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Scuola di Scienze Mediche e Farmaceutiche
CORSO DI LAUREA IN MEDICINA E CHIRURGIA

Tesi di Laurea

Aromatic L-Amino Acid Decarboxylase deficiency (AADCd): a multicenter study evaluating 3-OMD through dried blood spots

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INDEX

Abstract

Background: Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare neurometabolic disorder. Owing to certain similarities with other conditions (e.g., cerebral palsy and epilepsy), cases of AADC deficiency are often misdiagnosed. We evaluated the utility of a minimally invasive method -dried blood spots (DBS)- to evaluate the levels of 3-O-Methyldopa (3-OMD) for AADC deficiency detection in a cohort of symptomatic pediatric patients.

Methods: Children with global developmental delay (DD) of undetermined cause were recruited through national collaboration. Blood samples specimen were collected through DBS and mass spectrometry was performed to determine 3-OMD levels. Clinical data, imaging, and electroencephalographic results were collected through a structured questionnaire.

Results: 58 patients (30 females) with a mean age of 3.2 years (range 0.66-5) were enrolled. The mean age at symptoms onset was 10 months (range 5-12). Eighteen (31.5%) patients had seizures, including 15 (26.3%) cases of generalized tonic-clonic seizures and 3 (5.2%) cases of seizures with

impaired awareness. Eleven (18.9%) patients had primary and 3 (5.2%) secondary microcephaly. Eight (14%) patients had a diagnosis of autistic spectrum disorder. Hypotonia was present in 50 (86.2%) patients (38.6% cases axial; 45.6% cases global). Fifteen (26.3%) patients had nystagmus and 3 (5.2%) had oculogyric crises. Autonomic dysfunctions and sleep disturbances were present in 16 (28%) and 4 (7%) patients, respectively. Patient #57 had 3-OMD values (4314 nmol/L) over the mean of the population and AADC deficiency was confirmed by second-tier tests.

Conclusions: DBS is useful for the evaluation of the 3-OMD levels and the diagnosis of possible patients with AADC deficiency. Screening of larger pediatric populations and the application of this method as neonatal screening will allow early diagnosis given the possibility of targeted gene therapy.

1. AROMATIC L-AMINO ACID DECARBOXYLASE DEFICIENCY

Aromatic L-amino acid decarboxylase deficiency (AADCd) is a rare, autosomal recessive neurometabolic disorder that is biochemically characterized by a deficiency of catecholamines (dopamine, epinephrine, and norepinephrine) and serotonin. Onset is early in life, and key clinical symptoms are hypotonia, movement disorders (oculogyric crisis, dystonia, and hypokinesia), developmental delay, and autonomic symptoms.

Owing to certain similarities in clinical presentation with other conditions (e.g., cerebral palsy and epilepsy), cases of AADC deficiency are often misdiagnosed. This means that the number of patients is low and that diagnostic delay, resulting in late-onset treatment initiation, is common.

It has an estimated prevalence of 1:90,000 births in the USA, 1:118,000 in the European Union, 1:182,000 in Japan, and 1:32,000 in Taiwan due to the presence of a founding mutation caused by the presence of a founder splice variant, c.714 + 4A> T (IVS6 + 4A> T), which causes 37 nucleotides insertion from intron 6 into the DDC mRNA.[1,2,3,4]

1.1 Pathogenesis

Aromatic L-amino acid decarboxylase (AADC) is a lyase enzyme present in brain tissue involved in catalyzing the synthesis of dopamine and serotonin from their respective precursors, Levodopa (L-DOPA), and 5-Hydroxy-tryptophan (5-HTTP).

AADC deficiency is caused by an inborn error of neurotransmitter biosynthesis, resulting from pathogenic variants in the dopa decarboxylase gene, DDC, encoding for the AADC enzyme. Lack of the AADC enzyme leads to a severe combined deficiency of dopamine, serotonin, and other catecholamines (noradrenaline and adrenaline). The absence of these neurotransmitters inhibits neuronal signaling in the central nervous system (CNS), which is required for motor development, cognitive function, behaviour, and autonomic function.

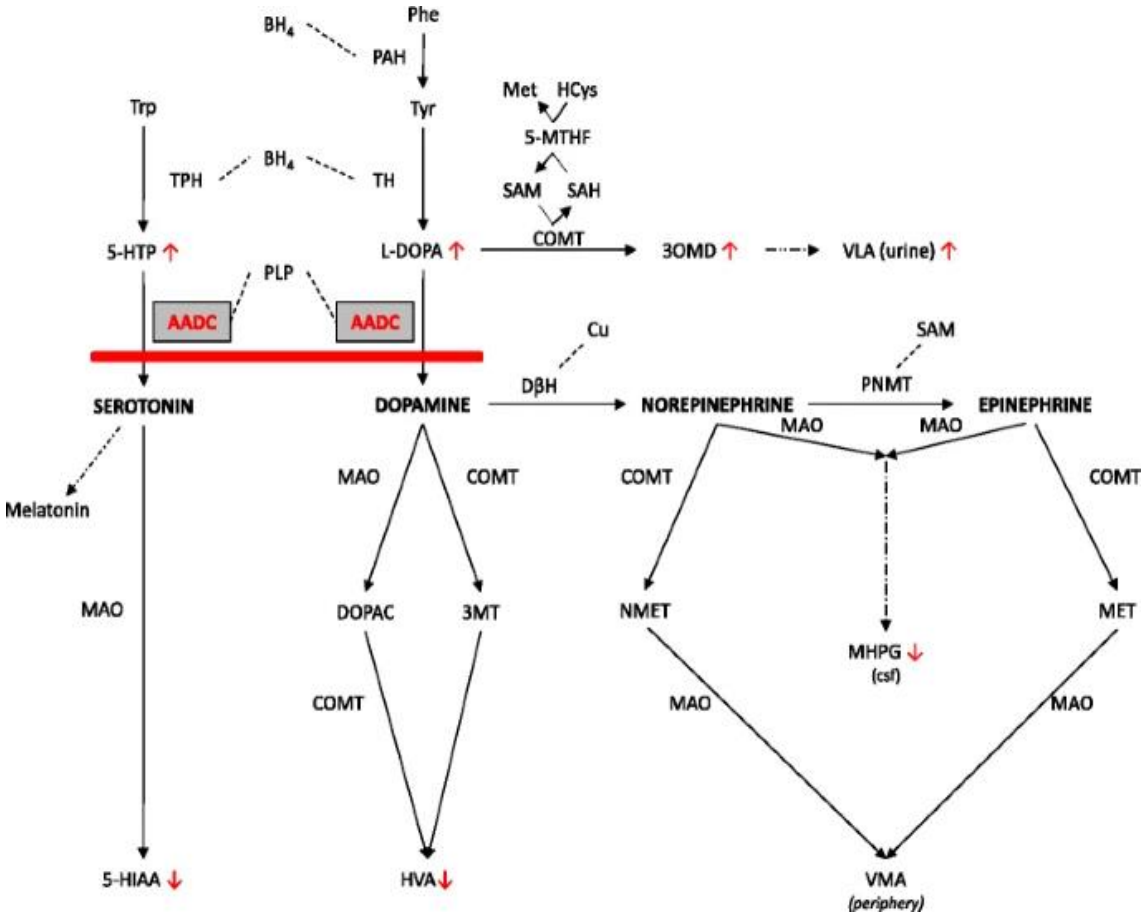

Fig. 1 *Biosynthesis and breakdown of serotonin and the catecholamines, and the metabolic block in AADC deficiency. Simplified scheme of the biosynthesis and breakdown of serotonin and the catecholamines (dopamine, norepinephrine, and epinephrine), and melatonin synthesis. Cofactors (BH4, PLP, Cu) and methyldonorr (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, and 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: copper; DβH: dopamine beta-hydroxylase; DOPAC: dihydroxyphenylacetic acid; HCys: homocysteine; 5-HIAA: 5-hydroxy indole acetic acid; 5-HTP: 5-hydroxytryptophan; HVA: homovanillic acid; L-Dopa: 3,4-dihydroxyphenylalanine; MAO: monoamine oxidase; MET: metanephrine; Metmethioninene; 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 hydroxylasTRHTrH: tryptophan hydroxylase; Tryp: tryptophan; Tyr: tyrosine; VLvanillictic acid; VMA: vanillmandelic acid: Vit B6 vitamin B6 (pyridoxine)(Wassenberg et al., 2017)

AADC is involved in catalyzing various decarboxylation reactions.

