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L-AMINO ACID DECARBOXYLASE DEFICIENCY: INDICATIONS FOR EARLY DIAGNOSIS AND INSIGHTS ON MANAGEMENT STRATEGIES

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A mamma, papà e Gio

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1. INTRODUCTION

1.1 The *DDC* gene

Aromatic L-amino acid decarboxylase deficiency (AADC-deficiency, AADCD, OMIM # 608643) is a rare autosomal recessive disorder caused by biallelic pathogenic variant in *DDC*. The AADC enzyme, whose co-factor is pyridoxal phosphate, is responsible for catalysing the final step in the synthesis of the monoamine neurotransmitters, serotonin and dopamine. Its deficiency in the brain causes low levels of these molecules. Additionally, the levels of norepinephrine and epinephrine are also affected since dopamine is their precursor. The lack of these neurotransmitters is directly responsible of the clinical features observed in this neurometabolic disease.

DDC is located on chromosome 7p12.3 -p12, consists of 15 exons, and encodes a protein of 480 amino acids with a molecular mass of 53.9 kD. More than 50 pathogenic variants have been correlated with AADCD[1]. AADC, also known as DOPA decarboxylase (DDC) or tryptophan decarboxylase or 5-hydroxytryptophan decarboxylase, is a lyase which is active as a homodimer and needs pyridoxal phosphate (PLP) as cofactor[10]. Before the addition of the pyridoxal phosphate cofactor, the apoenzyme exists in an open conformation. Upon cofactor binding, a large structural transformation occurs as the subunits pull closer and close the active site. This conformational change results in the active, closed holoenzyme[7].

In the active state of the enzyme, PLP is bound to lysine-303 of AADC as a Schiff base. Upon substrate binding, Lys-303 is displaced by the substrate's amine group and the carboxylate of the PLP is positioned within the active site, thus favouring decarboxylation.

This reaction produces a quinonoid intermediate, which is subsequently protonated to produce a Schiff base adduct of PLP and the decarboxylated product. Lys-303 can then regenerate the original Schiff base, releasing the product while retaining PLP. Probing this PLP-catalysed decarboxylation, it has been discovered that there is a difference in concentration and pH dependence between substrates. DOPA is optimally decarboxylated at pH 5.7 and a PLP concentration of 0.125 mM, while the conditions for optimal 5-HTP decarboxylation were found to be pH 8.3 and 0.3 mM PLP. In PLP-deficient murine models, it has been observed that dopamine levels do not significantly deviate from PLP-supplemented specimens. However, the concentration of serotonin in the deficient brain model was significant. This variable effect of PLP-deficiency indicates possible isoforms of AADC with differential substrate specificity for DOPA and 5-HTP. Dialysis studies also suggest that the potential isoform responsible for DOPA decarboxylation has a greater binding affinity for PLP than that of 5-HTP decarboxylase[11].

AADC regulation, especially as it relates to L-DOPA decarboxylation, has been extensively studied. AADC has several conserved protein kinase A (PKA) and protein kinase G recognition sites, with residues S220, S336, S359, T320, and S429 acting as potential phosphate acceptors. *In vitro* studies have confirmed that PKA and PKG can both phosphorylate AADC, causing a significant increase in activity. In addition, dopamine receptor antagonists have been shown to increase AADC activity in rodent models, while activation of some dopamine receptors suppresses AADC activity. Such receptor mediated regulation is biphasic, with an initial short-term activation followed by long-term activation. The short-term activation is thought to proceed through kinase activation and subsequent phosphorylation of AADC, while the sensitivity of long-term activation to protein translation inhibitors suggests a regulation of mRNA transcription.

1.2 Metabolic pathway

AADC catalyses several different decarboxylation reactions:

- **L-DOPA** → *dopamine* (a neurotransmitter)
- **L-Phenylalanine** → *phenethylamine* (a trace amine which functions as a neuromodulator)
- **L-Tyrosine** → *tyramine* (a trace amine neuromodulator)
- **L-Histidine** → *histamine* (a neurotransmitter)
- **L-Tryptophan** → *tryptamine* (a trace amine neuromodulator)
- **5-HTP** → *serotonin* (5-hydroxytryptamine, a neurotransmitter)

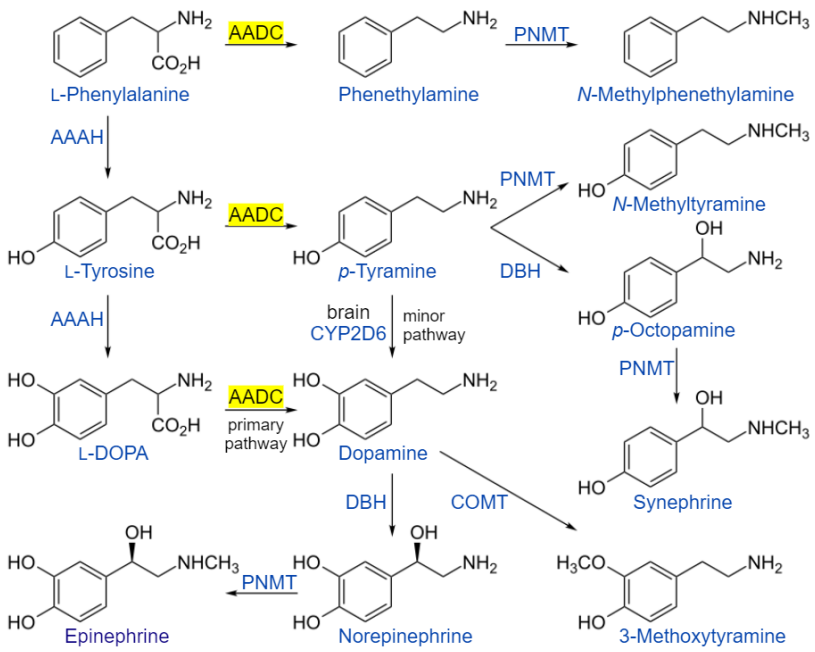

Figure 1 AADC metabolic pathway (part 1)

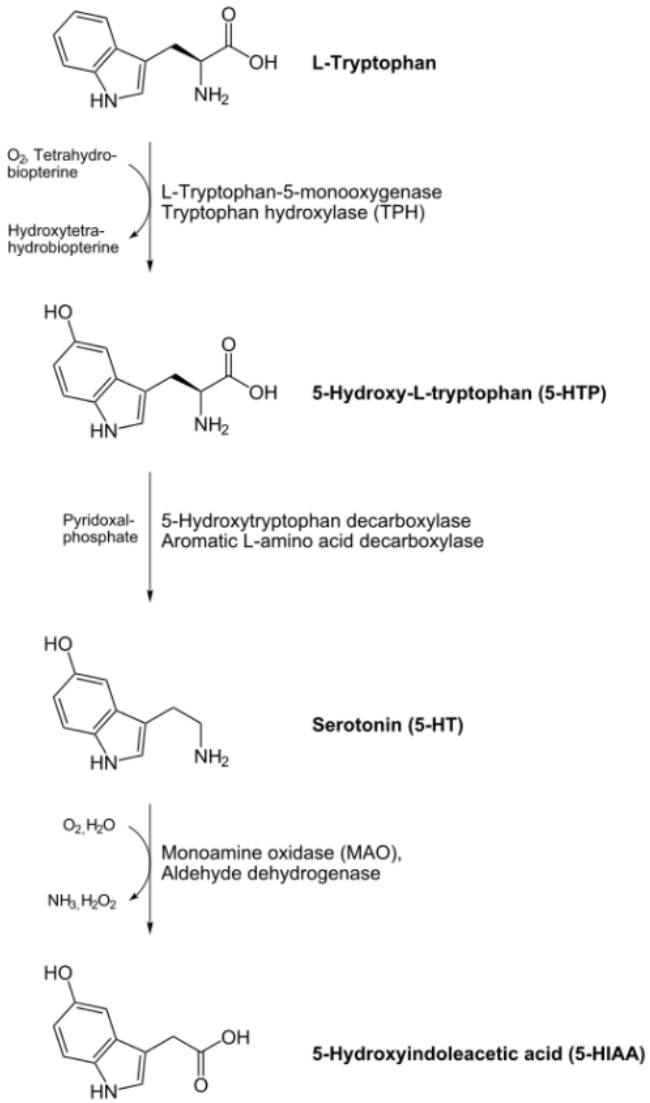

Figure 2 AADC metabolic pathway (part 2)

However, some of these reactions do not seem to bear much or any biological significance. For example, histamine is biosynthesised strictly via the enzyme histidine decarboxylase in humans and other organisms.

1.3 Clinical aspects

AADC deficiency is a rare recessive autosomal neurometabolic disorder. Since the enzyme is involved in the synthetic pathway of dopamine, serotonin and, ultimately, epinephrine and norepinephrine, the clinical presentation is heterogeneous. Low levels of dopamine affect voluntary movements, emotion, cognitive function, and the release of various hormones. Norepinephrine and epinephrine deficiency affects attention, mood, sleep patterns, cognition, and stress hormone levels. The lack of serotonin, which is a substrate for melatonin, can affect gastrointestinal function, the respiratory and cardiovascular systems, circadian rhythm, body temperature, and the sensation of pain and mood.

AADC-deficient patients usually show symptoms during the first months after birth, usually at 3 months according to *Pearson et al., 2020*. The most common initial symptoms are hypotonia, oculogyric crises, developmental delay and feeding problems. Non-motor problems are also sleepiness, irritability, excessive sweating, and nasal congestion[2]. Since one of the most prominent and consistent features are oculogyric crises, AADC-deficient patients (especially if small children) tend to be misdiagnosed with an epileptic disorder and it is not uncommon that these subjects undergo antiepileptic drug treatments, resulting in the lack of benefit in most cases. Epilepsy must be always ruled out in these patients, despite the fact that some of them also suffer from seizures. Therefore, in most cases epilepsy and AADCD should be considered as mutually excluding, although they may coexist in some patients[5]. According to *Wassenberg et al.*, the phenotypical spectrum is broad, but can be roughly divided into mild (mild delay in developmental milestones, ambulatory without assistance, mild intellectual disability), severe (no or very limited developmental milestones,

fully dependent), and moderate (in between). Most patients tend to present with a severe phenotype, are often bedridden, and rely on their caregivers for their day-to-day life.

Since the first report in 1990[13], only 135 patients have been identified so far, but many affected individuals are likely to be undiagnosed. Nearly half of the reported patients are Asian, from Taiwan and Japan in particular, which is likely explained by a founder mutation in these populations. The most common *DDC* variant identified in Taiwanese subjects and in the whole cohort of AADC-deficient patients is the c.714+4A>T. This variant is always associated with a severe phenotype and is the only variant with a clear genotype-phenotype correlation. Other common variants are S250F, with an allele frequency of 10%, and G102S, with an allele frequency of 8% [8]. Currently, more than 80 different types of pathogenic variants have been identified[10]. However, the severity of the disease depends not only on the variant type, but also on the inheritance pattern (homozygosis vs compound heterozygosis).

