient had an asymptomatic subdural haemorrhage, and apnoeic episodes were described in one patient, which resolved within months. Hence, the procedure was well tolerated. (53) (59) (60) (61)

The main mechanism of action appeared to be the direct transduction of MSNs in the putamen and consequent AADC production. (53) (55) Animal studies have shown that the neurotropic AAV-2 vector, infused into the striatum, primarily targets the MSNs, which subsequently express AADC, replacing the degenerating nigra afferents in PD. L-DOPA is administrated systemically and enters MSNs, probably through neutral amino acid transporter. The mechanism of dopamine release has not been fully characterized, however, microdialysis experiments conducted in L-DOPA-treated parkinsonian rodents have shown that MSNs released dopamine. In AADCd patients, AAV2-hAADC infusion allows endogenous L-DOPA to be used to synthesize dopamine. Dopamine could leak into the extracellular space and act on dopamine receptors or activate intracellular dopamine signalling. (53) (55) (62)

The striatum also contains bienzymatic non dopaminergic neurons that, expressing tyrosine hydroxylase (TH) and AADC, can produce dopamine under

normal conditions, and monoenzymatic nondopaminergic neurons, expressing TH or AADC. After infusion of AADC gene therapy, the monoenzymatic neurons that express only TH could gain new functions by producing AADC and then dopamine, while the AADC-producing monoenzymatic neurons could become active and restore dopamine production. (53) (63)

AADC gene therapy leads to motor improvement; the basis of the therapeutic effects is persistent dopaminergic restoration in the prefrontal cortico-putaminal network, demonstrated by *Onuki et al.*, in 2021. The human prefrontal cortex is primarily affected by AADC deficiency, and the study suggested that putaminal dopamine promotes the development of the immature motor control system, particularly in the prefrontal cortex. The prefrontal area belongs to the fronto-parietal control network, which contributes to cognitive-motor control function (motor initiation and planning). PET scans with 6-[^{18}F]-fluoro-L-m-tyrosine (FMT) performed in 8 patients with AADC deficiency, after receiving AADC gene therapy in the bilateral putamen in an open-label phase 1/2 study, showed that FMT uptake increased in the putamen over years. In PD, AADC gene therapy promotes the functional recovery of a well-developed motor system. (53) (64)

Eladocagene Exuparvovec: first approved disease-modifying treatment

Eladocagene Exuparvovec (Upstanza™), was approved on July 20, 2022, in the EU, for the treatment of patients aged 18 months and older with a clinical, molecular, and genetically confirmed diagnosis of AADC deficiency with a severe phenotype. (42)

Eladocagene Exuparvovec is a gene therapy and the first approved disease-modifying treatment for AADC deficiency. It is a non-replicating recombinant AAV2 based vector containing the cDNA of the human *DDC* gene under the control of the cytomegalovirus immediate-early promoter. Eladocagene exuparvovec is produced in human embryonic kidney cells by recombinant DNA technology. Gene therapy is a one-time treatment, in which the functioning *DDC* gene is delivered directly into the putamen through stereotactic surgery resulting

in the expression of the AADC enzyme, the restoration of dopamine synthesis, and the subsequent motor improvement. The AADC enzyme could be expressed through the transduction of medium spiny neurons and/or monoenzymatic/dienzimatic neurons in the putamen. (42) (65)

Specifically, patients receive a total one-time dose of 1.8×10^{11} vg delivered as four 0.08 mL (0.45×10^{11} vg) infusions directly into the putamen during a single minimally invasive stereotactic neurosurgical procedure. It should be administrated in a specialised stereotactic neurosurgery centre by a qualified neurosurgeon and should only be infused with the SmartFlow® ventricular cannula. (65) (66)

After the procedure, patients should be monitored closely. Exacerbations of symptoms of underlying AADC deficiency may occur. The risk of viral shedding is considered low; however, precautions for handling waste materials are recommended for 14 days after treatment. Patients should not donate blood, organs, tissues, and cells for transplantation. There was no evidence of detectable viral vector in urine or blood either at baseline or in the 12 months after treatment, because eladocagene exuparvovec is infused directly into the brain and does not distribute externally. (42) (65)

According to the clinical trials, after eladocagene exuparvovec treatment of patients with AADCd, key motor milestones were achieved, cognitive and communication skills, body weight, hypotonia, and dystonia improved, and OGCs frequency and duration were reduced. The primary endpoint was the number of patients who achieved PDSM-2 motor milestones 24 months after gene therapy: full head control, sitting unassisted, standing with support, and walking with support. In all patients treated with eladocagene exuparvovec total PDSM-2 scores improved compared with baseline; furthermore, higher final total PDSM-2 scores and more rapid response were observed in patients given eladocagene exuparvovec at a younger age. (65)