Following the hydroxylation of tyrosine to L-dihydroxyphenylalanine (L-DOPA), catalyzed by the enzyme tyrosine hydroxylase, DDC decarboxylates L-DOPA to form dopamine.

The latter is subsequently converted by the enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) to homovanyl acid (HVA), the main urinary catabolite.

The tryptophan hydroxylase enzyme produces OH-tryptophan 5, which is decarboxylated by DDC, giving rise to serotonin. The latter is converted by the monoamine oxidase (MAO) enzyme to 5-hydroxy indoleacetic acid, the

main end product of serotonin metabolism.[5,6,7] 3-OMD accumulates in AADC-deficient patients due to the inability to use L-dopa generated by tyrosine hydroxylase; it can exert toxic effects by increasing the generation of reactive oxygen species, as well as a direct neurotoxic effect.[8] Furthermore, high concentrations of 3-OMD have been associated with dyskinesia and increased side effects in patients with Parkinson's disease receiving L-dopa therapy.[9,10]

1.2 Clinical presentation

The clinical picture is characterized by high phenotypic variability and by the typical association of neurological and non-neurological symptoms, which can make diagnosis difficult.

The onset occurs in the first months of life with delayed psychomotor development of varying degrees (mild, moderate, severe), axial or global hypotonia, oculogyric crises, dystonia, dyskinesias, irritability, autonomic dysfunctions including increased sweating, nasal congestion, diarrhea, hypersalivation, orthostatic hypotension.[11,12,13]

Sleep disturbance appears to evolve with age: children experience excessive sleepiness, while many children and adolescents exhibit prominent insomnia.[12]

Drug-resistant seizures (absences or tonic-clonic) and non-diabetic hypoglycemic seizures are also reported. During the neurological examination, the newborn appears mostly to collaborate with the presence of a social smile and visual pursuit.

However, severity can range from mild to severe, resulting in wide variability in presentation. Patients with more severe forms of AADCd may never meet developmental milestones such as head control, sitting, standing, walking, or talking.[1,3]

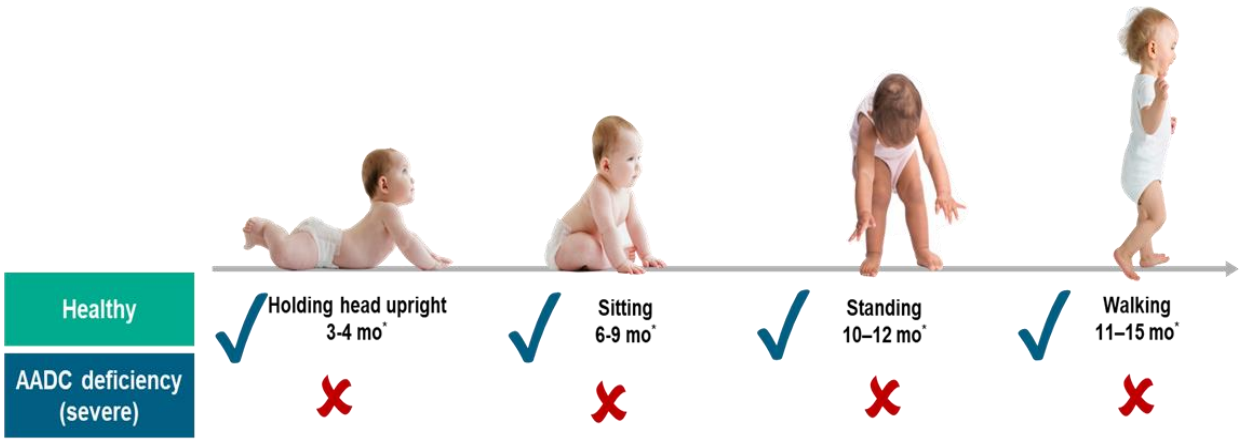

***Fig. 2 Denver** II Developmental Milestone chart*[14]

A milder phenotype has also been described[15], including spontaneous improvement during the second decade of life, with independent walking and feeding and syndromic intellectual disability with autonomic dysfunction but without dystonia or eye crisis.

Especially in early-onset forms, nonspecific neurological symptoms and signs such as developmental delay and hypotonia/hypokinesia may be underestimated or misinterpreted.[15] It is, therefore, necessary to increase awareness of both neurological and non-neurological symptoms, such as non-diabetic hypoglycemic crises and autonomic dysfunctions, to avoid incurring a diagnostic delay.[4,13]

The disease itself is progressive, even if the progressiveness is not so aggressive, it seems that there is a period of fluctuations in which the patient is better.

2. DIAGNOSIS

2.1 Analysis of cerebrospinal fluid (CSF), AADC activity, and genetic testing

To diagnose AADCd two of the three criteria represented below (Fig3) must be confirmed.

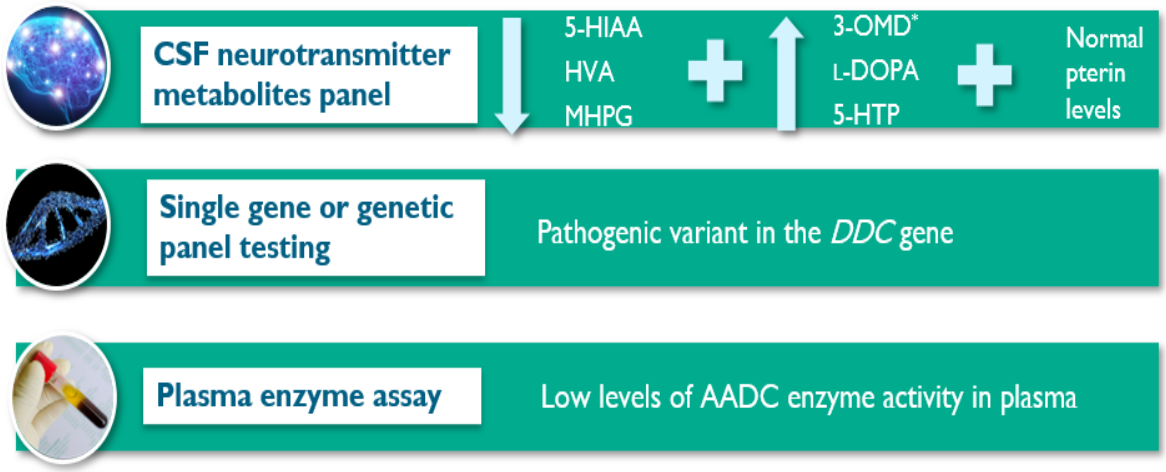

Fig. 3 *Core diagnostic tests to confirm AADC deficiency.*[1,4]

Lumbar puncture

CSF in AADCd shows low levels of 5-hydroxy indole acetic acid (5-HIAA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenyl glycol (MHPG), normal pterins including neopterin and biopterin, and high concentrations

of 3-O-methyldopa (3-OMD), L-dopa and 5-OH tryptophan (5HTTPP). These results reflect metabolic blockade at the AADC level.

Normal CSF pterins (neopterin, dihydrobiopterin, and tetrahydrobiopterin) are essential to differentiate AADCd from tetrahydrobiopterin disorders.[16]

CSF profile of AADCd may be like that found in pyridoxine 5-phosphate deficiency (PNPO), where AADCd is due to deficiency of its cofactor pyridoxalkyl phosphate (PLP). However, further results in this disorder are a very low PLP and an increase in glycine and threonine in CSF. In addition, the clinical picture of PNPO deficiency is characterized by severe neonatal epileptic encephalopathy, unlike AADCd.[17,18]

AADC activity in plasma

It is necessary to consider the assay of plasma AADC enzyme activity if the neurotransmitters of the cerebrospinal fluid show insignificant abnormalities.[11]

AADC deficiency in plasma can be measured using both L-Dopa and 5-HTP as a substrate. Since L-Dopa provides a higher analytical yield, it is used as

a standard method. AADC activity is also reduced in heterozygous carriers (35-40% of normal)[4,19,20]

Molecular diagnosis

If the genetic diagnosis is performed as a first step (e.g. whole exome or affected familial sequencing), functional confirmation should be completed by measuring the activity of the AADC enzyme in plasma and/or neurotransmitter metabolites in cerebrospinal fluid. If local resources allow, it is recommended to perform all three key diagnostic tests in patients with this rare disease.[4]

The genetic investigation is carried out through the sequencing of the entire housings of Sanger analysis.[21,22]

About 50 disease-causing dopa decarboxylase (DDC) gene variants have been described: 39 substitution variants, 2 nonsense, 5 deletions, 1 insertion, and 4 splicing variants.[23]

2.2 Misdiagnosis

Owing to certain similarities in clinical presentation with other conditions (e.g cerebral palsy[24], epilepsy[1,4,25]), cases of AADC deficiency are often undiagnosed or misdiagnosed. For this reason, it is important never to underestimate the symptoms and perform a correct differential diagnosis, also aided by instrumental tests (MRI, EEG) which are often negative in the AADC deficiency. The presence of hypotonia, hypertonia, dyskinesias, dystonia, oculogyric crises, and hypokinesia makes it necessary to distinguish the clinical picture from movement disorders or cerebral palsy.