Clinical phenotype and response to treatment are variable, and the long-term and functional outcome is far from being clearly established. A patient registry was established by the non-commercial International Working Group on Neurotransmitter Related Disorders (iNTD) to provide a basis towards the improvement of the understanding of epidemiology, genotype–phenotype correlations, outcome, impact on the quality of life, and diagnostic and therapeutic strategies.

1.4 Diagnosis

According to the guidelines written by *Wassenberg et al. 2017*, AADC deficiency can be diagnosed through clinical, metabolic, and genetic tests.

More specifically, these guidelines suggest performing *at least two tests* among:

- **CSF testing**
- **AADC enzyme plasma activity**
- **Genetic testing**

If the first two tests are performed and are positive, *Wassenberg et al*.[1] suggest a further confirmation with genetic testing, which is ultimately crucial in any case. More recently, *Brennenstuhl at al. 2019* proposed a new diagnostic test on dried blood spots which analyses the concentration of 3-O-methyldopa[3]. This test is presented as a neonatal screening and its main purpose is to identify AADCD as early and as minimally invasive as possible. In 2020, the same group proposed a new diagnostic test consisting of measuring the concentrations of VLA and VMA in urine samples[4]. Their ratio is considered reliable for the diagnosis of AADC deficiency.

1.5 Purpose of the study

We aimed to investigate the presence of pathogenic variants in *DDC* in patients with neurodevelopmental conditions with heterogeneous clinical presentation through the critical revision of genomic data obtained from exome sequencing. Additionally, the current guidelines for AADC diagnosis date back to 2017 and are not updated. Therefore, a further aim of this study was to update these guidelines and suggest a diagnostic flowchart based on the most recent clinical and genetic findings.

2. METHODS

2.1 Study rationale

AADC deficiency comes with a burden of disability. Many patients tend to have a severe form. Even though some of them stabilize, a regression of motor and developmental skills with age is observed in others. On the other hand, patients with milder phenotypes seem to improve with age and tend to have a better response to symptomatic drugs[23]. However, these subjects represent a very small percentage of AADC-deficient patients. AADC deficiency is an extremely rare disease, with an estimated prevalence at birth of 1:90.000 in the US, 1:118.000 in Europe and 1:182.000 in Japan[36], but it is still important to search for it, since gene therapy can modify the natural course of this disorder. Indeed, early diagnosis means early treatment, better care, and possible improvement of their quality of life and outcome. AADC deficiency may present with similar clinical manifestations to other neurodevelopmental conditions and developmental and epileptic encephalopathies (DEEs) (e.g., movement disorders, oculogyric crisis, early developmental delay, and behavioural problems). Also, brain MRI may be normal in line with what observed in some of these conditions. Considering this common clinical and radiological presentation, we reanalysed ES data to detect the possible presence of *DDC* variants in our cohort, since the early adoption of the appropriate management may significantly and positively affect the clinical course of AADC. The purpose of our study was to identify though Next Generation Sequencing techniques children affected by AADCD, treat them with the current symptomatic drugs available, and evaluate if they may be eligible for gene therapy.

2.2 Study design

This is a single-centre retrospective study, non-interventional, no-profit, non-randomized. This study was approved by the Regional Ethical Committee on 1st March 2021. We screened a cohort of 1,200 patients with neurodevelopmental conditions and DEEs for variants in the DDC gene. This cohort included patients aged 2-11 years presenting with non-lesional complex neurological conditions affecting psychomotor development and cognitive performances, who were previously investigated through exome sequencing (ES) for the identification of the underlying genetic cause. Indeed, despite ES led to the identification of causative variants in many cases, other individuals remained undiagnosed. The presence of *DDC* variants was therefore investigated in these subjects without a specific genetic diagnosis with the purpose of identifying possible causative pathogenic variants.

2.3 Inclusion and exclusion criteria

Inclusion criteria:

The inclusion criteria used in this study are the same of the previous study (003.003.152):

- A clinical diagnosis consistent with a neurodevelopmental condition or DEEs (according to international criteria based on ILAE classification of epileptic syndromes);
- Informed consent provided from the parents or legal guardians of the enrolled subjects

Exclusion criteria:

- Confirmed genetic diagnosis involving pathogenic variants in different known disease genes;
- Brain MRI supportive of a secondary neurological condition with a lesional/structural aetiology.

2.4 Endpoints

The endpoints of the current study are:

- Identification of patients with genetically confirmed AADC deficiency;
- Electro-clinical evaluation of identified patients;
- Genotype-phenotype correlations (if possible)

2.5 Genetic data analysis

Candidate variants in *DDC* were screened according to allele frequency, the involvement of conserved amin acid residues was evaluated through GERP (http://mendel.stanford.edu/SidowLab/downloads/gerp/), and the impact of variants on protein function was assessed through several *in silico* tools.

The identified variants were filtered according to allele frequency (<0.001) in frequency databases, including gnomAD (https://gnomad.broadinstitute.org), dbSNP (https://www. ncbi.nlm.nih.gov/snp/), and Exome Aggregation Consortium–Exac

(http://exac.broadinstitute.org). ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) and pertinent literature were also screened.

The predicted impact on protein function was assessed through several *in silico* tools, including PolyPhen-2 (http://genetics.bwh.harvard.edu/ pph2/), DANN (https://cbcl.ics.uci.edu/public_data/DANN/), Mutation Taster (http://www.mutationtaster.org/), and Combined Annotation Dependent Depletion (CADD) (https://cadd.gs. washington.edu/snv). Candidate variants were eventually classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines[37].

2.6 Diagnostic Flowchart

To provide a valuable tool for the early identification of affected individuals based on clinical, radiological, metabolic, instrumental, and genetic data, we also developed a flowchart with a potential huge impact on the facilitation of the diagnostic process. In fact, with this purpose in mind, we decided to implement the current guidelines by *Wassenberg et al*. with the most recent findings, including the work of Brennenstuhl[3,4]. In 2020, *Pearson et al.* evaluated 63 confirmed AADC-deficient patients, thus reporting one of the largest and most recent cohorts of affected individuals. The patients and their caregivers were interviewed in order to delineate the characteristics and frequency of their clinical manifestations and collect the relative data.

Hypotonia	75 %	3
OGC	62 %	3
Developmental delay	62 %	3
Feeding problems	54 %	2
Sleepiness	50 %	2
Irritability	45 %	2
Excessive sweating	45 %	2
Nasal congestion	42 %	2
Stiffness	35 %	1
Insomnia	31 %	1
Temperature instability	29 %	1
Involuntary movements	25 %	1
Diarrhoea	13 %	0.5
Hypoglycaemia	9 %	0.5

Table 1: initial symptoms[2]

This table (Table 1) lists the symptoms identified by *Pearson and colleagues*. Each symptom, if present, appeared in the first year of life, with a mean onset at 2.7 months. Based on this collection, we decided to create a score to divide the patients into three categories:

- Very likely;
- Possible;
- Unlikely to be AADC-deficient patients.

Since the first three symptoms are the most common, with a frequency > 60% in the first year of life, we suggested 3 points for each. We assigned 2 points to symptoms with a frequency between 40 and 59 %, 1 point to symptoms with a frequency between 20 and 39 %, and 0.5 points to symptoms with a frequency under 20% in the first year of life. If the

score is ≥ 9 (e.g., the patient has just the first three symptoms), we consider him/her very likely to be AADC-deficient and a specific diagnostic workup is indicated. If the score is between 5 and 9, we consider the subject to be a possible AADC-deficient patient, whilst if the score is < 5, the patient is unlikely to be AADC-deficient. In addition to clinical aspects, we also considered anamnestic data, metabolic, genetic and instrumental findings. Since the age of onset for all the patients in this cohort is < 1 year of life, with a mean of 2.7 months, we gave an extra point to each symptom that occurred in the first year of life and 2 extra points if it occurred before 6 months. We also added one point based on the specific ethnicity (if the patient is from Taiwan, China, and Japan), because of the known presence of a founder mutation in *DDC* in these populations, and another point if the parents are consanguineous. If the child has older affected siblings and presents with one clinical feature, we considered him/her at high risk and neonatal screening on dried blood spot was indicated. One out of four AADCD patients may show brain MRI abnormalities (especially cerebral atrophy or white matter involvement). Therefore, if available, brain MRI may be helpful in the diagnosis. (Table 2)

Symptoms/signs	Prevalence	Points	Symptom onset: - +1 if it occured < 1 year of life - +2 if it occurred < 6 months of life
Hypotonia	75 %	3	+1 / +2
OGC	62 %	3	+1 / +2
Developmental delay	62 %	3	+1 / +2
Feeding problems	54 %	2	+1 / +2
Sleepiness	50 %	2	+1 / +2
Irritability	45 %	2	+1 / +2
Excessive sweating	45 %	2	+1 / +2
Nasal congestion	42 %	2	+1 / +2
Stiffness	35 %	1	+1 / +2
Insomnia	31 %	1	+1 / +2
Temperature instability	29 %	1	+1 / +2
Involuntary movements	25 %	1	+1 / +2
Diarrhoea	13 %	0.5	+1 / +2
Hypoglycaemia	9 %	0.5	+1 / +2
Ethnicity (Taiwan. China, Japan)		1	
Consanguinety		1	
Siblings affected (genetically identified)		6	
Metabolic findings - **VLA/VMA ratio on urine sample** - **3OMD blood spot**		 2 9	
Genetic findings		If positive, diagnosis is made	
Brain MRI		Normal : 1 Cerebral atrophy/white matter abnormalities: 2	
TOTAL			= _____

Table 2: diagnostic score

Through the collection of these data, we developed a complete and highly reliable diagnostic score for AADCD (Figure 3).