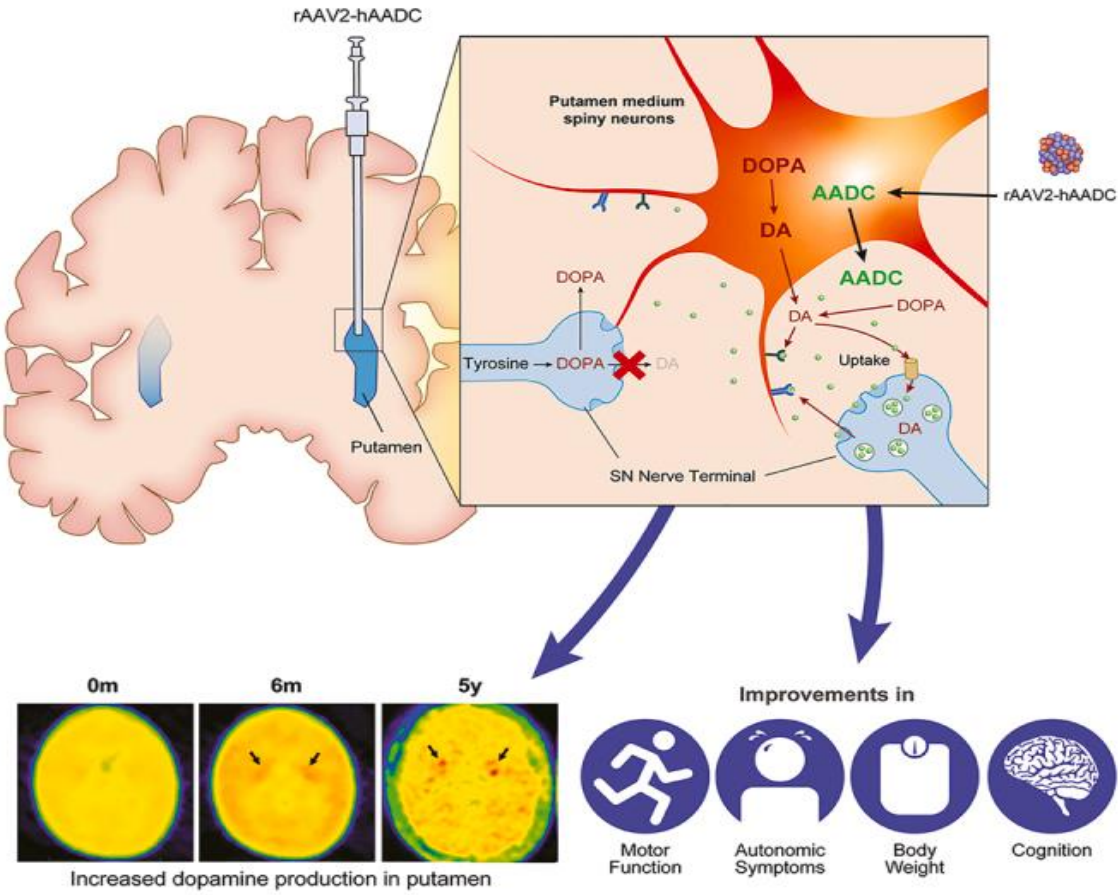

Figure 9. (Tai et al. – 2022). (67)

Tai et al., published the combined results of a long-term follow-up of the three eladocagene exuparvovec gene therapy trials. Eladocagene exuparvovec, a recombinant AAV2 vector containing the human aromatic L-amino acid decarboxylase gene (rAAV2-hAADC) was infused bilaterally into the putamen of a total of twenty-six patients with AADC deficiency, who had not achieved head control; the 26 patients completed 1-year evaluations. These analyses provided two important advancements: the results of the 1-year evaluations of 26 patients provided a better understanding of the potential connection between patient characteristics and dosage on outcomes; in addition, 11 of the 26 patients were followed for more than 5 years, allowing evaluation of long-term efficacy and safety. Motor and cognitive functions were assessed with PDMS-2, AIMS, CDIIT,

and Bayley-III scores; rapid motor and cognitive improvements were observed within 12 months after gene therapy with eladocagene exuparvovec and were maintained during follow-up for more than 5 years. Significant improvements in patient symptoms (mood, sweating, temperature, and OGCs) and growth, as well as in the quality of life of the caregivers, were also described after gene therapy. (67)

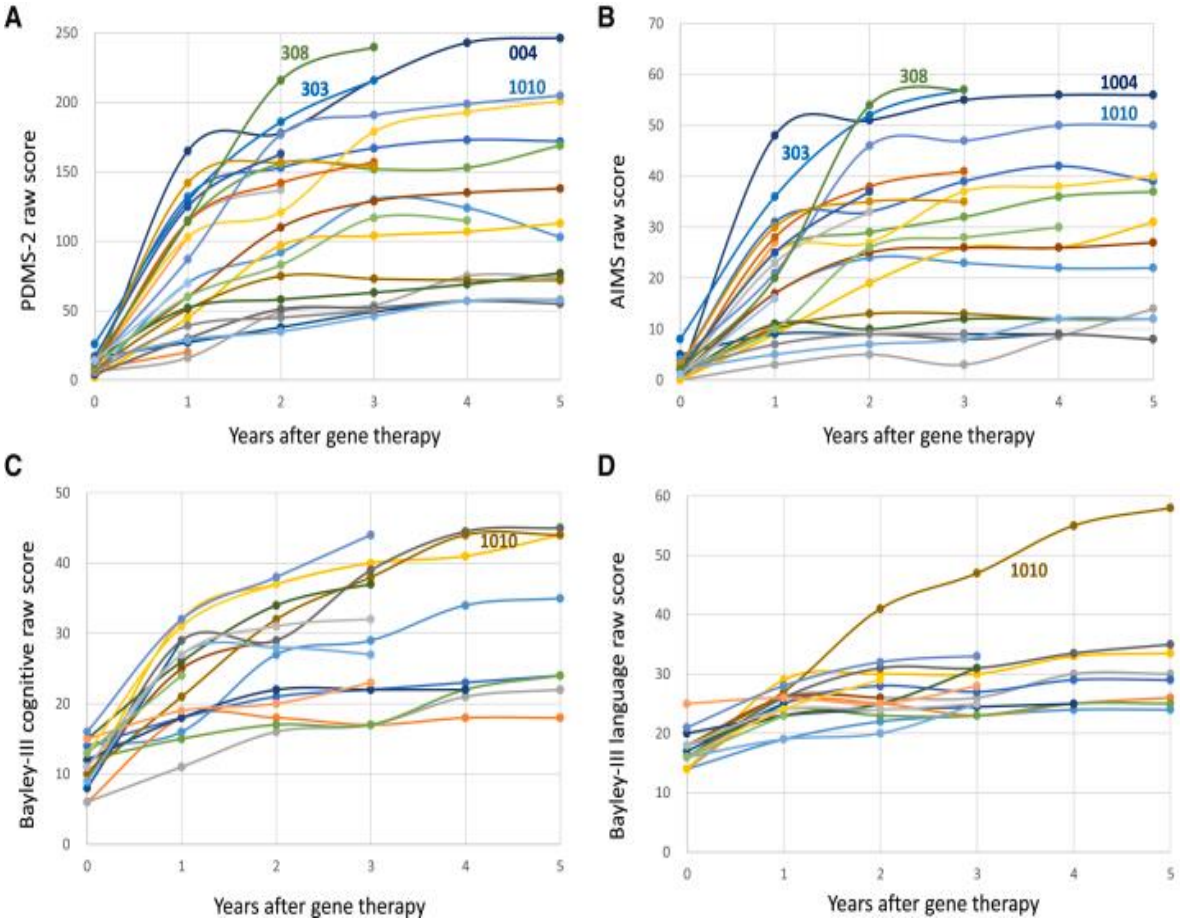

Figure 10. Improvements in developmental milestones after gene therapy. Patients were evaluated and data were plotted according to years after gene therapy. (A) All patients exhibited rapid increases in PDMS-2 score after gene therapy. Three patients who could walk without assistance are marked with patient numbers 303, 308, and 1004. (B) AIMS score. (C) Cognitive score of Bayley-III for the phase 1/2 and phase 2b patients. (D) Language score of Bayley-III; patient 1010 exhibited an extraordinary score (Tai et al. – 2022). (67)

PET scans with L-6-[^{18}F]-fluoro-3,4-dyhidroxiphenilanaline (^{18}F-DOPA), performed in patients with severe AADCd after eladocagene exuparvovec administration, showed a sustained increase in putaminal uptake compared with

baseline. Increased uptake of ^{18}F-DOPA was found as early as 6 months after treatment and was also evidenced 12 months, 2 and 5 years after treatment administration. PET data demonstrated the durability of the gene transduction effect at 5 years and were consistent with the durability of the motor milestones development. HVA levels in CSF before gene therapy were very low and 12 months after treatment with eladocagene exuparvovec were significantly increased compared with baseline, whereas 5-HIIA concentrations in CSF before and after gene therapy did not differ significantly. (65) (67)