The presence of epileptic seizures and an adequate differential diagnosis with epileptic syndromes should be evaluated.

Finally, the presence of irritability, dysphoria, excessive crying, speech problems, and intellectual disability may suggest a behavioral disorder/ autism.[26]

2.3 Neonatal screening

ADCDd is, therefore, a complex pathology and its t often confused with other clinical conditions that present overlapping symptoms. Therefore, it is common for patients to wait a long time before receiving a correct diagnosis, and this can lead to a worsening of the prognosis. The importance of an early diagnosis is evident, which can help promptly intervene on symptoms, and the prognosis.[27,28] Medical and especially gene therapy also benefit from early diagnosis.

Part of the research is focusing on identifying valid, simple, and low-cost methods for neonatal screening. Scientists from the National Taiwan University (NTU) have developed and tested a newborn screening test specific for AADCd, simple, fast, and inexpensive.

The results obtained from the experimentation of this test, published in the *Journal Molecular Genetics and Metabolism*, in addition to validating the effectiveness of the new diagnostic tool, allowed to establish the effective incidence of AADC deficiency in the Taiwanese population, estimated at 1: 32,000.[1,29]

3-OMD accumulates in AADC-deficient patients due to the inability to use L-dopa generated by tyrosine hydroxylase; it can exert toxic effects by increasing the generation of reactive oxygen species, as well as a direct neurotoxic effect.[30] Furthermore, high concentrations of 3-OMD have been associated with dyskinesia and increased side effects in patients with Parkinson's disease receiving L-dopa therapy.[9,10]

Neonatal screening is based on the search for the key biomarker 3-O-methyldopa (3-OMD), which is increased in AADCD, in dried bloodstains (DBS) using the tandem mass spectrometry of the flow-injection analysis with the Neo Base ™ 2 and 13C 6-Thyrosine reagents as internal standard, which is routinely used in neonates.[31]

In this way, it is possible to obtain an initial indication of the disease and proceed, in the event of a positive result, to more targeted and specific tests (the “second-tier tests”) such as genetic tests and CSF sampling.

An inverse correlation was highlighted between the age of patients and 3-OMD levels as the latter decrease with advancing age.

Further evaluation of 3-OMD DBS concentrations in adolescent and adult patients is required. Particularly because there are reports of mild phenotypes of patients with AADC deficiency, in which only alterations in

the concentration of 3-OMD in the biochemical profile of CSF have been noted.[32,33]

For these patients and patients up to the age of 18 years, the detection of 3-OMD in DBS is a non-invasive, simple, rapid, and valid method for detecting AADC deficiency. However, specific cut-off values should be applied and validated by repetitive longitudinal measurements of patients with AADC for selective screening approaches in adolescent and adult patients with unclear movement disorders and developmental delay. Until then, it is still recommended to undergo a lumbar puncture to determine a biochemical profile and enzymatic or genetic tests for a definitive diagnosis.[4]

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3.TREATMENT PARADIGM

3.1 Medical therapy

Experts agree to consider dopamine agonists, MAO inhibitors, and vitamin B6 as the current first therapeutic choice in patients with AADC deficiency. Additional symptomatic therapeutic agents are anticholinergic agents (for movement disorders), melatonin, benzodiazepines (dystonic state or oculogyric crisis,) and alpha-adrenergic receptor blocker.[11]

In general, multiple drug therapy is required and doses should be titrated individually and sequentially.

The general principles to follow are a gradual approach, starting low and going slow when increasing doses, and stopping/weaning cations that are not helpful.[4]

There is also agreement on the need for a multidisciplinary approach in the follow-up of patients with AADC deficiency.

Dopamine agonists

Dopamine agonists work by directly activating postsynaptic dopamine receptors. Pergolide and Cabergoline, ergot-derived dopamine agonists with a strong serotonergic (5HT2b) agonist action, are strongly associated with heart valve disease and other fibrous complications[34,35,36] and should therefore not be used in AADCd.

Ergot-derived dopamine agonists without 5HT2b agonist action (Bromocriptine) have a lower risk, although incidental cases of pulmonary, retroperitoneal, and (peri) cardiac fibrosis have been reported, with a dose-effect relationship.[37]

It is, therefore, necessary to perform a cardiac screening before and during treatment.

On the other hand, dopamine agonists not derived from ergot including pramipexole, ropinirole, rotigotine (transdermal patches), and apomorphine (subcutaneous), presented a very low risk of complications and fibrosis.[38]

The use of bromocriptine, pramipexole, rotigotin patches, and pergolide obtained positive responses such as improved head control, hypotonia, oculogyric crisis, voluntary movements, and autonomic symptoms.

Side effects included irritability, weight loss, worsening of growth failure, vomiting, and even mild to severe dyskinesia.[39]

Overall, the benefits outweighed the side effects in most cases. It is important to consider that many patients have been treated concurrently with more than one drug class, therefore assessing the impact of a single drug is often problematic.

MAO inhibitors

MAO inhibitors work by preventing the breakdown of dopamine and serotonin, thereby increasing the availability of monoamines. They are associated with dopamine and/or pyridoxine agonists. Most studies described improvement in at least one clinical endpoint (e.g., hypotonia), with no effect on the others.[4,25,40]

Biochemically, there is a strong recommendation to administer MAO inhibitors to AADCd patients, although there is little evidence of clinical benefit.

Pyridoxine / pyridoxalphosphate (PLP)

Pyridoxal phosphate (PLP), the active form of pyridoxine, is a cofactor of AADC. Therefore, it is used to increase the residual activity of the AADC enzyme. As pyridoxine is more readily available and cheaper than PLP, it is used more often. Side effects include gastrointestinal disturbances, sleep disturbances, and extreme motor restlessness in patients using very high doses of pyridoxine and concomitant treatment with L-DOPA. Additionally, pyridoxine and PLP can cause reversible polyneuropathy when used in high doses for lonperiodsme.[41,42]

Anticholinergic drugs

Anticholinergic drugs in AADCd can be used to treat autonomic symptoms, dystonia, and oculogyric crises. They are commonly used to treat certain movement disorders, particularly parkinsonism[43] and dystonia.[44]

Although their exact mechanism of action is not known, they are believed to affect the relative imbalance between the dopaminergic and cholinergic pathways.

Melatonin

There is very limited evidence for the use of melatonin in AADCd.[45]

From a pathophysiological point of view, supplementation for sleep induction disorders is reasonable because melatonin is formed from serotonin and therefore can be reduced in AADCd. Hitherward, it is recommended for use in patients with sleep disorders.

Benzodiazepines

Benzodiazepines (e.g., rectal diazepam), especially when used intermittently, can be considered in specific contexts, such as oculogyric or sustained dystonic crises.[4,11]

Drugs to Avoid in AADC deficiency

Centrally acting dopamine antagonists, used for their antiemetic and antipsychotic properties, should be avoided in AADCd because they have the potential to worsen dopamine deficiency symptoms. Metoclopramide should not be used for the treatment of nausea. It is important to realize that many drugs have antagonistic properties for different neurotransmitters and before introducing any drug to AADCd patients, its potential benefits and harms should be carefully considered.

In case of nausea and vomiting in patients with AADCd, supportive care to avoid dehydration and hypoglycemia is very important. If possible, anti-dopaminergic and anti-serotonergic agents should be avoided. If medical therapy is required, low-dose domperidone may be considered. It is essential to follow local guidelines on availability, heart problems, and dose recommendations. Although domperidone is a dopamine antagonist, it does not cross the brain barrier and therefore side effects in AADCd are expected to be limited.