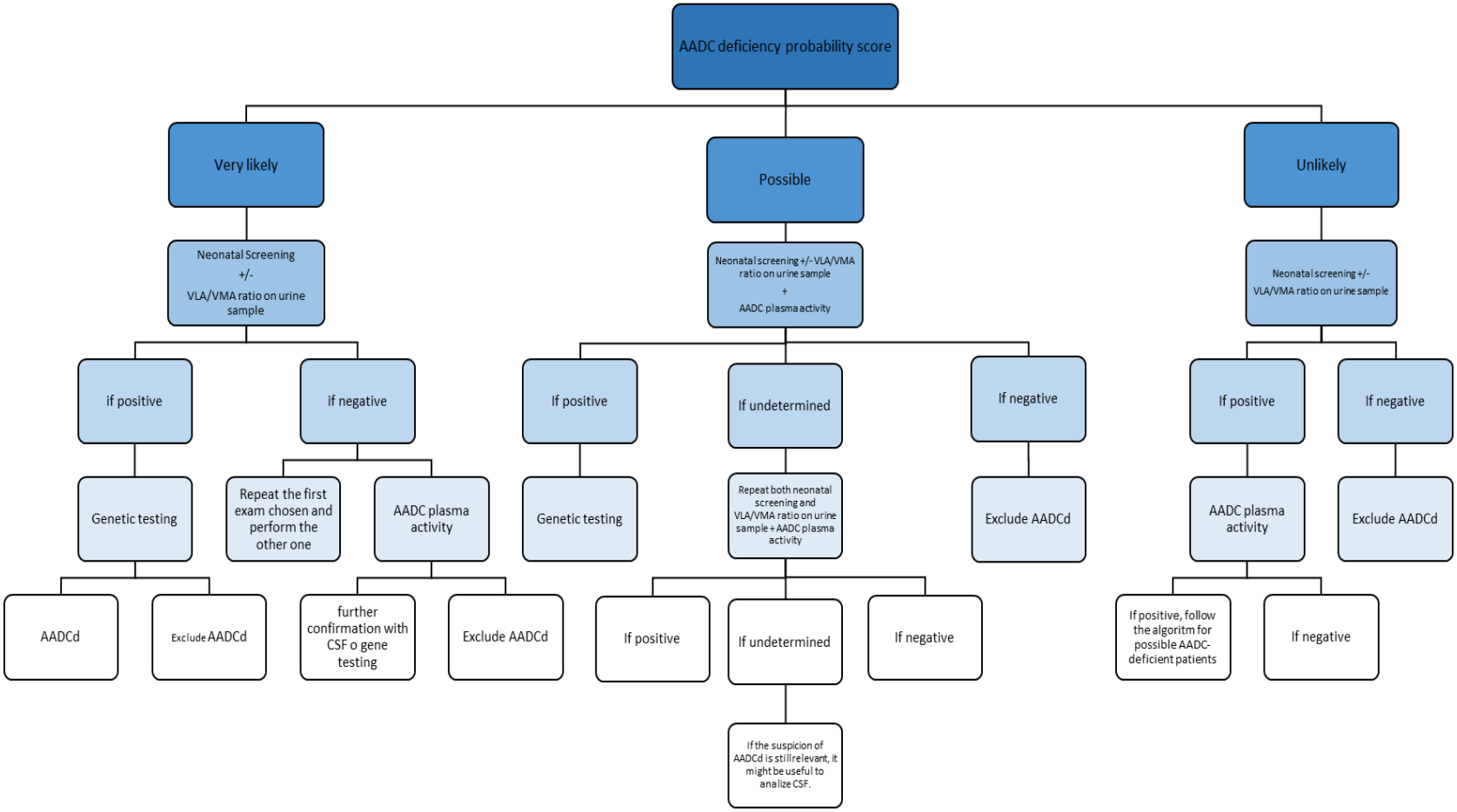

Figure 3 diagnostic flowchart

3. RESULTS

3.1 Phenotypic features of the studied cohort

The population in which *DDC* variants were detected included individuals presenting with a neurodevelopmental condition with or without epilepsy and/or syndromic features, whose phenotypic features were included in the AADCD spectrum. The epileptic phenotype encompassed focal (symptomatic or asymptomatic) and/or generalized seizures, often in the context of an underlying DEE. Syndromic features, when present, consisted of dysmorphic facial features, brain MRI abnormalities (e.g., white matter abnormalities, cerebellar hypoplasia, abnormal gyration), and additional multisystem manifestations (e.g., gastrointestinal symptoms, endocrinological abnormalities, cardiovascular involvement). Global developmental delay and/or intellectual disability was present in most subjects, especially in patients with DEEs. In these subjects, psychomotor regression was also observed occasionally. Additional neuropsychiatric involvement consisted of abnormal tone (hypotonia or appendicular spasticity), movement disorders (e.g., tremors, dyskinesia, choreic movements, dystonia), behavioural problems (e.g., irritability, autism spectrum disorder, psychotic manifestations), and sleep disorders (e.g., hypersomnia/insomnia).

3.2 Genetic variants characterization

Reanalysis of WES data led to the identification of *DDC* variants in 2 subjects, both lacking a specific genetic diagnosis.

One individual (ITAIGI-16-S-0158) harboured two compound heterozygous variants in DDC (NM_000790.4): c.436-12T>C and c.435+24A>C. The c.436-12T>C variant has a GnomAD exomes allele frequency = 0.00631 (exomes coverage = 76.3), which is greater than 0.00335 (threshold derived from the 138 clinically reported variants in gene DDC). The variant is reported in 1,584 subjects in heterozygous state and in 8 subjects in homozygous state. Although affecting a conserved residue (GERP 4.88), it is predicted benign by *in silico* tools (CADD score 12.47) and does not directly affect splicing. According to the ACMG/AMP criteria, this variant is therefore classified as benign (class I: BS1, BS2, BP4, BP6). The c.435+24A>C variant has a GnomAD exomes South Asian allele frequency = 0.00637 (exomes coverage = 94.5), which is greater than 0.00335 (threshold derived from the 138 clinically reported variants in gene DDC). This variant was observed in 370 individuals in heterozygous state and in 4 individuals in homozygous state. It has benign predictions in terms of pathogenicity (CADD score 0.13) and is classified as benign (class I: BS1, Bs2, and BP4).

The other subject (ITAIGI-16-S-0621) was found to harbour three distinct variants in homozygous state: c.1385G>A; p. (Arg462Gln), c.234C>T; p. (Ala78=), and c.201+37A>G. The p. (Arg462Gln) is a missense variant has a GnomAD exomes African allele frequency = 0.153 (exomes coverage = 73.7), which is greater than 0.05 threshold. It has a low predicted pathogenic impact (CADD score 1.13), is reported as benign in the ClinVar database, and is classified as benign according to the ACMG/AMP criteria (class I: BA1, BP4, BP6, PP2). The p. (Ala78=) variant is a synonymous variant with GnomAD

exomes African allele frequency = 0.139 (exomes coverage = 71.9), greater than 0.05 threshold. It has partial pathogenicity (CADD score 16.1) but is classified as benign in ClinVar and according to ACMG/AMP criteria (classI: BA1, BP4, BP6, and BP7). Eventually, the c.201+37A>G variant has GnomAD exomes allele frequency = 0.0116 (exomes coverage = 81.9), which is greater than 0.00335 (threshold derived from the 138 clinically reported variants in gene DDC). It is observed in healthy adults (heterozygous in 2,895 and homozygous in 37), has a low CADD score (0.3), and is considered benign (class I: BS1, BS2, and BP4).

In line with the abovementioned considerations, none of the detected variants should be considered as possibly causative of the clinical phenotype observed in the two subjects.

3.3 Phenotype of the patients harbouring DDC variants.

Patient 1

The patient carrying the compound heterozygous variants in *DDC* (ITAIGI-16-S-0158) presented with global psychomotor delay with incontinence. She was born at 38th weeks, after an uneventful pregnancy. There was positive family history of epilepsy from her father's side. She started walking independently at 14-15 months of age and showed speech delay. At 20 months of age, she was admitted to the Child Neuropsychiatric Ward due to tonic spasms with loss of responsiveness, either alone or in series, typically at awakening. She was treated with valproic acid and showed mild psychomotor developmental delay, motor hyperreactivity, restlessness, and repetitive hand movements and fidgeting. At 7 years old, was diagnosed with a mild mental impairment (WISC-R: QIT 65; QIP 67; QIV 68). When she was 11, she started suffering from episodes of psychomotor arrest, chewing automatisms, unresponsiveness lasting few seconds and subsequent drowsiness, with an increasing frequency (up to multiple times a day). Carbamazepine was started. At 12 years of age, there was persistence of episodes of psychomotor arrest (occurring weekly), tendency to isolation, stereotypical hand and mouth gestures, aggressiveness towards her parents and classmates. EEG showed relevant slow and paroxysmal anomalies, mainly located in the central frontal lobe and right frontal temporal lobe, which were continuous and diffuse to all brain areas during sleep. Brain CT was normal. Her blood carbamazepine levels were 6.1 μg/ml and CBZ was then associated with topiramate (up to 100 mg/day). The brain MRI performed at 11.5 years was normal. She currently suffers from daily episodes of absence and confusion with speech impairment, anomia, uncompleted sentences, and words inserted out of context, lasting 30 – 40 seconds, which recede spontaneously and are followed by

post-critical drowsiness. She still shows irritability, dysphoria, oppositional and verbal heteroaggressive behaviour. Additional clinical manifestations included polymenorrhea and mild hirsutism.

Patient 2

The second patient (ITAIGI-16-S-0621) is a 7-year-old boy harbouring three distinct *DDC* variants in homozygous state. His growth was regular, but a global psychomotor delay was noticed when he was 1 year old. He could not hold his head properly and was unable to sit unassisted. At the age of 4 years, he started suffering from seizures. EEG during sleep showed generalized epileptic anomalies, that were ascribed to child myoclonic epilepsy. He started valproic acid (50 mg at evening and 25 in the morning). A month later he was hospitalized due to a generalized tonic-clonic seizure that lasted 2 minutes. During his monitoring he suffered another generalized crisis characterized by loss of consciousness, eyes rolled back and generalized hypertonia. Physical examination confirmed a global developmental delay and a cognitive impairment, and clinical and instrumental presentation were consistent with generalized myoclonic-astatic epilepsy.

3.4 Diagnostic categories according to the flowchart

According to the flowchart we developed (Figure 3), three diagnostic categories can be identified:

1. Patients very likely to be AADC-deficient.
2. Patients with possible AADCD
3. Patients unlikely to be AADC-deficient.

The subdivision in three classes was functional to the assignment of the patients to the most appropriate diagnostic pathway. For example, a patient falling into the 'unlikely to be AADC-deficient' category will not need to undergo the same number and type of diagnostic tests of a patient classified as 'very likely to be AADC-deficient'. Also, it is likely that these tests will not need to be as invasive as the ones considered in the second case. In fact, according to the scored points, a specific diagnostic flowchart can be followed based on the aforementioned categories.

Class 1.

Patients considered **'very likely to be AADC-deficient'** (score **≥ 9** points)**,** should undergo 3OMD neonatal screening on dried blood spots (possibly associated with semiquantitative detection of VLA/VMA ratio on urinary sample):

- If this test results are positive, the patient is referred to the genetic laboratory that will perform genetic testing, whose results can confirm or deny the diagnosis;

- If this test is negative, we suggest repeating it and, at the same time, perform the semiquantitative detection of VLA/VMA ratio on urine samples:
    - if these tests are positive, the patient is directed to genetic testing;
    - if negative, we recommend dosing the AADC enzyme plasma activity on blood, which can be also assessed directly, thus avoiding the repetition of 3OMD screening.

In fact, AADC plasma activity evaluation is known to be reliable and has a major value in the diagnosis:

- if AADC plasma activity test is positive, further confirmation might come with CSF analysis or genetic testing, as indicated originally by *Wassenberg and colleagues* in their 2017 guidelines;
- if AADC plasma activity is negative, we exclude the diagnosis of AADCD.

Class. 2

Patients **'with possible AADCD'** (score **between 5 to 9** points) should be assessed with a combination of 3OMD dried blood spot screening and AADC enzyme plasma activity (possibly associated also with semiquantitative detection of VLA/VMA ratio on urinary sample):

- If the tests result positive, the patient undergoes genetic testing.
- If the tests result negative, we can exclude AADCD.