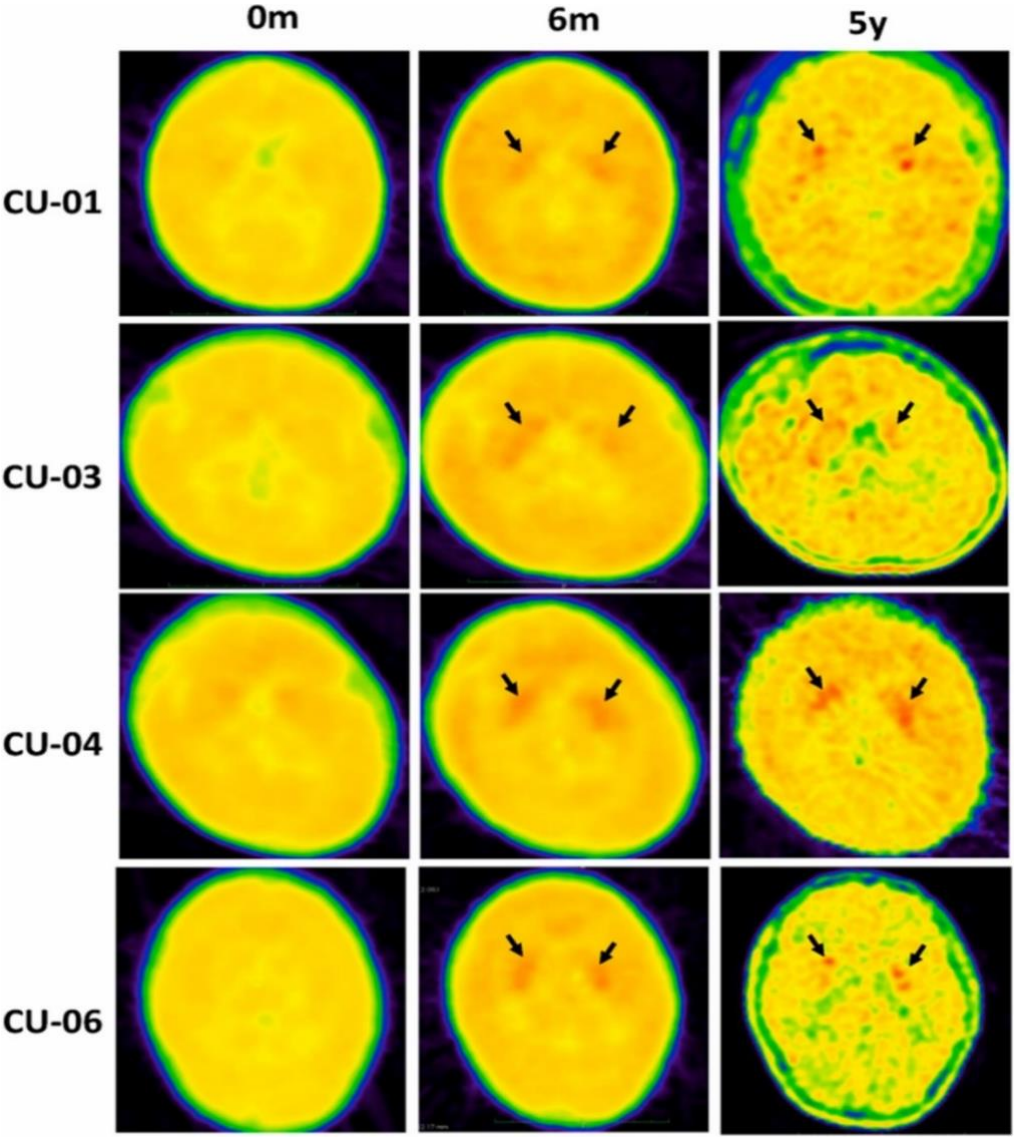

Figure 11. De novo dopamine production: visualized 18F-DOPA PET increases in four patients. Each row shows 18F-DOPA PET scans of the putamen at baseline (0 months), 6 months (except for patient CU-06 at 12 months), and 5 years. Black arrows indicate the observed signal. (Tai et al., - 2022). (67)

The analysis showed a significant correlation between age at the time of treatment and response to therapy as measured by the PDMS-2 scale at 1 year and 2 years after gene therapy; younger patients had faster and greater improvements, the increase in PDMS-2 total scores had a negative correlation with age, this is probably due to a higher degree of neuronal plasticity in younger patients. A strong correlation between the HVA levels in CSF after treatment and PDMS-2 scores was observed, suggesting that dopamine production enabled by the administered gene therapy resulted in improved motor function. A positive correlation between pre-treatment HVA levels and post-treatment PDMS-2 score was also observed, the presence of pre-treatment HVA may indicate a slight decrease in disease severity and, although not clinically recognizable, may be associated with better treatment outcomes, but data need further investigation.