3.2 Gene therapy

Medical supportive therapies provide variable results and do not treat the underlying cause of the disease, which is related to the insufficiency or absence of AADC activity. Studies in adults with Parkinson's disease have shown that intraputaminal infusion of adeno-associated virus type 2 (AAV2) mediated by the human AADC gene vector increases the enzymatic activity of AADC, with good safety and tolerability profiles.[46,47,48]

Since the main symptoms of AADC deficiency include reduced levels of brain dopamine and impaired motor functions, this disease would be a promising candidate for a gene therapy approach like that used in Parkinson's disease.[49]

The goal of intraputaminal AADC gene therapy is to provide a functional copy of the human DDC gene (hAADC) directly to the striatal regions affected by the disease, which subsequently leads to increased conversion of L-Dopa to Dopamine in targeted striatal neuronal cell bodies.[50]

The recombinant AAV2 vector containing the human AADC gene (rAAV2-hAADC, eladocagene exuparvovec), was developed as gene therapy with sterile parenteral formulation, containing the recombinant active biological

substance and compendial excipients, administered to cells within the putamen bilaterally to drive the production of the AADC enzyme.[29,51,52]

As previously highlighted, studies on the intraputaminal infusion of gene therapy against Parkinson's disease (PD) served as the basis for the development of therapy against AADC deficiency.

The rationale for the selection of putamen as an anatomical target in PD for DDC gene therapy is that transduction of post-synaptic cells expressing dopamine receptors in the putamen may provide sufficient AADC activity to cause an increase in local metabolism of exogenous levodopa (i.e., dopaminergic drugs) and a subsequent increase in striatal dopamine levels.[53,54]

Dopamine is a key monoamine neurotransmitter that acts within the striatum to modulate the output of neurons in brain regions involved in voluntary motor movements, learning, memory, cognition, and emotions.

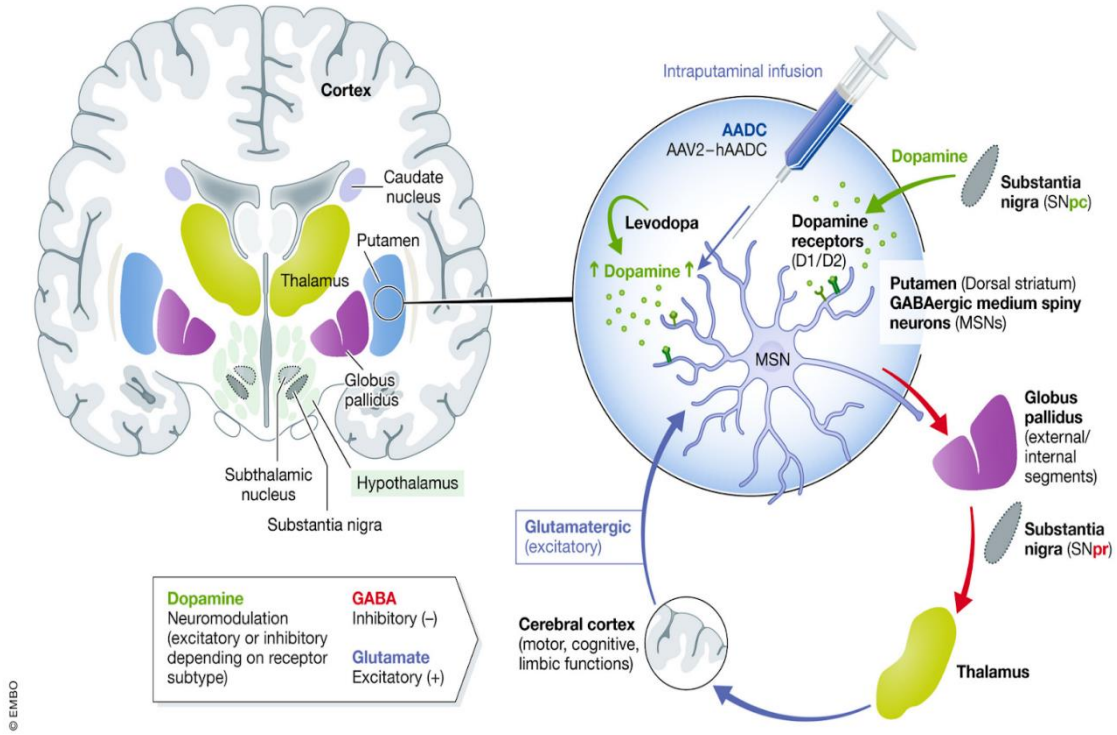

Fig. 4 *Simplified circuit diagram illustrating the key afferent and efferent connections of the dorsal striatum (putamen) and their role in the control of the motor, cognitive, and limbic functions. Dopaminergic inputs to the putamen originate from the SNpc. The dopaminergic terminals release dopamine, 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 the 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 neuromodulator effect on the 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.*[50]

The study published in Molecular Therapy by the researchers of the Taiwan group demonstrated transformative and lasting neurological and neuromuscular improvements, which continued for over 9 years.

All patients treated with intraputaminal AADC gene therapy gained head control, and some were able to stand, walk, and even talk.

After the first year of birth, children with AADC deficiency usually stop gaining weight[55], with serious repercussions on their growth rate. Instead, 12 months after the administration of gene therapy, a weight gain was observed in line with the age tables.

In addition, a significant reduction in the severity of symptoms associated with AADC deficiency was reported in treated children, including mood disorders, excessive sweating, temperature instability, and eye crises.

Significant was also the improvement, reported by parents, in the quality of life of caregivers who daily assist sick children.

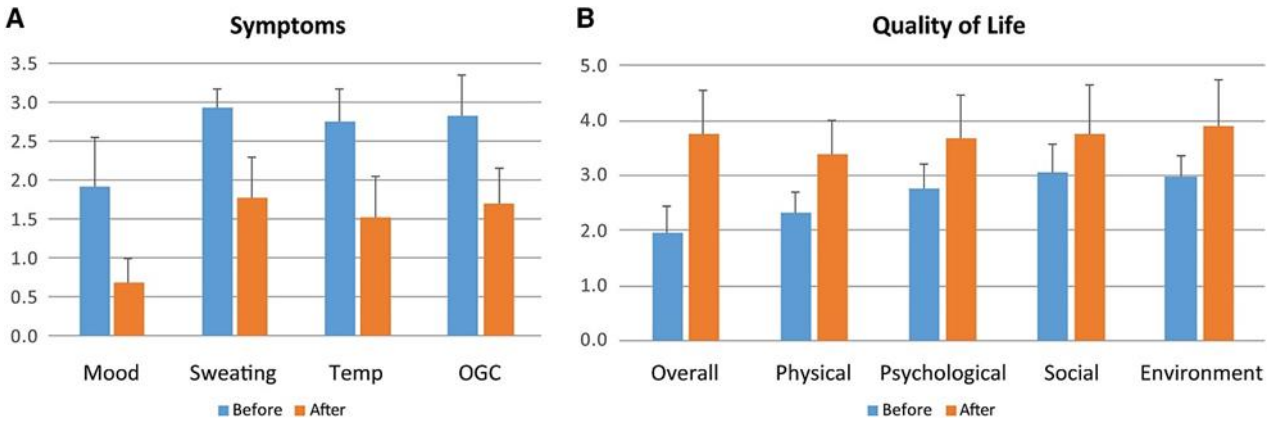

Fig. 5 *Improvements in patients' symptoms and caregivers' quality of life*
Mothers were asked to assess their quality of life and patients' symptoms at the end of 2020 (after) and to remember the conditions before gene therapy (before). (A) Patients' symptom severity score results (a higher score indicates more severe). B) Results of the WHOQOL-BREF Taiwan version (a higher score indicates better quality) of caregivers. The bars above the column indicate 1 standard deviation.[49]

During the study, two universal scales were used to measure the children's motor skills. Abnormal Involuntary Movement Scale (AIMS) focused on gross motor skills, while Peabody Developmental Motor Scales (PDMS-2) included fine motor function.

A strong correlation was observed between post-treatment HVA level and PDMS-2 scores, suggesting that improvements in motor function were made by dopamine production, as enabled by the gene therapy product provided.

Younger patients showed faster and greater improvements in PDMS-2 scores after gene therapy, a similar finding to previous publications.[49] This may be due to a higher degree of neuronal plasticity in younger patients.