- If the tests result undetermined, we repeat both 3OMD screening and AADC enzyme plasma activity, but this time associated with semiquantitative detection of VLA/VMA ratio on urinary sample:
    - if all three tests are positive → genetic testing
    - if all three tests are negative → we exclude AADCD
    - if all three tests are undetermined and the suspicion for AADCD is still relevant, we suggest performing the CSF analysis.

Class.3

Patients with low scoring (**< 5** points) are considered **'unlikely to have AADCD'**. However, despite the diagnosis is unlikely, the patient is undiagnosed and has some features consistent with AADCD, at least dried blood spot 3OMD screening should be performed. Indeed, this test is rapid, non-invasive, and non-expensive:

- if negative, we can exclude AADCD (also based on the low score)
- if positive, the patient should undergo AADC enzyme plasma activity evaluation:
    - if negative, we can exclude AADCD;
    - if positive, the patient follows the original diagnostic algorithm proposed by *Wassenberg et al.*

4. DISCUSSION

4.1 Neurotransmitters

AADCD is a rare autosomal recessive disorder caused by biallelic loss-of-function variants in the *DDC* gene. The AADC enzyme, whose co-factor is pyridoxal phosphate, is responsible for catalysing the final step in the synthesis of the monoamine neurotransmitters, serotonin and dopamine. Its deficiency in the brain causes low levels of these molecules as well as norepinephrine and epinephrine (whose precursor is dopamine), the lack of these neurotransmitters explaining the clinical features observed in affected individuals. Brain neurotransmission is key to neuronal differentiation and growth, as well as for brain circuitry development[9].

In AADCD, our main points of interest are serotonin and dopamine: these neurotransmitters are now known to have crucial roles in the brain, especially regarding control of locomotion, mood, and behaviour. In fact, up until the 1950s, they were thought to have few roles in the brain and the scientists at the time did not know that dopamine is widespread across the entire Central Nervous System. Mapping studies showed dopaminergic cells in the ventral midbrain and an extensive distribution in the striatum, leading to the hypothesis that dopamine was involved in motor function and that lower dosages in the striatum might lead to Parkinson's Disease. Later mapping studies identified further location of dopamine producing cells, such as substantia nigra pars compacta (which has projections towards the striatum trough the nigrostriatal pathway), ventral tegmental are of the midbrain (with the mesocorticolimbic pathway) and the hypothalamus (via the tuberoinfundibular pathway) (Figure 4).

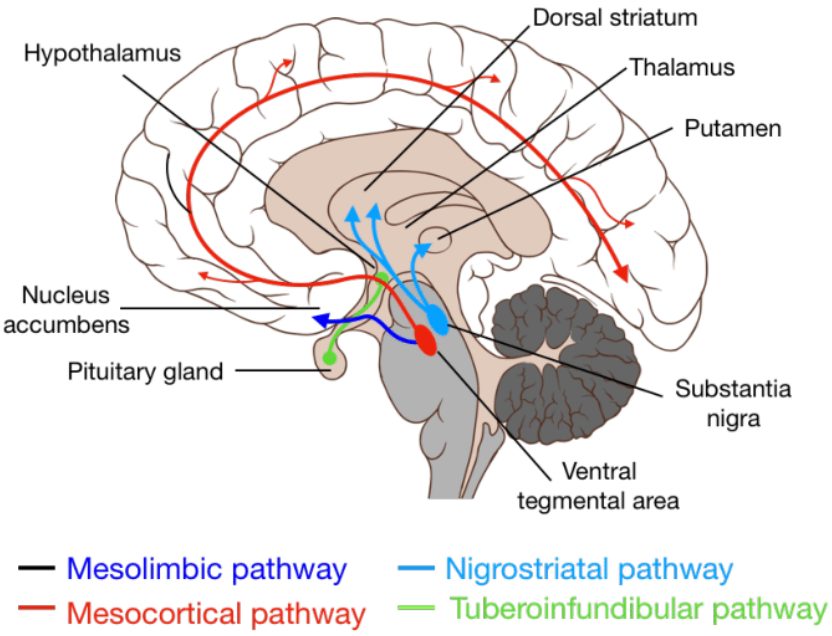

Figure 4 Dopaminergic pathways

Therefore, dopamine is not only implied in motor function, especially in the control of voluntary locomotion, but also in cognitive processes (including attention and memory), neuroendocrine secretion (e.g., dopamine secretion inhibits prolactin secretion from the pituitary gland), control of motivated behaviours (emotion, affection, reward mechanisms).

Dopamine is also the precursor of noradrenaline (norepinephrine) and adrenaline (epinephrine). Norepinephrine is released when a host of physiological changes are activated by a stressful event. In the brain, this is caused in part by activation of an area of the brain stem called the locus coeruleus. This nucleus is the origin of most norepinephrine pathways in the brain.

Noradrenergic neurons project bilaterally from the locus coeruleus along distinct pathways to many locations, including the cerebral cortex, limbic system, and the spinal cord, forming a neurotransmitter system. Norepinephrine is also released from

postganglionic neurons of the sympathetic nervous system, to transmit the fight-or-flight response in each tissue respectively. The adrenal medulla can also be counted to such postganglionic nerve cells, although they release norepinephrine into the blood. Anatomically, the noradrenergic neurons originate both in the locus coeruleus and the lateral tegmental field.

The axons of the neurons in the locus coeruleus act on adrenergic receptors in:

- Amygdala
- Cingulate gyrus
- Cingulum
- Hippocampus
- Hypothalamus
- Neocortex
- Spinal cord
- Striatum
- Thalamus

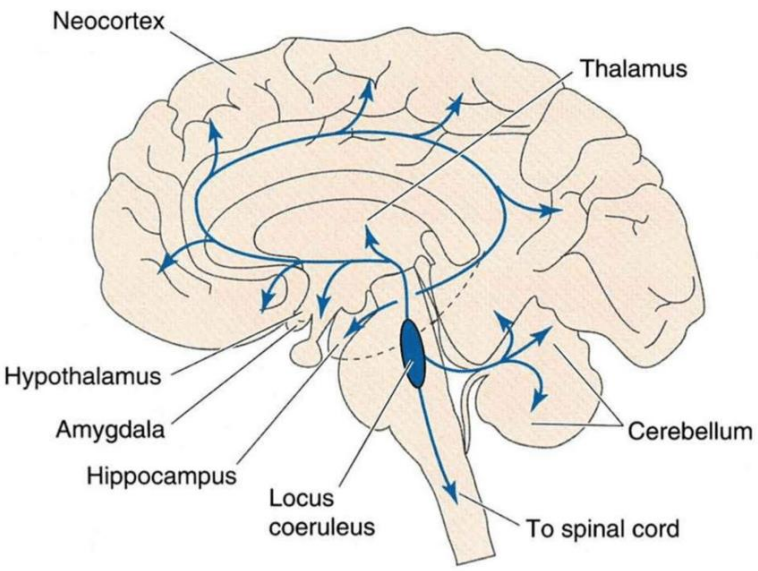

Figure 5 Noradrenergic pathways

On the other hand, axons of neurons of the lateral tegmental field act on adrenergic receptors in hypothalamus, for example. This model explains some of the clinical uses of norepinephrine, since a modification of the system affects large areas of the brain (Figure 5).

Epinephrine derives from norepinephrine and is primary synthetized in the adrenal gland, but it is a brain neurotransmitter as well. In the brain, epinephrine is mainly present in the medulla, pons, and hypothalamus.

Serotonin neurons have been also mapped (Figure 6). They are mainly found in the dorsal and ventral raphe nuclei of the midbrain, which are part of the reticular formation, and project widely to cortical areas (e.g., supplementary motor area, premotor cortex, primary motor cortex), and to cerebellum and spinal cord. Serotoninergic innervation is quite high also in the globus pallidus, and reduces progressively in putamen, substantia nigra, and is quite low in the basal ganglia. This implies that serotonin has an impact on motor control as well. This data seems to be supported by the finding of motor abnormalities during a serotoninergic syndrome access, where there are myoclonic and dystonic postures.

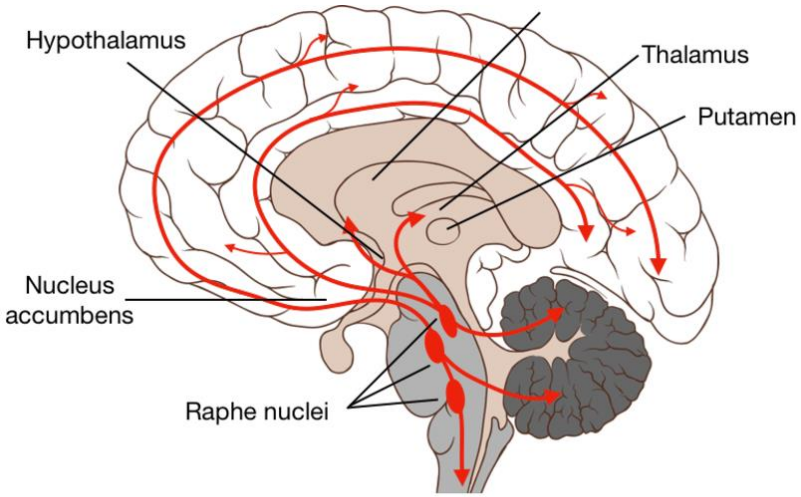

Figure 6 Serotoninergic pathways

Therefore, we can affirm that low levels of dopamine affect voluntary movements, emotion, cognitive function, and the release of various hormones. Norepinephrine and epinephrine deficiency affects attention, mood, sleep patterns, cognition, and stress hormone levels. The lack of serotonin, which is a substrate for melatonin, can affect gastrointestinal function, respiratory and cardiovascular systems, circadian rhythm, body temperature, pain

sensation, and mood. Interestingly, all these symptoms are present in variable combination and extent in AADCD (Figure 7).