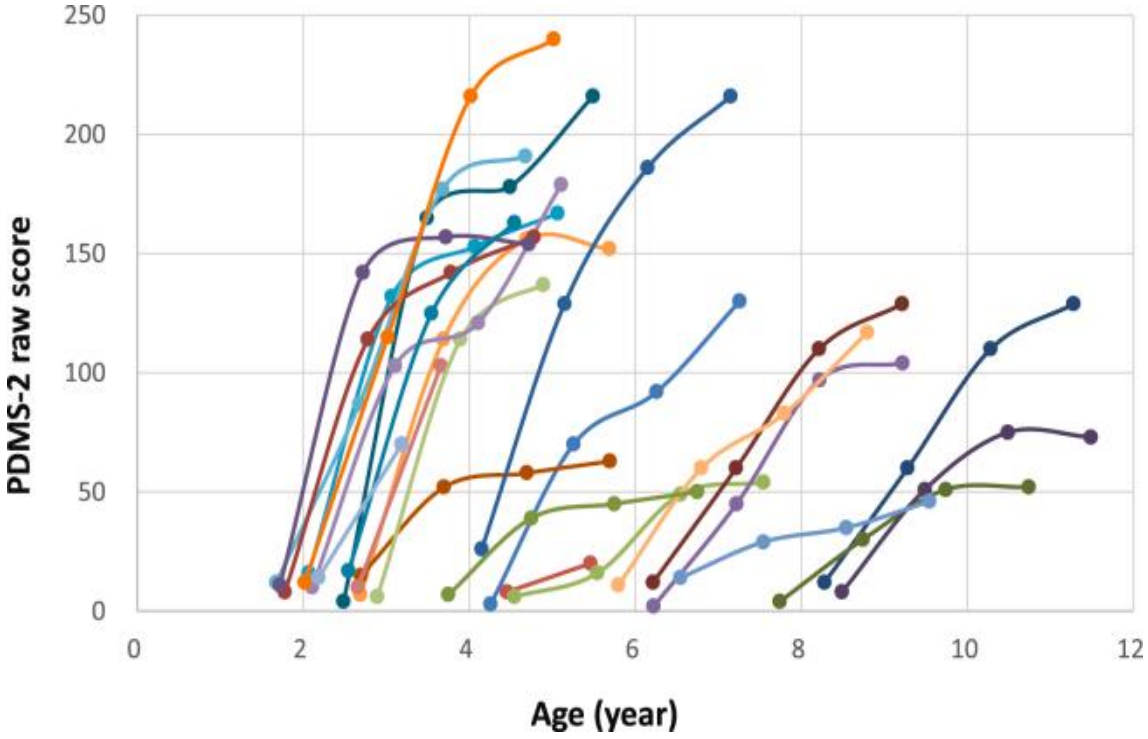

Figure 12. PDMS-2 score, by patient and chronological age. PDMS-2 scores of individual patients (N = 26) at baseline and 1 year after gene therapy. Each line graph shows PDMS-2 total score in each patient. The first data point for each patient indicates baseline score at the time of eladocagene exuparvovec administration. (Tai et al. – 2022). (67)

The study demonstrated the safety of eladocagene exuparvovec. Short- and long-term (≥5 years) tissue damage due to surgery was evaluated; MRI of one

patient, 7 years after treatment, showed evidence of tracts due to surgery, with no further tissue damage, and no treatment-associated brain injury occurred. At least one treatment-emergent adverse event (TEAE) occurred in all patients, the most common of which were pyrexia and dyskinesia. Dyskinesia was generally mild to moderate in severity, while it was more severe and prolonged in older treated patients, was not dose-related, appeared 4 weeks after treatment with a peak at 8 weeks, and resolved within a few months; only one dyskinesia event occurred more than 12 months after treatment. The dyskinesia events were transient and probably related to dopamine receptor hypersensitivity. Post-surgery complications, including CSF leakage, were observed in ten patients, which were managed with standard of care, and all resolved. The study demonstrated the safety and sustained efficacy of intraputaminal infusion of eladocagene exuparvovec in patients with AADCd; administration of gene therapy immediately after newborn screening could potentially cure the disease. (42) (67)

All patients, before treatment with eladocagene exuparvovec, had anti-AAV2 antibody titers ≤ 1:20, while most patients in the first 12 months after gene therapy administration had a positive anti-AAV2 antibody response, which stabilized or declined over time. Immune responses are not expected to affect localized brain gene therapy. No correlation was found between anti-AAV2 antibodies and decreased efficacy or increased severity or frequency of adverse events. (65) (67)

Correlation between age at the time of treatment and response to gene therapy has been shown in clinical trials. Early diagnosis is considered necessary to expect the best improvements from AAV vector-based gene therapy, even if it is not required to be eligible for AAV vector-based gene therapy. (3)

4. AIMS OF THE STUDY

Our study aimed at evaluating the real-life applicability of a new method for AADCd early identification on dried blood spots (DBSs) and, hence, the 3-O-methyldopa (3-OMD) reference levels in the Ligurian neonatal population, in view of setting-up reference values (cut-offs) for a regional AADCd newborn screening program.

5. PATIENTS AND METHODS

5.1 Patients' selection

Dried Blood Spots (DBSs) were collected in the Ligurian neonatal population, as part of the normal collection process for expanded newborn screening and were blindly analysed at LABSIEM – Neonatal Screening Centre part of the Paediatric Clinic and Endocrinology Unit of IRCCS Istituto Giannina Gaslini.

We included the samples collected under current regulations (Law 167/2016) between the first 48-72 hours of life, and, considering the samples collected according to the "special protocols" procedure, applied by our Centre (IRCSS Giannina Gaslini), we also included the first sample collected in each infant.

The "special protocols" procedure involves performing serial DBSs in case of special conditions, including:

- Infants weighing less than 1800 g and/or gestational age less than 38 weeks.
- Infants on parenteral/enteral nutrition and/or on carnitine/MCT therapy in the presence of antibiotic therapy.
- Infants undergoing transfusions.
- Non-feeding infants.

- Infants born to mothers on hormone replacement therapy for thyroid disease/cortisone therapy.
- Infants transferred to another inpatient ward and/or discharged before 48 hours.
- Infants with exitus in the first 48-72 hours.

5.2 Sample collection and method

A blood sample was taken by a puncture on the newborn's heel and applied directly on the Guthrie Card (Dried Blood Spot, DBS), according to current newborn screening procedures.

Sampling was performed between the first 48-72 hours of infants' life, except for special protocols. Infants had to have been fasting for at least three hours.

Blood was laid on the card and had to pass through the entire thickness of the paper in one direction only.

The card then had to air dry, avoiding blood contact with any surface for at least two hours before being placed in a plastic bag.

Only once dry, the card could be stored in the refrigerator inside a plastic bag.

Levels of 3-OMD on DBS were assayed by flow-injection analysis tandem mass spectrometry (FIA-MS/MS) using labelled tyrosine (^{13}C6-Tyr) as an internal standard, routinely used as a reagent in expanded newborn screening. The assay was based on the protocol routinely used for the expanded newborn screening program, employing the commercial kit already in use and supplementing the procedure. (45)

The spot was obtained by punching dried blood on the cards, using a Multi Puncher instrument (PerkinElmer, USA).

Spots with a diameter of 3.2 mm were subjected to extraction using the commercial "NeoBase 2 Non-derivatized MSMS kit" (PerkinElmer, USA) with

labelled tyrosine as the internal standard and were injected directly into the tandem mass spectrometry system (FIA-MS/MS).

To achieve the best sensitivity and signal-to-noise ratio, after incubation, 15 µl of the eluted samples were injected at a flow rate of 190 µl/min by direct injection. The source temperature was set at 260° C.

MRM mass transition (212.2/153.1 m/z Multiple Reaction Monitoring) specific to 3-OMD was simply added to the kit acquisition method without invalidating the IVD certification.

Parents/guardians of the infants were properly informed about the expanded newborn screening procedures and the storage of DBSs for 5 years for verifications related to the purpose of newborn screening, they did not express dissent. Parents/guardians gave written informed consent for prolonged storage of samples (total of 10 years) for clinical or research purposes.

5.3 Statistical analysis

Descriptive statistical analyses were performed to evaluate DBSs features, clinical characteristics of infants, and 3-OMD concentrations, with continuous data presented as mean – standard deviation (SD) or median (interquartile range [IQR]) as appropriate, and ordinal data expressed as number (percentage).