A positive correlation between PDMS-2 score and pre-treatment HVA levels has also been demonstrated. This suggests that 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.

Transient dyskinesia that resolved within months and episodes of apnea that decreased after ten months were observed as adverse events.[51,52] Pyrexia was also commonly observed (16%).[29] One case of subdural bleeding without clinical symptoms has been documented.

Cerebrospinal fluid analyses of HVA and 5-HIAA reflect dopamine and serotonin levels in the brain. Before gene therapy, patients had very low levels of HVA in the cerebrospinal fluid, and levels increased 12 months after gene therapy.

Efficacy was further confirmed by positron emission tomography (PET) which revealed an increase in the absorption of tracers l -6-[18 F] fluoro-3, 4-dihydroxyphenylalanine or 6-[18 F] fluoro-l-m-tyrosine (FMT) after gene

transfer.[29,51] In addition, analysis of cerebrospinal fluid showed an increase in the metabolites of dopamine and serotonin.

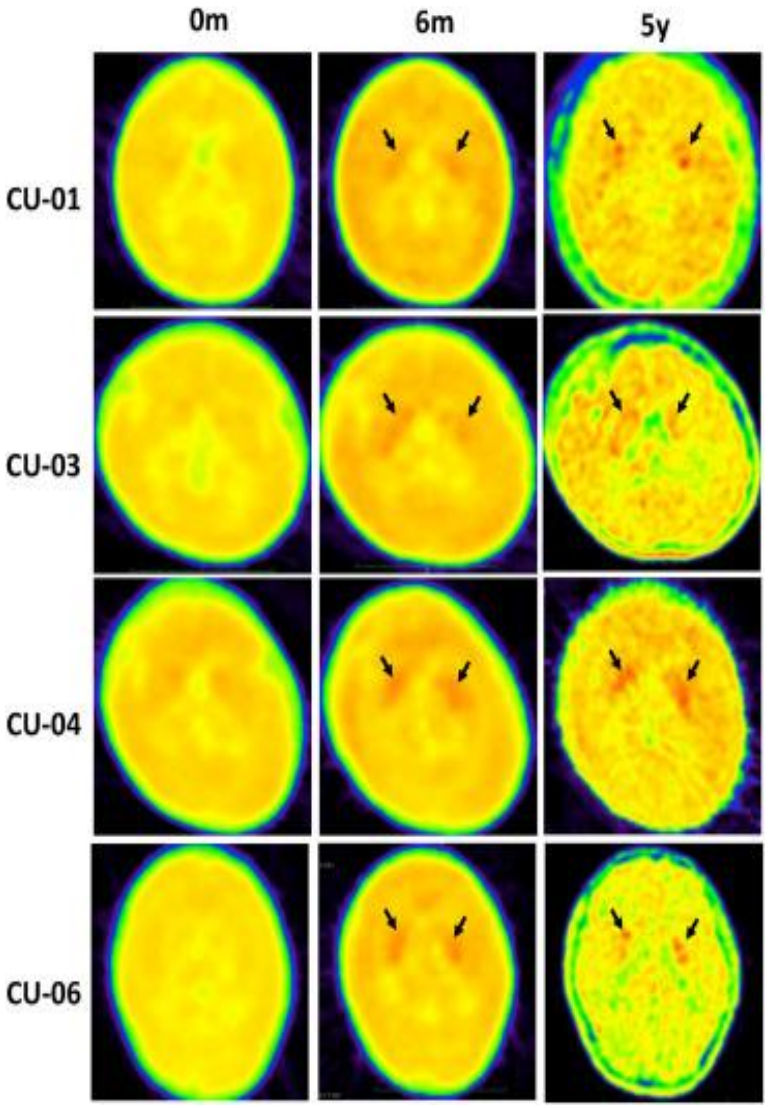

Fig. 6 *De novo dopamine production: visualized 18F-DOPA PET increases in four patients*

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.[49]

A few years ago, a new study was launched focused on the use of gene therapy on a new neuronal target: the dopaminergic neurons of the

midbrain. This study was reviewed and approved by the Institutional Review Boards of the University of California San Francisco (Protocol No. 15-17756, approved June 24, 2016) and Ohio State University Wexner Medical Centre (Protocol No. 2018H0269, approved November 29, 2019).[54]

In contrast to previous studies, it has been hypothesized that patients with AADC deficiency would benefit from administering AAV2-hAADC to two specific regions of the midbrain: the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA).

The rationale for selecting the midbrain target concerning the putamen is that dopaminergic neurons in the midbrain and their axonal projections are structurally intact in children with AADC deficiency.[56]

In addition, the mood and autonomic symptoms that accompany severe motor impairment may be partly attributable to dopamine deficiency beyond the nigrostriatal system, and therefore would not be treated by putaminal transduction.

By providing AAV2-hAADC to SNc and VTA, the goal was to increase the activity of the AADC enzyme in the dopaminergic neurons of the midbrain, thus saving dopamine biosynthesis and dopaminergic neurotransmission in

the nigrostriatal, mesolimbic and mesocortical pathways. Midbrain delivery also takes advantage of anterograde axonal transport of AAV2 from these regions to deliver AAV2-hAADC to neuroanatomically appropriate brain regions like the striatum.[57,58]

Direct infusion of AAV2-hAADC into SNc and VTA was performed by identification of anatomical targets, accurate placement of an intracranial catheter, and subsequent confirmation of carrier delivery. This approach was achieved using real-time MRI imaging in combination with co-infusion of an MR contrast agent.[59,60,61] Preoperative identification of SNc and VTA using high-resolution MR imaging enabled the planning of catheter trajectories designed to prevent passage through eloquent regions. An MRI-guided navigation system enabled direct intraoperative confirmation of catheter placement in selected targets. Finally, the infusion of the vector was monitored with continuous MRI imaging which confirmed the accurate and reproducible distribution of the infusion.

The primary objectives of this study were to demonstrate the safety of the procedure and to detect evidence from biomarkers of increased AADC brain activity. The secondary objectives were to evaluate clinical improvements in OGC and motor function after gene delivery.

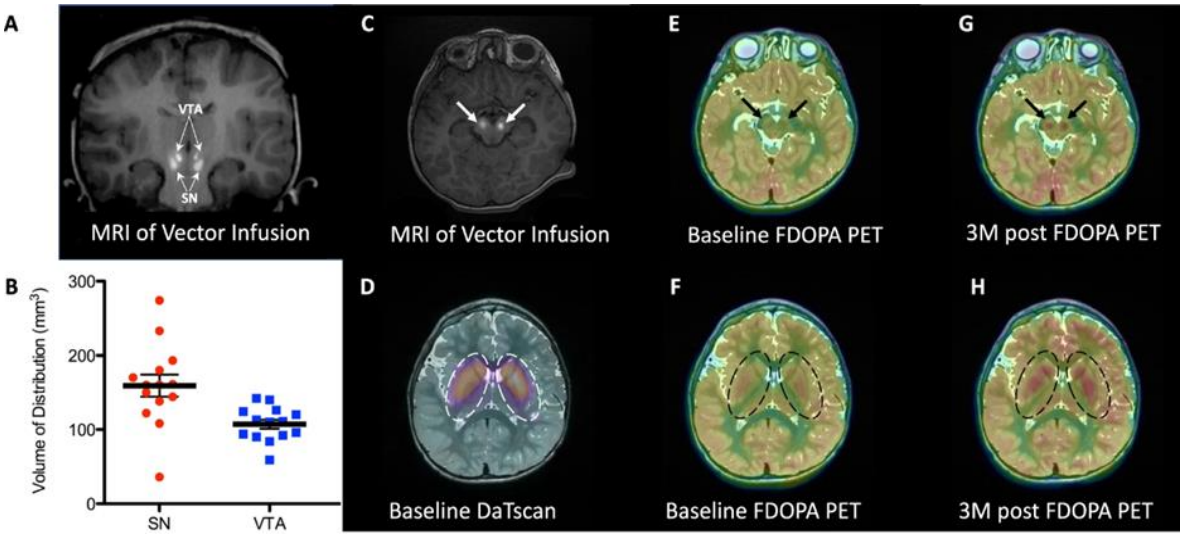

Fig. 7 *MR-guided delivery of AAV2-hAADC into the midbrain, baseline DaTscan and changes in FDOPA PET biomarker after gene delivery.*