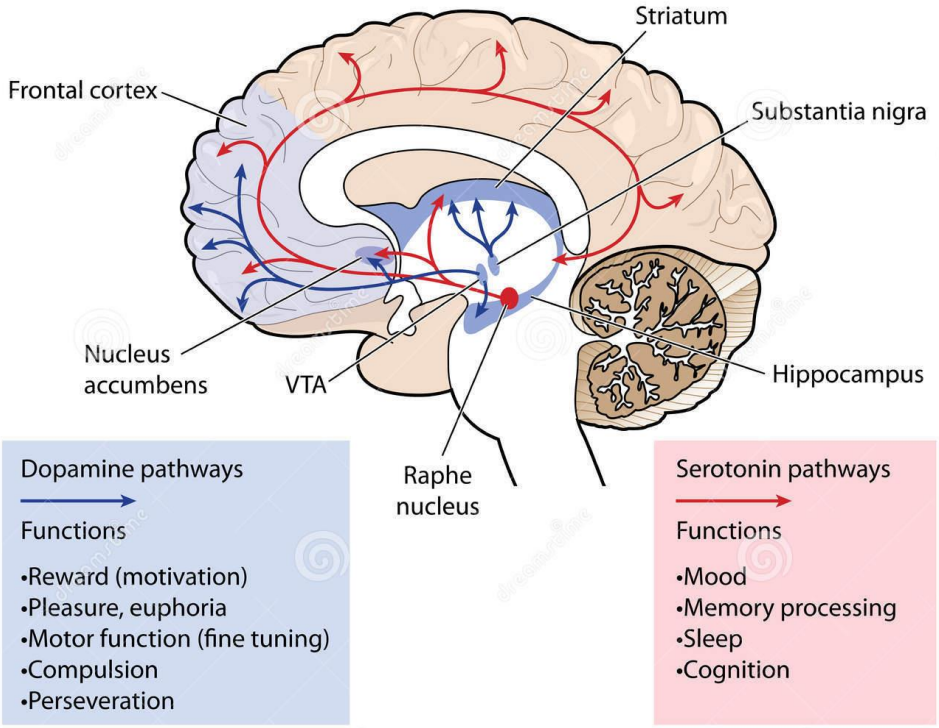

Figure 7 Dopaminergic and serotoninergic pathways

4.2 Enzyme

The AADC enzyme is a homodimeric pyridoxal 5′-phosphate (PLP)-dependent α-decarboxylase, which converts L-dopa and 5-HTP into dopamine and serotonin, respectively. Each monomer consists of a large domain of 309 residues containing the PLP binding site, an 86-residue C-terminal domain, and an N-terminal domain of 85 residues. Previous studies have revealed that some residues are important for enzyme catalysis, including: (1) residues participate in PLP binding (Ser-147, Ser-149, His-192, Asp-271, Asn-300, His-302, Lys-303, and Phe-309); (2) the localization of substrate binding and cofactor stabilization (Trp-71, Tyr-79, Phe-80, Thr-82, Ile-101, Phe-103, and Thr-246). Interestingly, the active sites are different between the apoenzyme form of AADC and the holoenzyme form, and some flexible loop regions play important roles during the apoenzyme to holoenzyme transition, including loop1 (residues 66–84), loop2 (residues 100–110), and loop3 (residues 323–357)[12].

This characterisation of the enzyme was first started in the 1950s, through the cloning of the pig kidney (pkAADC) and rat liver (rlAADC) isoforms in E. Coli. Only recently, the human isoform (hAADC) was cloned in E. Coli as well and finally studied properly. All the three isoforms have been characterized. The substrate specificity, the steady state kinetic parameters, the spectral properties of the enzyme (either by itself or with substrates/substrates analogues) and the susceptibility to the protease are now well-defined. We can affirm that the natural and the recombinant mammalian enzyme share structural and functional features, can be reproduced in the laboratory, and the effect of pathogenetic mutation on enzymatic function and expression can be easily studied.

In 2021, *Montioli et al*. revised the available biochemical data and interpreted them on the basis of the structure-function relationship, with the ultimate goal of identifying relevant residues in the enzyme for catalysis and/or folding, also aiming to define the enzymatic phenotype of patients with pathogenic variants and propose a specific therapy. Among other tests, this group analysed the crystal structure of the pkAADC in the holo form, which was solved at 2.6 Å. The overall conformation of AADC consisted in a tightly associated dimer. Each subunit included: (1) a large domain (86–360 residues) containing the PLP binding site and consisting of a central, seven-stranded mixed β-sheet surrounded by eight α-helices in a typical α/β fold; (2) a C-terminal domain (361–480 residues) comprising a four-stranded antiparallel β-sheet packed against the face opposite to the large domain; (3) an N-terminal domain (1–85 residues), comprising two parallel helices linked by an extended strand, packing on the top of the large domain (Figure 8A). The short stretch of 11 amino acids (residues 328–339), representing a mobile loop important for the catalysis, was not ordered enough to be built in the crystal structure of the enzyme. PLP, covalently bound through a Schiff base to the active site Lys 303 (internal aldimine), was located at the subunit interface and interacted with residues of the dimer subunits. Besides the interaction with Lys303, the PLP molecule was non-covalently bound to the active site by a base stacking interaction. This involves the His192 side chain and the PLP pyridine ring, a salt bridge between the Asp271 and the N1 of PLP, as well as several possible hydrogen bonds connecting the phosphate group of the coenzyme with three residues (Ala148, Asn300, and His302) and a water molecule (Figure 8B). The crystal structure of pkDDC complexed with the anti-Parkinson drug carbidopa was also solved. The binding of carbidopa, occurring through a hydrazone linkage with PLP and its hydrazinic group, being the carboxylate moiety approximately orthogonal to the PLP ring, mimicked the external aldimine enzyme–

substrate intermediate, even if the carbidopa–PLP adduct, compared to the Schiff base complex with L-DOPA, contained an additional N–N bond. The catechol ring of carbidopa, deeply buried in the active site cleft, was in contact with the active site residues Ile101 and Phe303 provided by the adjacent monomer, while the 3' and 4' catechol hydroxyl groups were hydrogen bonded with the coenzyme phosphate ant the hydroxyl groups of Thr82 respectively.

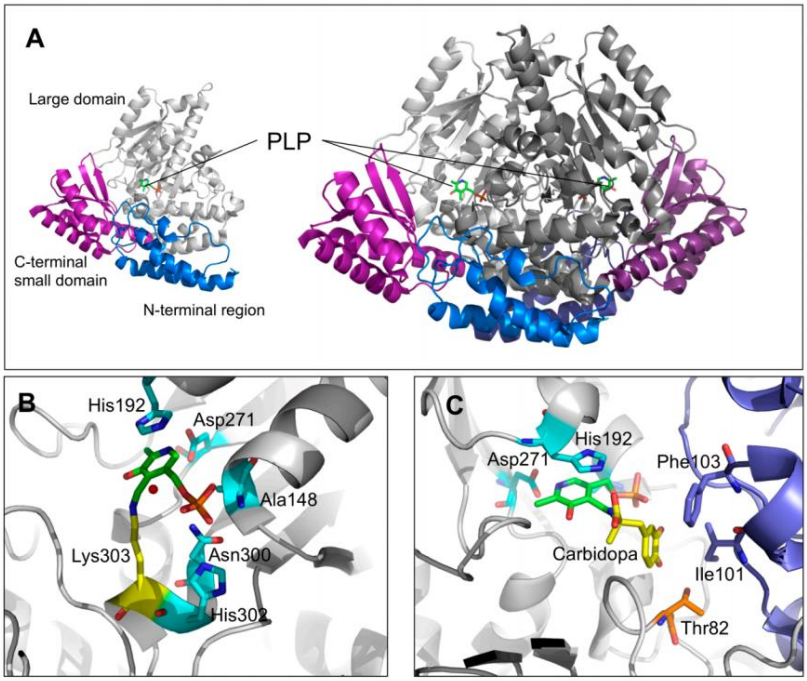

Figure 8 DDC structural features and active site architecture. (A) Ribbons represent the DDC monomeric and dimeric units. The N-terminal, large, and C-terminal domains are coloured in blue, grey, and magenta, respectively. (B) Active site of unliganded holo-DDC or (C) in complex with the inhibitor carbidopa. PLP molecules are represented as green sticks, the PLP binding residues are represented as cyan sticks. The residues in the proper position to interact with the carbidopa catechol ring are highlighted. Image was rendered by PyMol software (Schrödinger).

An unexpected and relevant finding was that the crystal structure of human apoDDC was very different from that of the pkDDC in the holo form. The comparison between these two structures was plausible considering that hDDC is the close orthologue of pkDDC (89% sequence identity). Indeed, the resolution of the apo revealed that the structure appeared as

an open bivalve shell in which the active site and the hydrophobic regions were partly exposed to the solvent, and the contact region between monomers comprised only the N-domains helices, which acted as the hinge. This crystal structure together with those of three alternative conformations of the active site at different PLP saturations allowed to suggest that the PLP binding to the apoenzyme triggered an initial conformational change at the level of the active site, yielding the rearrangement of loop1 (residues 66 to 84) transmitted to loop2 (residues 100 to 110) and then to loop3 (residues 323–357), with both loops 2 and 3 belonging to the adjacent subunit (Figure 9).

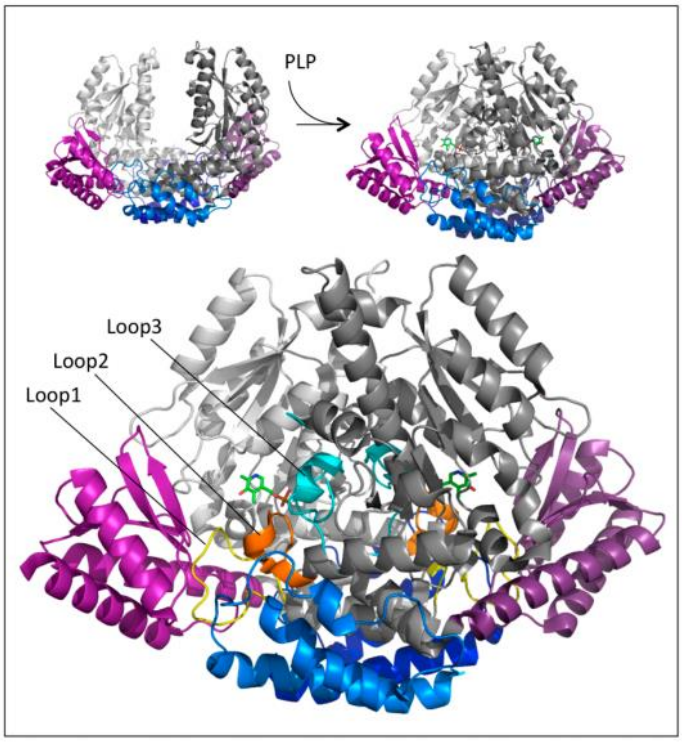

Figure 9 Structural regions involved in the apo-to-holo transition of DDC. Global conformational change accompanying the transition from the apo to the holo form of DDC. The N-terminal, large, and C-terminal domains are coloured blue, grey, and magenta, respectively. The two monomers are distinguished by dark and light colours. The PLP molecules are represented as green sticks and loops 1, 2, and 3 are highlighted in yellow, orange, and cyan, respectively. Image was rendered by PyMol software (Schrödinger).