6. RESULTS

6.1 Assay validation

To validate the method of measuring 3-OMD concentration on DBS by flow-injection analysis-tandem mass spectrometry (FIA-MS/MS) using labelled tyrosine as an internal standard, we measured 3-OMD concentration on DBSs of a patient with a genetically confirmed diagnosis of AADC deficiency. In detail, we

retrospectively measured the 3-OMD concentration on neonatal DBS of the patient, collected by the patient's Birth Centre, and we also assayed 3-OMD concentration on the DBS collected at our Institute, at 2 years of age.

The patient was born from a physiological pregnancy. The gestational age at birth was 38 weeks + 6 days. The patient had a birth weight of 3,090 g, a length of 51 cm, and a head circumference of 34 cm. The Apgar score was 9/10.

The family history was negative for consanguinity, while positive for neuropsychiatric disorders; within the paternal line, one case of mild language delay and autism spectrum disorder was reported.

At 5-6 months of age, the patient manifested the first symptoms, head control was not achieved.

Incomplete head control and absence of parachute reflexes were found on neurological examination; the patient had global hypotonia associated with upper limbs and trunk tonic fluctuations. Osteotendinous reflexes were hypoelicitable in the four limbs. Plantar skin reflex in bilateral flexion was also observed.

Oculogyric crises and autonomic symptoms, such as fluid dysphagia and hyperidrosis, were found.

The patient had a mild-moderate psychomotor delay.

MRI revealed a thinned corpus callosum, and the EEG found diffuse or more frequent slow abnormalities in the bi-hemispheric occipital regions.

Appropriate diagnostic tests for AADCd confirmed the diagnosis, specifically neurotransmitter metabolites assay on CSF, and genetic analysis was performed. Genetic analysis found a homozygous c.749C> T p. (Ser250Phe) mutation in the exon 7 of the *DDC* gene, confirmed by the segregation analysis.

Using flow-injection analysis tandem mass spectrometry (FIA-MS/MS) with labelled tyrosine as the internal standard we found a concentration of 3-OMD on the neonatal DBS of 15.4 µmol/L.

Using the same method, we repeated the 3-OMD assay on DBS collected in the same patient at 2 years of age, the concentration of 3-OMD was found to be 4.6 µmol/L.

We also measured the concentration of 3-OMD on the DBS collected at 2 years of age by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using 3-OMD-d3 as an internal standard. The concentration of 3-OMD was 3.8 µmol/L.

6.2 DBSs' features

A total of 9,876 DBSs were collected and subsequently analysed between March 2022 and May 2023.

The first DBS analysed was collected on March 2, 2022, and the last DBS was collected on May 28, 2023.

Among the 9,876 samples, 6,847 (69.3%) DBSs were part of the 2022 newborn screening program, while 3,029 (30.7%) were part of the 2023 newborn screening program.

Furthermore, 2,272 (23%) out of 9,876 samples were performed according to the "special protocols" procedure.

The number of DBSs collected between the first 48-72 hours of infants' life was 9,664 (97.8%), while 146 (1.5%) samples were collected before the first 48 hours, and sixty-six (0.7%) samples were collected beyond the first 72 hours.

Among the 9,664 samples collected between the first 48-72 hours, from 9,664 infants (4,614 females), 6,702 (69.4%) DBSs were part of the 2022 newborn screening program, while 2,962 (30.6%) were part of the 2023 newborn screening program.

Furthermore, 2,130 (22%) out of 9,664 samples were performed according to the "special protocols" procedure.

6.3 Newborns' features

The total number of newborns was 9,876, of which 4,715 (47.7%) were females and 5,161 (52.3%) were males.

The median gestational age was 276 (IQR, 270-282) days.

The mean gestational age was 273.91 days, SD ± 16.75, with a range from 23 weeks + 1 day to 42 weeks + 4 days.

Precisely 9,096 (92.1%) infants were born between 37 and 41 weeks, while fifteen (0.15%) infants were born at 42 weeks, and 750 (7.6%) were born before 37 weeks. The gestational age of fifteen (0.15%) newborns was unknown.

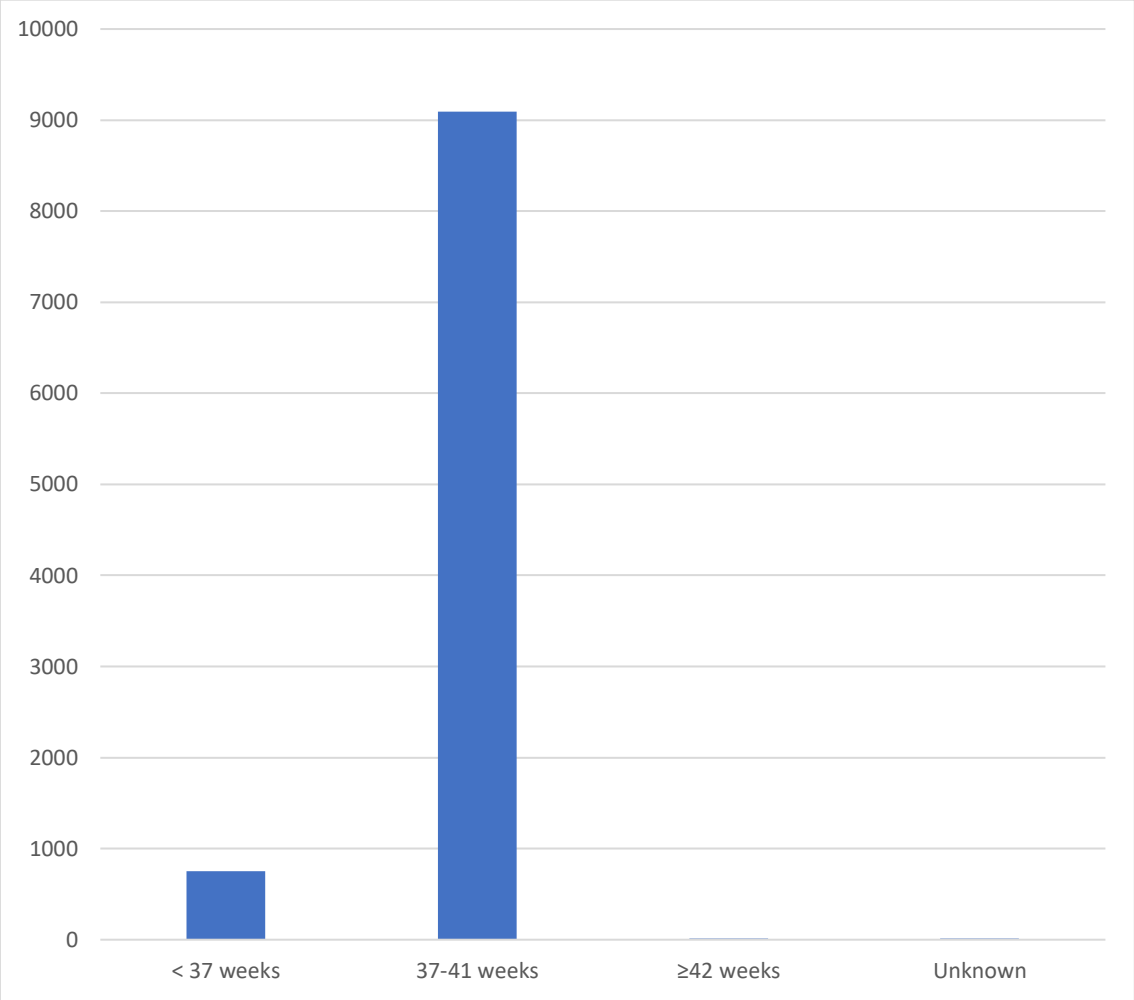

Figure 13. Gestational age of 9,876 infants.