Coronal (A) and axial (C) MR images after the vector infusions into SN and VTA regions (white arrows). The bright signal corresponds to gadoteridol admixed with AAV2-hAADC. Infusions are performed sequentially while imaging, starting with the right SN. Please, note accurate targeting and coverage in respective anatomical regions. B Coverage (volume of distribution, Vd) of all infusions performed into the SN and VTA in 7 subjects (n = 2 independent infusion sites (left and right) examined per participant (n = 7) for each target structure (SNc and VTA), for a total of n = 14 independent infusions per target structure). SN infusion (50 µL) achieved coverage of ~160 ± 60 mm3 (mean ± SD, n = 14 infusions (two infusions per participant)) with one suboptimal infusion due to a leak a withlong the perivascular space. VTA infusion (30 µL) resulted in coverage of 103 ± 22 mm3. Coverage volume of gadoteridol (Vd) suggests almost 80% anatomical coverage of both SN and VTA in all subjects (except single SN in one patient). D DaTscan imaging of the striatum at baseline confirmed a normal pattern of dopaminergic innervation in all study subjects, indicative of preserved nigrostriatal pathway. E, F Baseline FDOPA imaging of the midbrain regions (SN and VTA, black arrows in E) and nigrostriatal projection (caudate nucleus and putamen, dotted line in F). Lack of signal in both regions represses ents impaired conversion of FDOPA to F-dopamine due to absent AADC activity. G, H Increased FDOPA PET uptake 3 months after AADChAADC administration

in the midbrain and striatum, respectively. Images for Subject 4 are shown as representative of the group; see Supplementary Fig. S1 for images for each subject.[54]

Postoperative images at both month 3 and month 24 demonstrated increased absorption of FDOPA in the midbrain and in regions of the brain that receive dopaminergic projections from the midbrain. A diffuse increase in FDOPA signal was detected in both the putamen nucleus and the caudate (striatum) nucleus.

The detection of increased FDOPA absorption in the striatum is consistent with the hypothesis that the AAV2-AADC vector and the AADC protein both undergo anterograde axonal transport via the intact nigrostriatal pathway in nigrostriatal terminals with the enzymatic conversion of L-dopa to dopamine.

The concentration of HVA significantly increased 3 months after gene delivery compared to the average of the two basic measurements.

The concentration of 5-hydroxy indole acetic acid (5-HIAA) in the cerebrospinal fluid did not change after the release of the gene, consistent with expectations since serotonergic nuclei were not targeted in this procedure. 3-OMD was elevated in all tests and did not increase or decrease consistently after gene release (Figure 6).

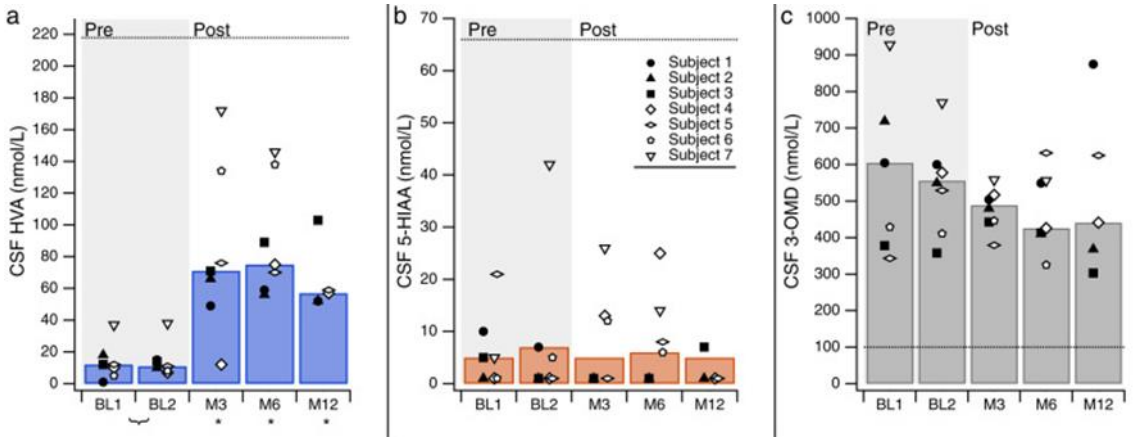

Fig. 8 *Concentrations of CSF metabolites measured at 2 separate baselines (BL) timepoints, Month 3, Month 6, and Month 12, in individual subjects in Cohort 1 (low-dose, black markers); and Cohort 2 (high-dose, white markers) and summarized at each time point as the group median (bars). A Homovanillic acid (HVA), the dopamine metabolite, was significantly higher at each post-operative point compared to the baseline mean (inverted bracket; (*p = 0.0078 at Months 3 and 6, p = 0.0313 at Month 12, one-tailed Wilcoxon signed-rank test). The lower limit t of the normal range: 218 nmol/L (dotted linhydroxy indole acetic acid (5-HIAA), the serotonin metabolite, did not change after gene The lower. The lower limit is of the normal range: 66 nmol/L (dotted line). C 3-O-methyldopa (3-OMD) was elevated in all subjects at time points. Normal: <100 nmol/L (dotted line).*[54]

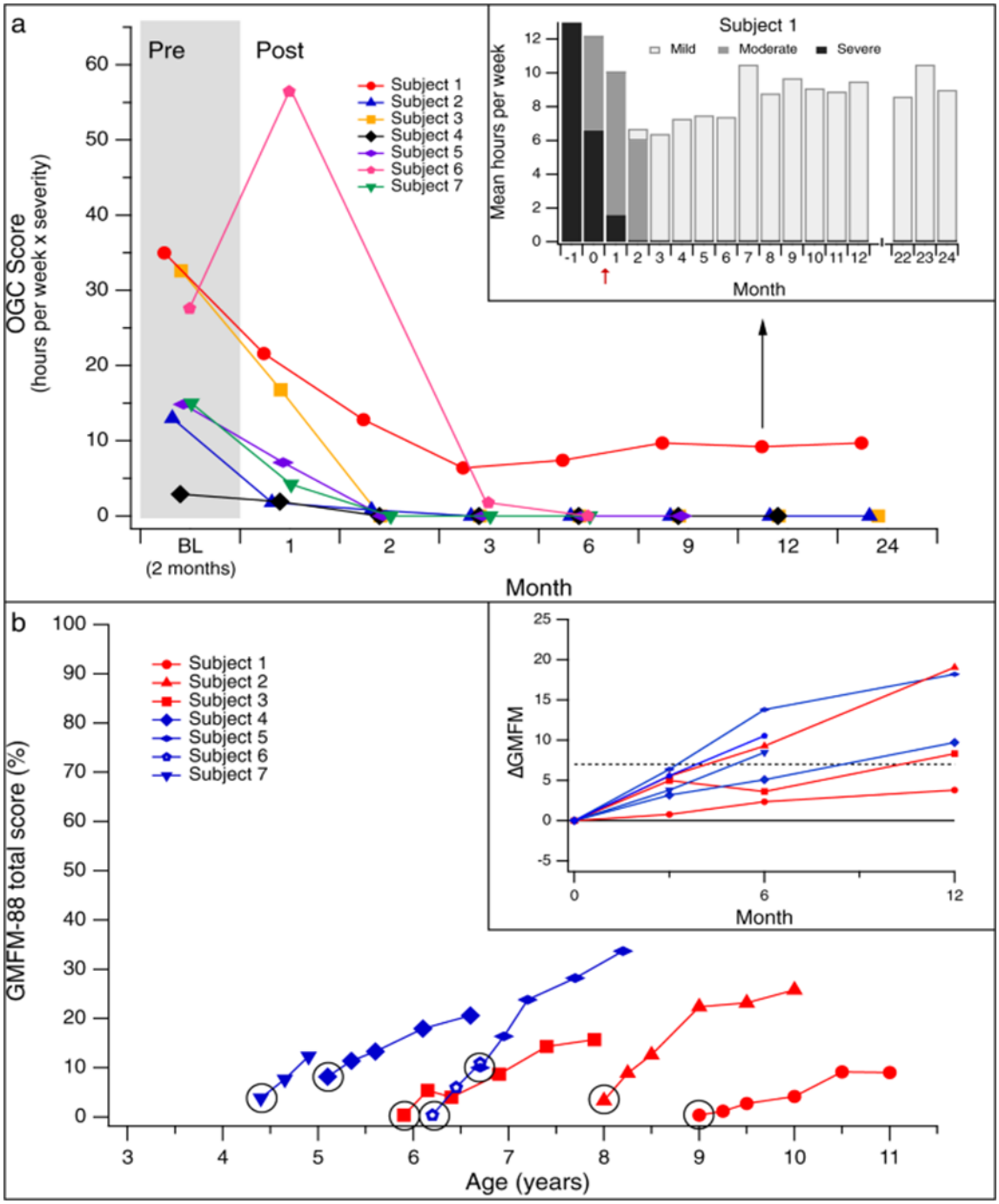

Fig. 9 *Changes in Oculogyric Crises (OGC) and motor function after gene delivery. The OGC score was calculated monthly and represents the weekly average of the duration of episodes (hours per week), weighted by severity (grade 1–3: 1 = mild/eye deviation only; 2 = moderate/eye deviation + dystonia or dyskinesia of the face and/or neck; 3 = severe/dystonia or dyskinesia involving the trunk and/or limbs). By Month 3, OGCs were*