4.3 Effects of *DDC* pathogenic variants

The solved crystal structures of ADDC in the holo and apo forms provided structural scaffolds to explain the effects of some pathogenic variants. Quite interesting was the presence of four mobile loops in ADDC, since their rearrangements were crucial to catalysis. In particular, loop1 underwent conformational changes upon PLP binding, giving rise to a correct apo → holo transition. These conformational changes were transmitted to loops 2 and 3 of the adjacent monomer, these loops becoming structured only in the closed conformation. Again, the catalytic loop underwent conformational changes as a response to substrate binding, leading to a closed productive conformation that was characterized by distinct interactions between the catalytic loop and the active site. Thus, the proper function of ADDC required dynamic structural elements scattered across the surface of the protein. These structural determinants were very useful to interpret the molecular defects of the pathogenic variants of residues belonging to or interacting with these loops. On the other hand, mutations of residues located outside these loops, i.e., the large, N-terminal, and C-terminal domains, were interpreted with difficulty since the structure-function relationships of these regions are unknown. However, the impact of the residues belonging to these regions on the folding and catalytic activity of ADDC improved the comprehension of the role of some residues of the enzyme. The structural elements along with the resulting catalytic effects were considered when characterizing the impact of *DDC* pathogenic variants (Table 3).

Catalytic and Folding Mutations	*Catalytic Mutations*	*Folding Mutations*
T69M (loop1)	R34Q (loop3)	P201L (large domain)
H70Y (loop1)	R347G (loop3)	L222P (large domain)
F77L (loop1)	L353P (loop3)	P237S (large domain)
Y79C (loop1)		S250F (large domain)
R447H (C-term domain)		R285W (large domain)
R453C (C-term domain)		E328A (large domain)
R462P (C-term domain)		P47H (N-term domain)
L408I (C-term domain)		
R412W (C-term domain)		
G123R (large domain)		
F309L (large domain)		
V60A (N-term domain)		
L38P (N-term domain)		
G102S (loop2)		
A110E (loop2)		

Table 3: classification of pathogenic mutations in homozygosis

So far, the main strategy of treatment in AADC-deficient patients is a symptom-based rather than an aetiology-based approach. However, only a mild improvement can be achieved in most cases and the outcome is generally poor. Genetic testing and studies addressing the characterization of the enzyme structure and function are crucial to identify and develop a correct and effective therapy. Moreover, studies focused on the identification of the functional effect of a specific pathogenic variant are of paramount importance to recognize a precise therapeutic target. This approach will allow to identify the proper drug treatment among the ones already available and develop new ones, gradually moving to aetiology-based approach and precision medicine with patient-tailored therapy. An example of the latter is the administration of L-DOPA in patients with identified variants of the L-DOPA binding site (e.g., G102S, R347Q, R160W). In these patients, L-DOPA is considered a first line of treatment and has a positive response, while this is not beneficial in patients with pathogenetic variants not involving the L-DOPA site[1]. It is therefore evident how genetic testing is crucial to guide the choice of the best treatment option. Ultimately, the

future is represented by the very promising gene therapy approach. This consists of the replacement of the defective gene with a functioning copy, representing a true aetiological therapy. Specific trials have been made and currently are held on with positive outcomes[6,34,41].

4.4 Clinical presentation and phenotype dissection

AADCD is a rare autosomal recessive neurotransmitter, with only 135 cases worldwide and half of them being Asian, likely due to a founder mutation. The mean age at onset is 2.7 months, but a considerable latency in diagnosis is common and the mean age at the disease identification is 3.5 years, ranging from 2 months to 23 years of age[1].

Since the AADC enzyme activity is impaired, the symptoms can be ascribed to the lack of serotonin, dopamine, norepinephrine, and epinephrine in the brain. These neurotransmitters are responsible for most brain functions, and this explains the clinical heterogeneity of AADCD. Key symptoms and signs are developmental delay, hypotonia, movement disorders, oculogyric crisis, dystonia, hypokinesia, ptosis, and autonomic symptoms (e.g., excessive sweating and nasal congestion) (Figure 10).

Developmental delay is always present in variable degrees, globally involving motor, cognitive and speech function. Motor function is always impaired to some extent. The attainment of motor milestones (head control, independently sitting, walking) is delayed by years and might not be achieved in children with severe presentation, who are usually bedridden. Cognitive impairment is a prominent feature. Children with AADCD tend to have lower I.Q. than general population. Since the majority of them has a severe phenotype, caregivers are essential for their day care. A smaller percentage of patients has a milder cognitive impairment and can be partially or even totally independent in their activities of daily living, such as mobility, feeding, bathing, and dressing. Twenty-nine subjects out of the 63 examined by *Pearson et al*. were indeed able to attend school. Speech is achieved later than normal and patients with severe phenotypes never learn how to speak correctly,

whereas those with milder phenotypes are able to complete simple sentences. Subjects with severe phenotypes learn instead only few words and tend to produce verses.

Hypotonia is one of the most prominent features. Often, AADCD patients present with a severe phenotype and many subjects show symptoms as new-borns. Almost 75% of the patients examined by *Pearson and colleagues* had some degree of hypotonia at birth and many presented as floppy infants. The most severe cases also showed reduced foetal movements and required a differential diagnosis during pregnancy with neuromuscular disorders, such as Spinal Muscular Atrophy (SMA). Usually, hypotonia tends to have a mild decrement with aging, thus allowing the patients to attain some degree of motor developmental milestones, such as head control, independent sitting, and walking. However, these motor skills are typically slowly attained through the years, rather than in the first 12 months of life. In some rare cases, patients develop spasticity, which is mainly limited to the limbs.

Movement disorders are another typical feature of AADCD. Under this term are included oculogyric crises, which can be considered the most typical symptom of this disorder, with a prevalence of 97 % [2]. They present as paroxysmal crisis of involuntary eye movements in the upward or lateral direction, with backward and lateral flexion of the neck. It is a form of dystonia, that often starts unexpectedly and is limited to the eyes, but might involve progressively the head, neck, and trunk, up to whole-body dystonia. These episodes occur in almost all AADC-deficient patients and are more prominent between 2 and 12 years of age. These crises might last over 4 hours and present up to 3 times per week, thus being extremely debilitating. In some cases, patients present with dystonia limited to the trunk and limbs, or to head and neck, with or without eye deviation. Oculogyric crises are probably the

most prominent feature in AADCD but are often misinterpreted as epileptic seizures. Since epilepsy is more frequent than AADCD in children, AADC-deficient patients might be misdiagnosed as epileptic, thus receiving antiepileptic treatment which leads to very mild benefits, if none, but significant side-effects. The two conditions can be differentiated through ictal EEG, which should always be performed during the differential diagnosis. We can affirm that epilepsy and AADCD are mutually excluding in most cases, however some AADC-deficient patients may experience seizures as well[5], probably due to a more severe cerebral damage, and benefit from AED treatment. On neurological examination, deep tendon reflexes might be normal, decreased or increased; sometimes pathological reflexes such as Babinsky sign are reported.

Autonomic disfunctions are characterized by impairment of the sympathetic regulation of the eyes (ptosis, miosis), upper airways (nasal congestion, excessive drooling, stridor), thermoregulation (excessive sweating and temperature instability), and cardiovascular system (hypotension, orthostatic hypotension, bradycardia, abnormalities of the heart rate). These abnormalities might be life threatening, especially when affecting the cardiovascular system. It appears that the chronic lack of catecholamines and the persistent autonomic dysfunction lead to possible cardiac complications, especially during illness or stress (e.g., infections, surgery etc.). Hence, these patients are less resistant to stressful events and their vital signs should always be closely monitored with ECG[1].

Other associated symptoms include behavioural problems, mostly described as irritability, excessive crying, dysphoria[8,19] and autistic features. Sleep disturbances are common and might present either as insomnia or as hypersomnia. Interesting fact is that many patients tend to experience a variation in their sleep with growth, since infants have

excessive sleepiness, whilst many children and adolescents exhibit significant insomnia. Some patients might even present with severe sleep apnoea that might be fatal. Apparently, sleep also has an impact on the other aspects of this condition, as some patients experience diurnal fluctuations, and their symptoms show an improvement after sleep.

Additional clinical findings include gastrointestinal problems such diarrhoea, constipation, gastroesophageal reflux. All these conditions, especially in association with dysphagia, lead to feeding problems. If not adequately nourished, children's growth is impaired and failure to thrive and short stature are frequently reported. Initially, children may be fed trough nasogastric tube, however, if artificial nutrition is necessary for a long period of time, gastrostomy is required. Dysphagia is a symptom that must always looked after. It not only makes difficult to feed the child but might cause choking with subsequent possible death by asphyxia, or ab-ingestis pneumonia, which is one of the most frequent causes of death in these patients.

From a metabolic standpoint, AADC-deficient patients might present with hypoglycaemia, which tends to occur during intercurrent illness and is an expression of the overall sympathetic dysfunction. They might present also increased levels of prolactin, since its secretion from the pituitary gland is not inhibited by the dopaminergic tuberoinfundibular pathway due to dopamine shortage. No other hormones deficiencies have been detected.

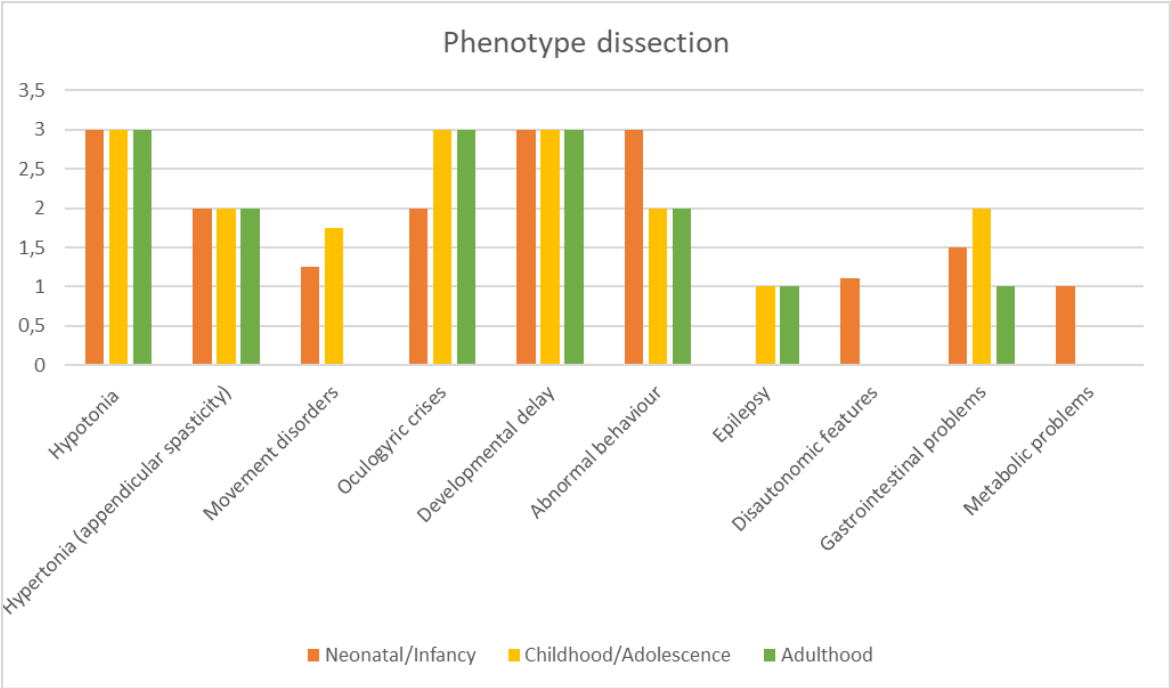

Figure 10 Movement disorders =, dyskinesia, dystonia, tremor, myoclonus; Dysautonomic features = ptosis, miosis, nasal congestion, excessive sweating, temperature instability, hypotension, bradycardia arrhythmias; Gastrointestinal problems = diarrhoea, obstipation, feeding problems; Metabolic problems = hypoglycaemia, hyperprolactinemia

AADCD presents with an extremely high clinical variability, not only between different patients, but also in the same subject during the course of life. According to *Wassenberg et al.*, AADC-deficient patients can be broadly classified as mild (mild delay in developmental milestones, ambulatory without assistance, mild intellectual disability), severe (no or very limited developmental milestones, fully dependent), and moderate (in between). Milder phenotypes tend to have milder symptoms and present predominantly with autonomic dysfunction, (diarrhoea, hypoglycaemic crises, nasal congestion), and without evident movement disorders[15,16], therefore hypotonia and oculogyric crisis might be almost undetectable or completely absent.