The median weight value was 3,230 (IQR, 2,925-3,535) g.

The mean weight was 3,198.89 g, ±SD 525.04, with a range from 400 to 5,110 (400-5,110) g.

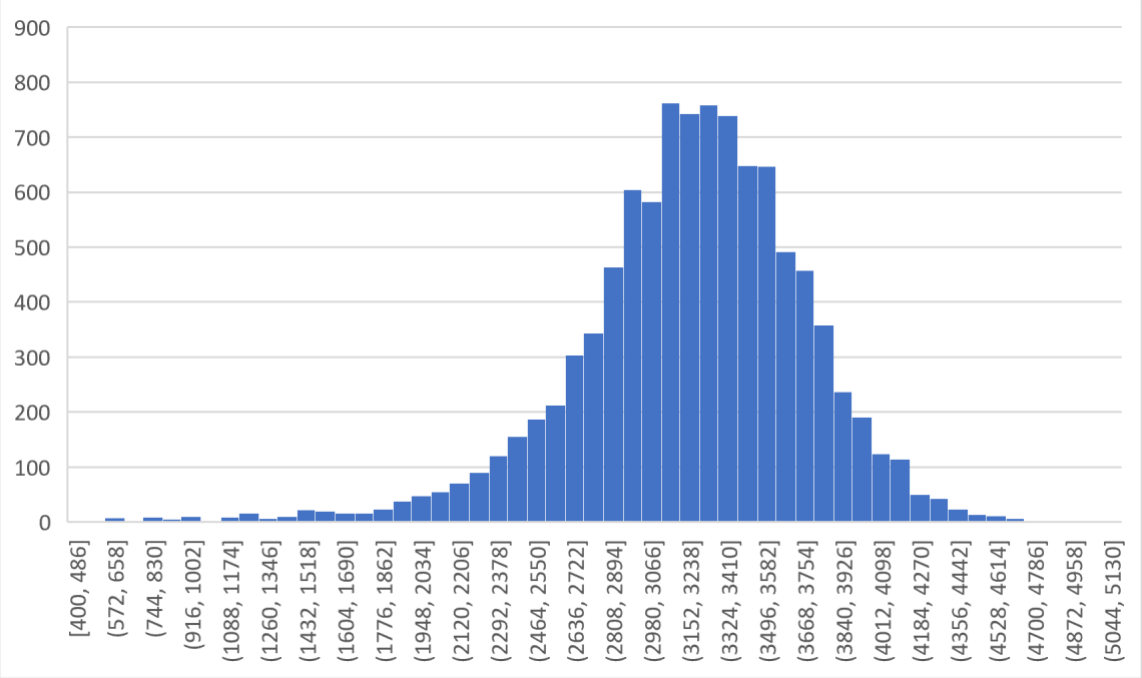

Figure 14. Distribution of body weight values of 9,876 infants.

6.4 3-OMD values

We considered 9,876 DBSs, of which 9,664 (97.8%) were collected between 48-72 hours, 146 (1.5%) were collected before the first 48 hours, and sixty-six (0.7%) were collected beyond the first 72 hours, (range: 0-116 hours). We then analysed the median value, interquartile range (IQR), mean value, and standard deviation (SD) of 3-OMD concentration on DBSs.

The median concentration of 3-OMD was 0.808 (IQR, 0.615-1.016) µmol/L.

The mean concentration of 3-OMD was 0.827 µmol/L, SD ± 0.286, with a range from 0.037 to 2.271 (0.037-2.271) µmol/L.

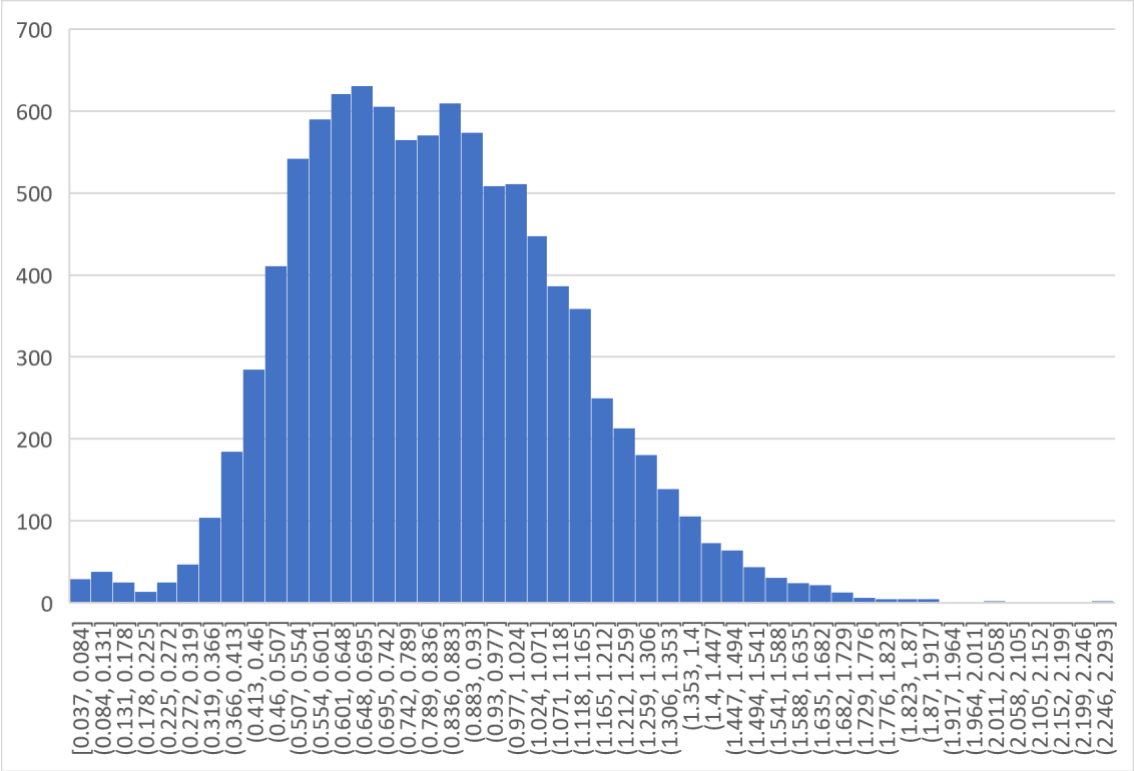

Figure 15. Distribution of 3-OMD values on 9,876 DBSs from 9,876 infants.