completely resolved in 6/ 7 subjects. Inset (black arrow): Subject 1 had residual episodes throughout the 24-month study period; the severity decreased after surgery (red arrow). B Gross Motor Function Measure-88 (GMFM-88) scores for Cohorts 1 (red) and 2 (blue). Data are shown through Month 24 for Subjects 1–3, Month 18 for Subjects 4 and 5, and Month 6 for Subjects 6 and 7. Baseline GMFM scores for all subjects (circled) were ≤10, consistent with severe motor impairment. Inset: changes in GMFM score between Baseline and Month 12. An increase of ≥7 points (dotted line, representing a clinically meaningful positive change) was observed in 6/7 subjects by Month 12. Source data are provided as a Source Data file.

Motor function was assessed using the measure of gross motor function (GMFM-88), a standardized tool designed to assess changes in motor function over time in children with motor impairment due to cerebral palsy.[62,63,64]

All subjects achieved recognizable gains in motor function after the procedure, namely an increase in tone and improvements in head and trunk control. The rate of improvement varied considerably from one subject to another.[65,66,67]

A marked improvement in sweating, feeding difficulties (such as vomiting), and upper airway obstruction due to abundant oral secretions, nasal congestion, and stridor have also been reported.

4. AIM OF THE STUDY

We aimed to evaluate the utility of a minimally invasive method -dried blood spots (DBS)- to evaluate the levels of 3-OMD for AADC deficiency detection.

5. PATIENTS AND METHODS

5.1 Patients' selection

Children aged 0-18 years with a global developmental delay of undetermined cause were recruited from Tertiary Pediatric Neurological Centres through a National Collaboration. Exclusion criteria included confirmed genetic diagnosis, well-established metabolic defect, or structural brain magnetic resonance imaging (MRI) abnormalities able to explain the clinical picture. Clinical and treatment data were retrieved through a structured questionnaire addressed to the referring clinicians.

5.2 Sample collection

DBS for the dosage of 3-OMD can be performed both on capillary and venous blood. It must be taken into account, however, that it is not possible to use blood-containing anticoagulants such as heparin or citrate.

To take the sample correctly, you must collect the venous blood, insert it into a test tube and then mix it gently.

It is necessary to apply a single drop of blood to the center of each dotted circle, making sure that the blood is well distributed covering the entire area delimited by the dotted circle, and that it spreads on both sides of the filter paper.

To preserve enzymatic activity, samples should be dried at room temperature for four hours, keeping the cardboard away from direct heat and light sources. In addition, to avoid contamination, the cards should not be stacked after collecting the sample.

Incorrect sample collection can result in a false positive or compromise the reliability of the test.

Once the blood has dried, you must cover the bands with the appropriate flap of paper and insert the kit inside the bag.

The cards are used to evaluate the values of 3-OMD through tandem mass spectrometry. In case of detected abnormal levels of 3-OMD, second-tier tests including next-generation sequencing (NGS) of the dopa decarboxylase gene (DDC) are performed. Eventually, the deletion/duplication analysis can be performed if no mutation is identified via NGS.

5.3 Statistical analysis

The 3-OMD values of each patient were sorted according to the age groups of the children who participated in the study (up to 6 months/ from 6 months-1 year/ 1 -2 years/ 2 - 6 years/ > 6 years).

6. RESULTS

6.1 Demographic features

58 children (30 females) with a developmental delay were recruited between May 2021 and May 2022 at the Pediatric Neurology and Muscular Diseases Unit of the IRCCS Istituto Giannina Gaslini, (Genoa), U.O.D. of Pediatric Neurology Sapienza University of Rome - Faculty of Medicine and Surgery, S.O.C of Child Neuropsychiatry of the Cesare Arrigo Children's Hospital of Alessandria, Pediatric Clinic IRCCS Policlinico S. Matteo di Pavia, Section of Neuropediatrics, Pediatric Clinic of Pisa, AOUP.

The mean age was 3.2 years (0.66-5).

Family history for neuropsychiatric disorders was positive in 7/58 (12%) cases, while a negative family history was reported in 51/58 cases (88%).

Only two children were born to consanguineous parents.

6.2 Clinical features

All 58 patients had DD and symptom onset was around 10 months.

On physical examination, 14 (24.1%) patients had microcephaly, in 11/58 (18.9%) cases microcephaly was present from birth (primary microcephaly), while in 3/58 (5.2%) it was acquired (secondary microcephaly).

There are 18 patients (31.5 %) with seizures of which 15 (26.3%) were tonic-clonic and 3 (5.2%) seizures with impaired awareness not better specified.

Only 8 patients (14%) had a diagnosis of autistic spectrum disorder.

At the neurological evaluation, 50 patients (86.2%) showed signs of hypotonia, which is 23/58 (38.6%) cases was axial and in 27/58 (45.6%) cases was global. Nystagmus was found in 15 (26.3%) patients, while 3 (5.2%) had oculogyric crises.

About the neuro vegetative sphere, 16 (28%) patients experienced autonomic dysfunction, and only 4 (7%) patients had sleep disorders present.

Below are the graphs with the percentages of clinical and neurological symptoms.