The course of the disease usually reaches a plateau and remains stable during the entire life of the individual. However, some patients tend to spontaneously improve during

puberty[23], and this happens more frequently with milder phenotypes. Despite rare, it is also possible that some patients experience a loss of skills, especially a regression of language skills[16,17]. A decline of motor function can appear as well, but it tends to be secondary to factors like joint contractions. In most cases, the disease is not progressive, even if the phenotype is severe[1].

4.5 Genotype-phenotype correlations

So far, more than 80 pathogenic variants involving the *DDC* gene have been described[8]. The severity of the disease depends on the type of pathogenic variant and how it is inherited, as compound heterozygosis or as homozygosis. Only 135 patients have been identified, but many are likely still to be diagnosed. Nearly half of them are Asian, from Taiwan and Japan in particular, and this phenomenon is probably due to a founder mutation in this populations. The most common DDC variant is c.714+4A>T (or IVS6 + 4 > T): this mutation is always associated with a severe phenotype, and it has a specific genotype-phenotype correlation, since it associated with the formation of a truncated AADC protein[18]. Other common mutations are S250F, with an allele frequency of 10%, and G102S, with an allele frequency of 8% [8]. Gender does not influence phenotypes, since both male and female have a comparable prevalence of severe presentations (77 % vs 72 %)[1].

Specific variants that were identified only in patients with a moderate phenotype were: c.367G>A (p. G123R), c.446G>C (p. S149T), c.734 C>T (p. T245I), c.853C>T (p. R285W), c.876G>A (p. E292E), and c.1337T>C (p. L446P)[2]. Other identified mutations, such as p.G102S, p.R347Q, p.R160W, affect the L-DOPA binding site and have a specific therapy represented by L-DOPA. Hence, it is clear how crucial it is to identify the correct mutation in order to recognize the severity of the disorder and provide the correct and most efficient treatment[1]. Studies in this direction have been made and are currently held on[10.]

Patient No.	Age	Sex	Geographic region	Allele 1 phenotype	Allele 2 phenotype	Motor phenotypic severity
1	8	F	North America	c.179T>C (p.V60A)	714+4A>T	Moderate
2	26.1	M	Europe	c.476C>T (p. A159V)	?	Moderate
3	6.6	F	North America	NA	NA	Severe
4	10.9	M	Europe	c.73G>A (p.E25K)	c.1073G>A (p. R358H)	Severe
5	15	M	North America	NA	NA	Moderate
6	4	M	North America	NA	NA	Severe
7	18.5	F	North America	c.140C>A (p. P47H)	Homozygous	Severe
8	8.8	M	North America			Mild
9	7.3	M	South America	c.1040G>A (p. R347Q)	Homozygous	Severe
10	3.7	M	Middle East	NA	NA	Severe
11	6.7	M	Europe	NA	NA	Mild
12	2.1	M	North America	c.19C>T (p. R7*)	c.214C>T (p. H72Y)	Severe
13	21	F	North America	NA	NA	Severe
14	1.6	F	South America	c.330_334dupCGATC (p.Q112fs*13)	Homozygous	Severe
15	4.3	M	Europe	c.231C>A (p. F77L)	Homozygous	Severe
16	3.9	F	North America	c.286G>A (p. G96R)	c.665T>C (p. L222P)	Severe
17	2.5	F	Asia	NA	NA	Severe
18	4.1	F	Europe	c.323G>A (p. S108N)	c.1041+1G>C	Severe
19	5.2	F	North America	714+4A>T	Homozygous	Severe
20	2.4	F	Europe	NA	NA	Severe
21	5.3	F	Europe	c.73G>A (p. E25K)	c.315G>C (p. W105C)	Severe
22	12.5	M	Europe	c.782G>T (p. C261F)	c.1060G>A (p. G354S)	Severe
23	1.4	F	Middle East	NA	NA	Severe
24	16.7	F	Middle East	c.242C>T (p. P81L)	Homozygous	Severe
25	10.7	F	North America			Severe
26	8.5	M	South America	c.568_569insCGAT (p. Q190Pfs)	c.1040G>A (p. R347Q)	Severe
27	0.8	M	South America			Severe
28	11.9	F	Middle East	c.1040G>A (p. R347Q)	Homozygous	Severe
29	7.9	F	Europe	c.139C>G (p. P47A)	?	Severe
30	24.2	F	Europe	c.367G>A (p. G123R)	c.876G>A (p. E292E)	Mild
31	4	M	North America	NA	NA	Severe
32	7	M	North America	NA	NA	Severe
33	5	M	Europe	NA	NA	Moderate
34	1.3	M	Europe	c.73G>A (p. E25K)	c.624delC (p. I209Sfs*26)	Severe
35	13.1	M	Europe	c.367G>A (p. G123R)	c.876G>A (p. E292E)	Mild
36	9	M	Europe	c.1A>G (p. M1V)	c.181G>A (p. E61K)	Severe
37	13	F	Europe	NA	NA	Severe
38	14.8	M	Europe	c.1073G>A (p. R358H)	Homozygous	Severe
39	3.4	M	Asia	714+4A>T	Homozygous	Severe
40	3.8	F	Asia	714+4A>T	c.1234C>T (p. R412W)	Severe
41	1.4	F	Asia	c.179T>C (p. V60A)	c.1234C>T (p. R412W)	Severe

42	3.3	F	Asia	714+4A>T	c.1297dupA (p. I433Nfs*60)	Severe
43	3.2	F	Asia	c.106G>A (p. G36R)	714+4A>T	Severe
44	4.6	F	Asia	c.170A>G (p. I57T)	c.1234C>T (p. R412W)	Severe
45	2	M	Asia	c.106G>A (p. G36R)	714+4A>T	Severe
46	1	F	Asia	714+4A>T	Homozygous	Severe
47	10.1	F	Europe	c.206C>T (p. T69M)	1337T>C (p. L446P)	Mild
48	25.4	F	Europe	c.206C>T (p. T69M)	c.439A>C (p. S147R)	Severe
49	5.7	F	Europe	c.260C>T (p. P87L)	c.799T>C (p. W267R)	Severe
50	19.7	F	Europe	c.214C>T (p. H72Y)	Homozygous	Severe
51	1.5	F	Europe	c.201+5G>C	Homozygous	Moderate
52	8.8	F	Europe	c.367G>A (p. G123R)	c.734C>T (p. T245I)	Mild
53	0.5	F	Europe	NA	NA	Severe
54	36.8	F	Europe	c.105delC (p. Y37T fs*5)	c.710T>C (p. F237S)	Mild
55	15	M	Europe	c.843C>G (p. C281W)	c.1085T>C (p. M362T)	Mild
56	0.5	F	Europe	c.322A>C (p. S108R)	c.812A>T (p. D271V)	Severe
57	0.8	M	Europe	NA	NA	Moderate
58	18.2	M	Asia	NA	NA	Moderate
59	25.4	F	Asia	714+4A>T	c.853C>T (p. R285W)	Mild
60	22.7	F	Asia	714+4A>T	c.853C>T (p. R285W)	Mild
61	14.2	F	North America	c.260C>T (p. P87L)	c.446G>C (p. S149T)	Mild
62	4.3	F	North America	c.260C>T (p. P87L)	c.446G>C (p. S149T)	Moderate
63	10.4	M	North America	NA	NA	Severe

Table 4: genotype-phenotype correlations (Pearson et al., 2020[2])

NA: not available

Previously published cases: 7; 50; 54; 58; 59;60.

Deceased: 13; 17; 20; 32; 37

Phenotypic severity: mild= able to walk independently; severe= minimal or no attainment of developmental milestones; moderate = intermediate.

*Denoting a nonsense variant in the gene.

4.6 Discussion of results and diagnostic process

AADCD can be diagnosed through clinical, biochemical, instrumental, metabolic, and genetic evaluations; however, the confirmed diagnosis can be obtained only through biochemical-metabolic and molecular testing. Since the clinical presentation is quite heterogeneous, it cannot be considered a diagnostic tool by itself, but some symptoms, such as oculogyric crisis, hypotonia and developmental delay, that occur in most AADC-deficient patients, render a high suspicion of the disease.

According to the consensus guidelines drawn up *by Wassenberg et al. in 2017*, there are three key diagnostic tools for AADCD diagnosis:

- ***CSF analysis*** (↓ 5-HIAA, HVA, MPHG; ↑ 3OMD, L-DOPA, 5HTP; normal pterins)
- ***Genetic diagnosis*** (compound heterozygous or homozygous pathogenic variants in *DDC*)
- ***AACD enzyme activity in plasma*** (decreased)

To diagnose AADCD, genetic testing should be performed and at least two out of three core diagnostic tests should be positive. All these tests (singularly and, even better, combined) are highly specific for the disease, but they are not screening exams. Lumbar puncture is quite invasive, especially for small children. Molecular testing is less invasive, since it requires a peripheral blood sample, but it takes months for the results to come and, in the meantime, patients might not be treated properly.

The mean age onset of symptoms in this disorder is at 2.7 months of age, according *to Wassenberg et al., 2017*, with a mean age of diagnosis around 3.5 years of age, despite

the early presentation. However, it is non rare that the diagnosis is made years later, even in adolescence or adulthood[23]. This happens especially in milder phenotypes and the patients might not receive a proper treatment or a treatment at all during these years. Even though the disease tends to reach a plateau and stabilize, some patients improve spontaneously. Other patients, unfortunately, experience a progressive deterioration with loss of skills[17].

Since the progression of the disease is quite unpredictable, early diagnosis and early treatment is decisive to avoid possible negative outcomes and improve the quality of life in patients, especially those with severe phenotypes, who are often bedridden and rely on their caregivers for their day-to-day life. Early diagnosis requires a screening tool, but the current guidelines are elusive. It is also important to notice that the work of *Wassenberg and colleagues* dates back to 2017. In the meantime, research progressed, and new tests have been developed.