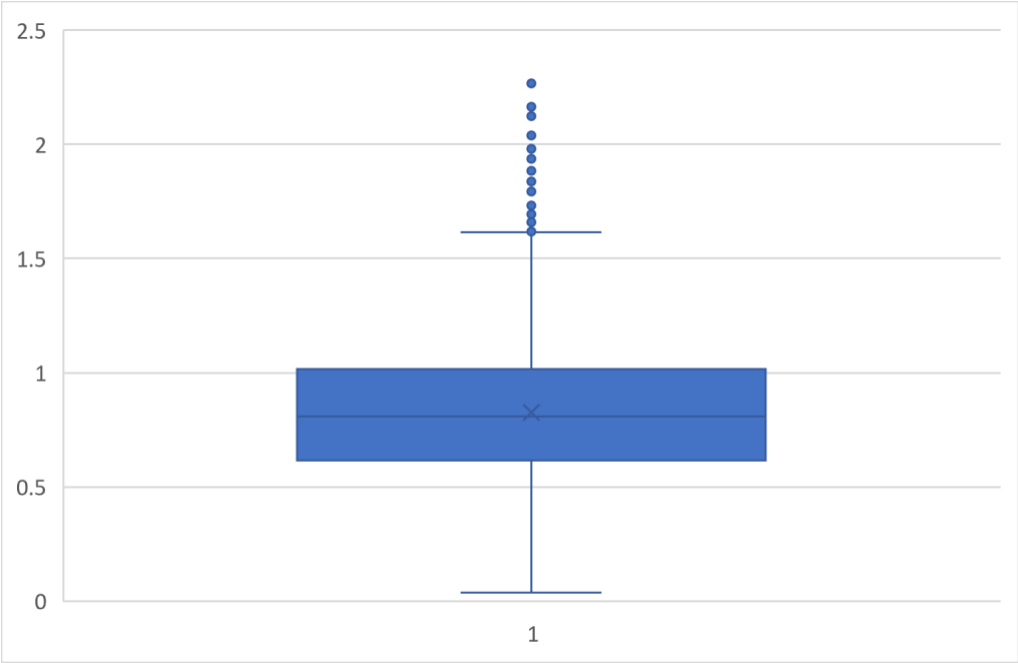

Figure 16. 3-OMD values on 9,876 DBSs from 9,876 infants. Representation of outliers.

We calculated the 95th percentile, 99th percentile, 99.5th percentile, and 99.9th percentile:

- 95th percentile: 1.32 µmol/L
- 99th percentile: 1.57 µmol/L
- 99.5th percentile: 1.66 µmol/L
- 99.9th percentile: 1.88 µmol/L

By approximating the 99th percentile value, we can identify 1.6 µmol/L as the cut-off.

Considering 1.6 µmol/L as the cut-off value, we found 3-OMD concentration greater than or equal to 1.6 µmol/L (≥ 1.6 µmol/L) on the DBSs of eighty-fife (0.86 %) infants, on which 3-OMD assay would be re-evaluated.

Considering only the 9,664 out of 9,876 samples collected between the 48th hour and 72nd hour of infants' life, we analysed the median value, interquartile range (IQR), mean value, and standard deviation (SD) of 3-OMD concentration on DBSs.

The median concentration of 3-OMD was 0.811 (IQR, 0.617-1.018) µmol/L.

The mean concentration of 3-OMD was 0.830, SD ± 0.285, with a range from 0.037 to 2.271 (0.037-2.271) µmol/L.

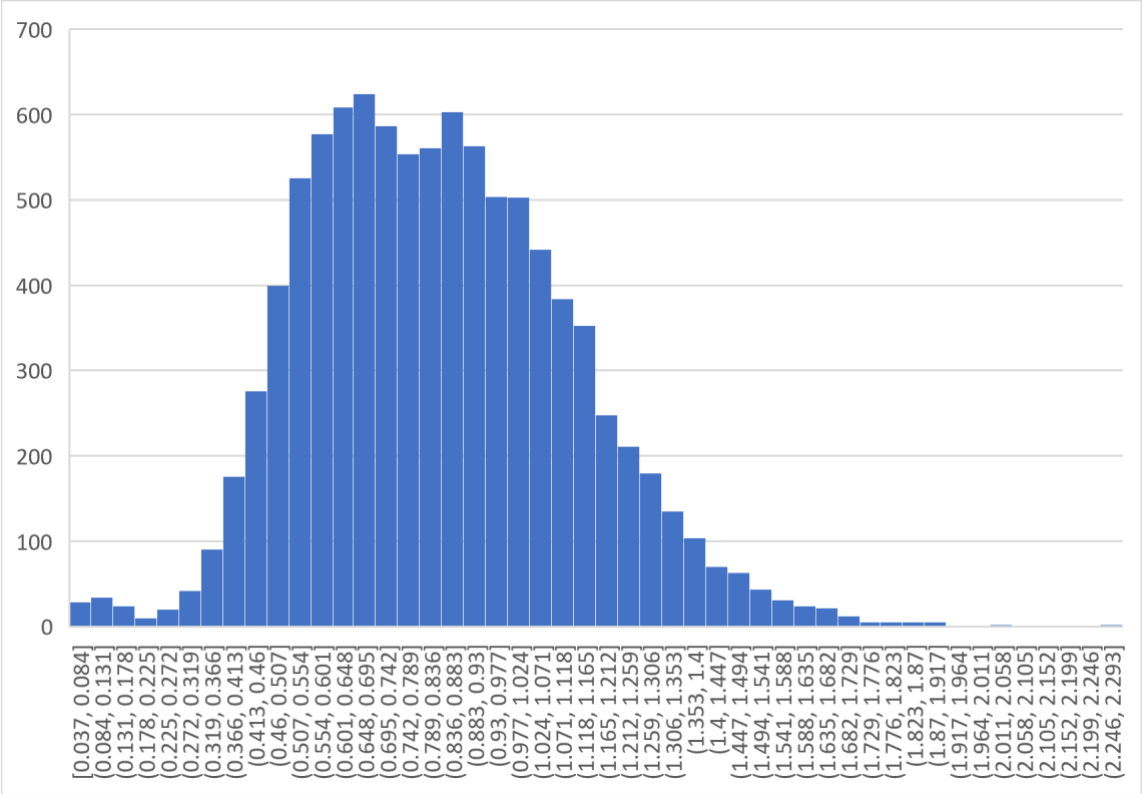

Figure 17. Distribution of 3-OMD values on 9,664 DBSs from 9,664 infants.