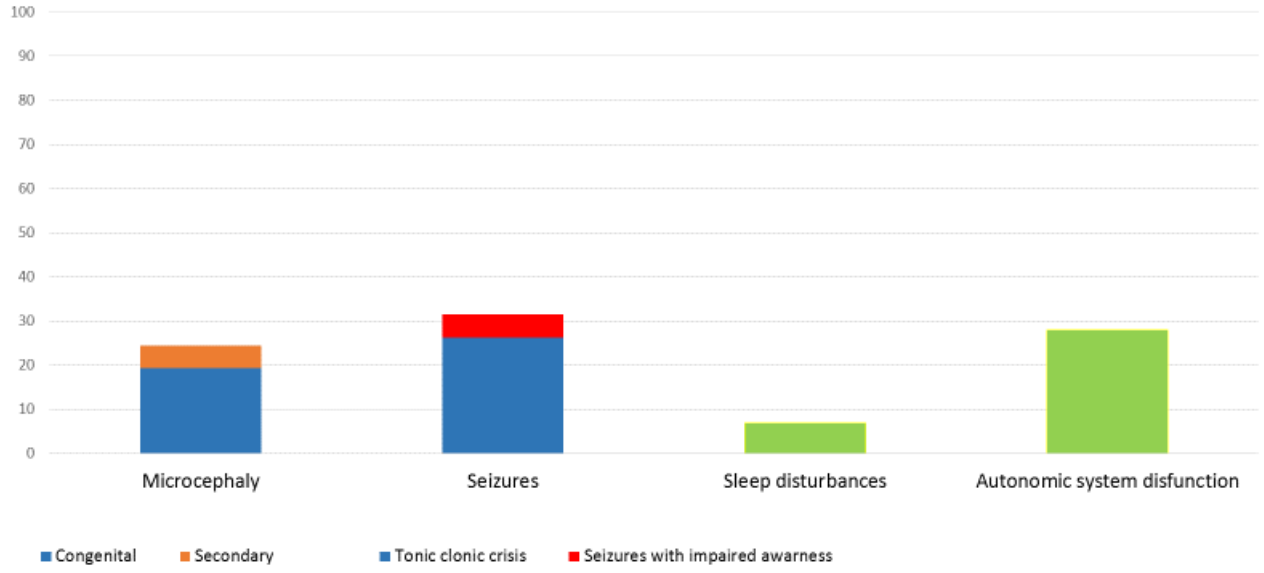

Fig. 10 *Clinical and neurological symptoms of the court of patients*

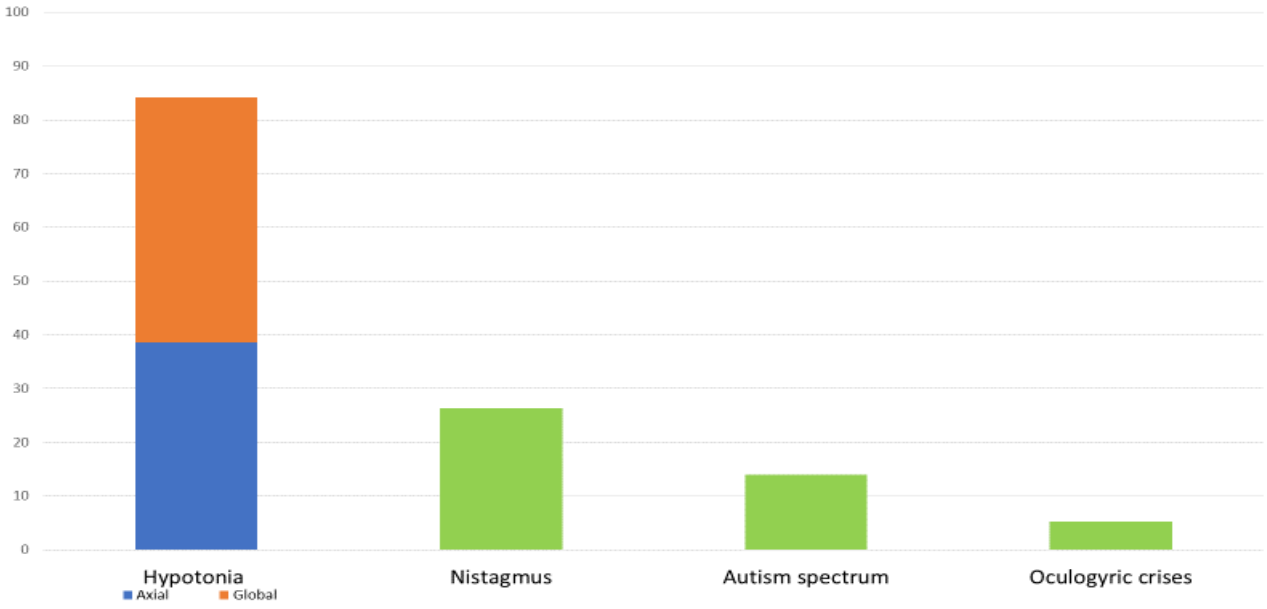

Fig. 11 *Clinical and neurological symptoms of the court of patients*

6.3 3-OMD values

The values of 3-OMD grouped by age:

- up to 6 months mean (range): 427 (373-481) nmol/L
- from 6 months - 1 year mean (range): 219 (120-392) nmol/L
- 1 -2 years mean (range): 168 (34-312) nmol/L
- 2 - 6 years mean (range): 187.7 (43-424) nmol/L
- 6 years mean (range): 62.4 (30-83) nmol/L

Only one child had 3-OMD values above the normal range (<1000 nmol/L).

For this reason, it was further investigated with second-tier tests. The remaining 57 patients tested negative, except one who has received the indication to re-dose for evidence of values of 3-OMD above normal.

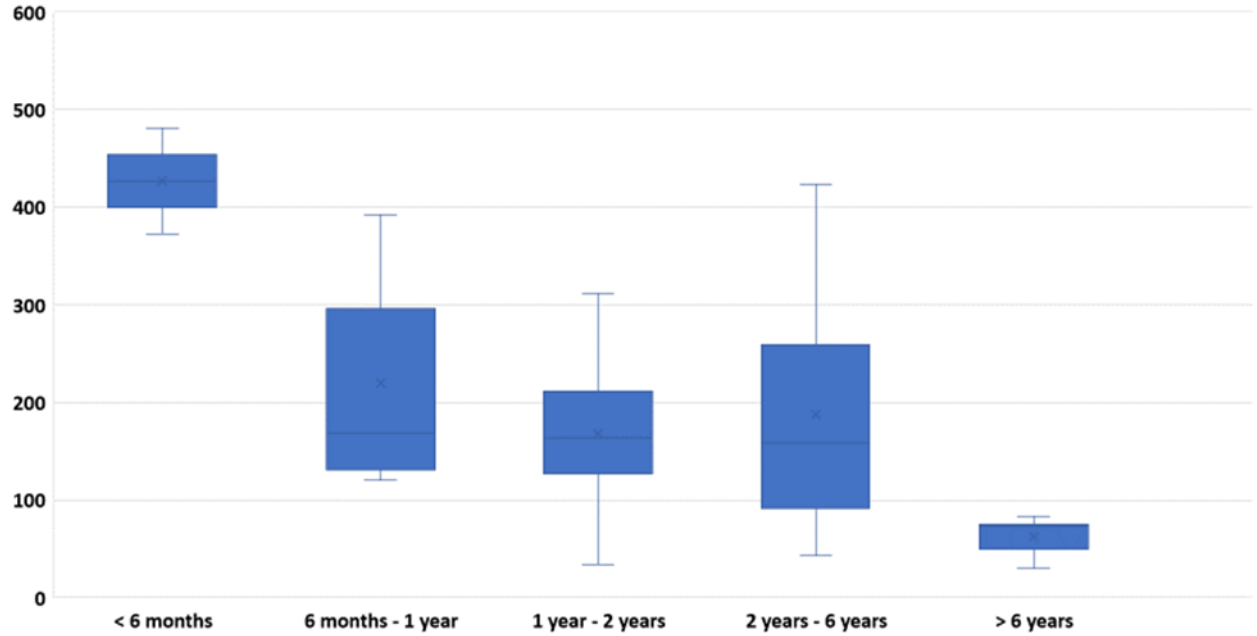

Fig.12 3-OMD values (nmol/L) by age group

6.4 Case report

Patient #57 was born from normal pregnancy at 38 weeks + 6 days of gestation. At birth he had: Apgar 9/10, weight 3090 gr, length 51 cm, and head circumference 34 cm.

He had a positive family history for neuropsychiatric disorders, including a case of autism spectrum disorder and mild language delay from the paternal line.

He began to show the first symptoms at 5-6 months due to a lack of control of the head, for which he was hospitalized for investigations.

The neurological examination revealed diffuse hypotonia associated with tonic fluctuations in the upper limbs and trunk. Hypoelicitable osteotendinous reflexes in the four limbs and plantar skin reflex in bilateral flexion.

Head control was incomplete, and no parachute reflexes were observable. The pupils were isocyclic, normally reactive to light stimulation. Gaze revulsion movements known as oculogyric crises were observed. Visual engagement and pursuit with gaze was present. Social smiles associated with vocalizations were also observed.

At the visit, a picture of mild to moderate psychomotor retardation was evident, accompanied by autonomic symptoms such as hyperhidrosis and fluid dysphagia.

On imaging, MRI showed only a picture of thinning of the corpus callosum, while on the EEG, diffuse or more frequent slow abnormalities on the bi-hemispheric occipital regions were detected.

The picture suggested the dosage of 3-OMD performed on a DBS card that has been executed at 1 year old. After the result of an increased (4314 nmol/L) level of 3-OMD, patient #57 was subjected to further diagnostic tests, through both the assay of the neurotransmitter metabolites on CSF and DNA extracted from peripheral blood giving a positive genetic result with a homozygous state for the mutation c.749C> T p. (Ser250Phe) in the exon 7 of the *DDC* gene, confirmed by the segregation analysis.

7. CONCLUSIONS

AADCd is a rare neurometabolic disease that, due to similarities with other diseases (e.g., epilepsy, cerebral palsy), is often accompanied by diagnostic delay.

The studies conducted have made it possible to understand the importance and effectiveness of the dosage of 3-OMD levels as a first-level method for reaching the diagnosis of AADCd in children presenting with the first symptoms. In fact, our work has allowed to early identify, within 1 year of life, an affected patient who has received the diagnosis of AADCd through appropriate in-depth investigations, making him the first Italian patient eligible to gene replacement therapy.

Our study also shows that the levels of 3-OMD clearly decrease over time in the various age group, eventually making the dosage of the metabolite less accurate for the diagnosis in elder children. With this in mind, it is pivotal to propose this screening as early as possible, eventually and ideally as new-born screening.

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