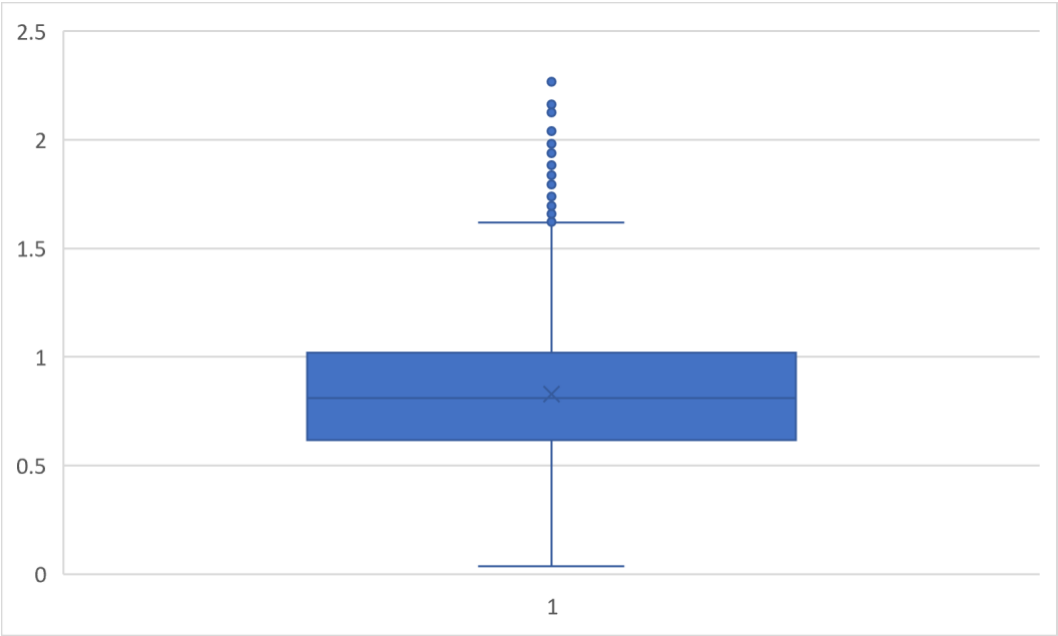

Figure 18. 3-OMD values on 9,664 DBSs from 9,664 infants. Representation of outliers.

We calculated 95th, 99th, 99.5th, 99.9th percentile:

- 95th percentile: 1.32 µmol/L
- 99th percentile: 1.57 µmol/L
- 99.5th percentile: 1.66 µmol/L
- 99.9th percentile: 1.88 µmol/L

6.5 Looking toward AADCd screening

Looking toward a future AADCd newborn screening program, the method of measuring 3-OMD on DBS by FIA-MS/MS using labelled tyrosine as an internal standard has been validated and can be proposed as a first-tier test.

After receiving written informed consent for AADCd newborn screening from the parents/guardians of infants, the first-tier test will be performed on the DBSs, collected according to the current expanded newborn screening procedure.

In case of a positive result in the first-tier test, the second-tier test will be performed on the same DBS, and the concentration of 3-OMD will be measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using 3-OMD-d3 as internal standard.

In case of a positive result in the second-tier test, infants will be recalled. Medical history will be collected, and clinical evaluation and biochemical and genetic diagnostic confirmatory tests on plasma and urine will be performed. DNA of presumed AADCd infants will be tested by direct sequencing of the *DDC* gene.

In case of a confirmed diagnosis of AADCd, the Pediatric Neurology and Muscular Diseases Unit of the IRCCS Istituto Giannina Gaslini, will provide for intake, treatment, and follow-up of the patients.

7. CONCLUSIONS

Aromatic L-Amino Acid Decarboxylase deficiency (AADCd) is a rare neurometabolic disorder, with autosomal recessive inheritance. Pathogenetic biallelic variants in the Dopa Decarboxylase (*DDC*) gene result in aromatic L-amino acid decarboxylase (AADC) deficiency. Pathogenetic mutations in the *DDC* gene and the resulting lack of function of the AADC enzyme led to the deficient synthesis of monoamine neurotransmitters serotonin, dopamine, and subsequently noradrenaline and adrenaline.

3-O-methyldopa (3-OMD), derived from the catabolism of L-Dopa, since it is an upstream metabolite of the reaction catalysed by the AADC-deficient enzyme, increases in patients with AADCd. Previous studies have shown that by flow-injection analysis tandem mass spectrometry (FIA-MS/MS) it is possible to perform the 3-OMD assay on neonatal DBS.

In our study, we assayed the concentration of 3-OMD on DBS in the Ligurian neonatal population by FIA-MS/MS using labelled tyrosine as an internal standard.

We identified a 3-OMD mean concentration of 0.827 µmol/L (SD ± 0.286; range: 0.037-2.271 µmol/L), a median concentration of 0.808 (IQR, 0.615-1.016) µmol/L, and a 99th percentile value of 1.57 µmol/L on a total of 9,876 DBSs collected between the first 0-116 hours of infants' life.

We also found a 3-OMD mean concentration of 0.830 µmol/L (SD ± 0.285; range: 0.037-2.271 µmol/L), a median concentration of 0.811 (IQR, 0.617-1.018) µmol/L, and a 99th percentile value of 1.57 µmol/L on 9,664 out of 9,876, collected precisely between the first 48-72 hours of infants' life.

The study made it possible to identify the approximate 99th percentile value 1.6 µmol/L as the cut-off, thus the percentage of DBSs with 3-OMD concentration above the cut-off was less than 1% (0.86%).

The method has been also validated through the measurement of 3-OMD on the neonatal DBS of a patient with a genetically confirmed diagnosis of AADCd and on the DBS collected in the same patient at 2 years of age.

Both the value of 3-OMD on the neonatal DBS (15.4 µmol/L) and the value of 3-OMD on the sample collected at 2 years of age (4.6 µmol/L) were widely above the established cut-off.

Given the recent approval of the first gene therapy for AADCd treatment, with good safety and efficacy profile, the greater the earlier it is administered to affected patients and given the possibility of assaying 3-OMD concentration on neonatal DBS, it is rational to talk about future newborn screening program for AADCd.

The method has proven to be valid as a first-tier test for AADCd newborn screening.

The analysis of reference values (cut-offs) will allow us to reduce the percentage of DSB on which to re-evaluate the 3-OMD assay using a second-tier test to below 1%. The second-tier test can be performed on the same neonatal DBS, this will avoid recalling infants and their families.

A newborn screening program for AADCd will allow a diagnostic assessment to be performed on patients who tested positive for screening. This will allow for early diagnosis of the disease and patients with a confirmed diagnosis of AADCd will benefit from the early administration of the recently approved disease-modifying gene therapy.

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