In 2019, *Brennenstuhl et al*. proposed a screening test based on the levels of 3-O-methydopa in dried blood spot[3]. Since the activity of the AADC enzyme is impaired, many substrates, including L-DOPA, cannot be metabolized, therefore they accumulate and are degraded by other enzymes. In this case, the analysis of 3OMD is considered reliable for the diagnosis of AADCD since the only way for this molecule to be produced is through COMT digestion of the L-DOPA that accumulated since not degraded by AADC enzyme.

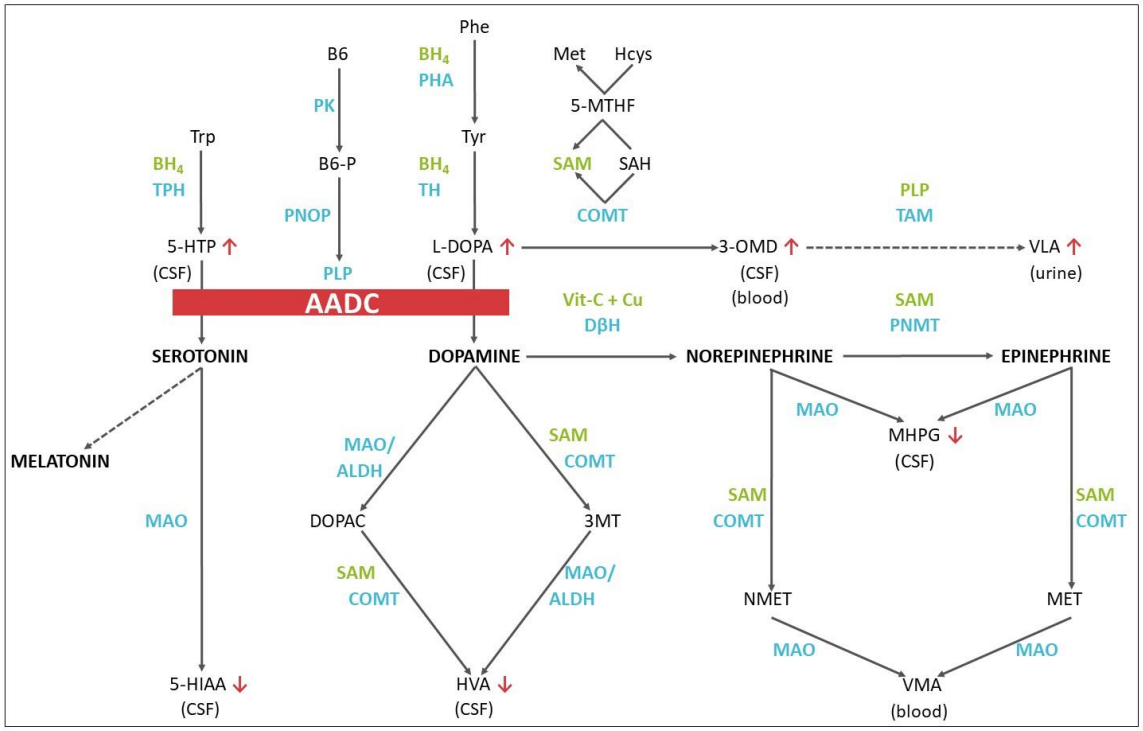

Figure 11 Biosynthesis and breakdown of serotonin and the catecholamines, and the metabolic block in AADC deficiency. Simplified scheme of the biosynthesis and breakdown of serotonin and the catecholamines (dopamine, norepinephrine, and epinephrine), and melatonin synthesis. Cofactors (BH4, PLP, Cu) and methyl donor (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: vanillmandelicacid: Vit B6 vitamin B6 (pyridoxine).

The advantages of this screening are reliability, rapidity, non-invasiveness, and cost-effectiveness. On the other hand, 3OMD levels are high in patients with deficiency of pyridoxine 5-phosphate oxidase (PNPO): both AADCD and PNPO present the same biochemical pattern and cannot be distinguished only through the analysis of 3OMD or CSF. However, the two disorders have different clinical presentations and can be easily

differentiated just through physical examination. Therefore, clinical evaluation is crucial and should never be ignored in favour of biochemical or instrumental exams. In 2020, the same group proposed a new screening test based on the semiquantitative of vanillactic acid (VLA) and vanillylmandelic acid (VMA) ratio on urine sample[4]. VLA derives from the degradation of 3OMD, a methylation product of L-DOPA, and its levels are higher in AADC-deficient patients.

Since Brennenstuhl identified 3OMD as a reliable disease marker, its degradation product appeared as an optimal marker as well. Urine samples were collected by 10,095 non-AADC deficient patients and 14 control patients with genetically proven AADCD and were analysed the levels of VLA and VMA individually and later was calculated their ratio. The mean urinary VLA concentration in non-AADC samples (n=10,095) was 0.3 mmol/mol creatinine (SD=1.18, range 0 – 57.79) and the mean VMA concentration was 5.59 mmol/mol creatinine (SD=3.87, range 0.04 – 60.62). Samples from the 14 AADC-deficient patients revealed a mean VLA concentration of 10.77 mmol/mol creatinine (SD=11.35, range = 0.37 – 33.06), whereas the mean VMA concentration was found to be 0.45 mmol/mol creatinine for VMA (SD=0.29, range 0.11 – 1.27). VLA concentrations did correlate negatively with age, while VMA concentrations were overall stable over age. The mean urinary VLA/VMA ratio in 10,095 control samples was found to be 0.07 (SD=0.37, range 0.0 – 23.24), while the mean ratio in 14 measurements from nine individual AADC deficient patients was 23.16 (SD=22.83, range 0.97 – 74.1), corresponding to a ~350-fold increase. For four patients, multiple measurements over time were available (up to n=3). In three of four cases a decrease of the VLA/VMA ratio was observed in the second measurement. In one case, the VLA/VMA ratio increased over time more than 3-fold. While the VMA concentration remained largely stable over multiple measurements within the same patient, the VLA

concentration seemed to decrease over time. Unfortunately, the group found out that the determination of individual concentrations of neither VLA nor VMA are reliable markers for AADCD diagnosis, but they demonstrated that VLA/VMA ratio is a reliable, easy, non-invasive, and cost-effective screening tool.

Considering the abovementioned recent findings, we suggested an update of the current guidelines by *Wassenberg et al*., which date back to 2017. With this purpose, we decided to implement the guidelines with the work of Brennenstuhl to create a diagnostic flowchart to help the diagnostic process in AADCD. Alongside this flowchart, we also developed a score to divide the patients into three categories (very likely – possible – unlikely to be AADC-deficient) and applied our score to the children in whom we identified *DDC* variants in our study. The patient identified as ITAIGI-16-S-0158 (patient 1), who harboured two compound heterozygous variants in *DDC,* presented with developmental delay, irritability and involuntary movements and would have scored 6 points, therefore being classified as a 'possible AADC-deficient' patient. The patient identified as ITAIGI-16-S-0621 (patient 2), with three distinct variants in homozygous state for the *DDC* gene, presented with developmental delay and crises involving ocular movements that might have resembled oculogyric crises, therefore scoring 6 points, and being classified as a 'possible AADC deficient' patient. According to our diagnostic score, both patients are 'possible to be AADC-deficient' and in fact, both presented with mutations involving *DDC*. However, in our study, after filtering variants for frequency, conservation, and predicted pathogenicity, these variants (in homozygous or compound heterozygous state) appear to be weak in terms of pathogenicity and the phenotype of these subjects was not consistent with a diagnosis of AADCD.

4.7 Updated diagnostic flowchart

We designed this flowchart to be an easy tool to assess patients with neurological and neurometabolic diseases that are yet to be diagnosed and whose clinical manifestations may be suggestive of AADCD. Our main purpose was to simplify the diagnosis, prioritizing the application of rapid, non-invasive, and relatively unexpensive tests, that are, yet, reliable. This is crucial to screen the susceptible population and to obtain an early diagnosis and start the appropriate treatment in time, with a positive outcome for the patients and their family/caregivers. These criteria are successfully met by 3OMD screening.

Brennenstuhl et al.[3] tested 3OMD in 38,888 unaffected new-borns, 14 heterozygous DDC variant carriers, seven known AADC deficient patients, and 1,079 healthy control subjects. 3-OMD concentrations in 38,888 healthy new-borns revealed a mean of 1.16 μmol/L (SD = 0.31, range 0.31-4.6 μmol/L). 1079 non-AADC control subjects (0-18 years) showed a mean 3-OMD concentration of 0.78 μmol/L (SD = 1.75, range 0.24-2.36 μmol/L) with a negative correlation with age. Inter- and intra-assay variability was low, and 3-OMD was stable over 32 days under different storage conditions, which marks another advantage of this test. They identified seven confirmed AADC deficient patients (mean 3-OMD 9.88 μmol/L [SD = 13.42, range 1.82-36.93 μmol/L]). The highest concentration of 3-OMD was found in a filter card of a confirmed AADC deficient patient with a mean 3-OMD of 35.95 μmol/L. 14 DDC variant carriers showed normal 3-OMD concentrations. With the results from the new-born screening cohort and with the knowledge of an 8-fold increase in concentrations in NBS cards of a confirmed AADC deficient patient, they defined a 5 μmol/L the cut-off value for the age group of 0 to 28 days. Preterm infants have been reported to reveal high concentrations of 3-OMD and should undergo regular follow-up

measurements in DBS after 32 weeks of gestation before raising the suspicion of AADC deficiency. However, by using the proposed cut-off score, they did not identify elevated 3-OMD concentrations in any preterm new-born. The cut-off was later adjusted according to age: at 5 μmol/L for neonatal screening; at 3 μmol/L for children aged from 28 days to 10 years, and 2 μmol/L for children between 10 and 18 years old. Cut-offs for patients above 18 years old were not identified. Hence, in patients older than 18 is not a validated screening tool, but rather a research method that should be performed as well to collect more data and expand the knowledge of this disorder.

Even though 3OMD screening is an extremely useful test, it is also altered and positive in patients suffering from PNPO deficiency. In these patients, the CSF analysis shows the same alterations of AADCD as well. However, the clinical presentation of this disease is far from the one of AADCD: in fact, PNPO is characterized by severe neonatal epileptic encephalopathy. Moreover, other laboratory exams allow the differential diagnosis, such as low CSF pyridoxal phosphate concentration and high glycine and threonine concentrations; also, AADC enzyme plasma activity is normal in PNPO deficient patients.

The other screening tool proposed by the same Brennenstuhl, the semiquantitative detection of VLA/VMA ratio on urinary sample[4], meets the criteria of rapidity, non-invasiveness, and inexpensiveness. However, the ratio is less reliable than the 3OMD dosage since the levels of VLA and VMA might greatly vary among patients and in the same subject is different. Also, it might be altered in other neurometabolic diseases or more general medical conditions (such as pheochromocytoma and neuroblastoma). The ratio is more specific than the dosage of the single acids, but it remains a sensible though not specific

laboratory parameter for AADCD diagnosis. Therefore, we decided not to rely much on this test as a screening tool and preferred 3OMD evaluation on dried blood spot.

According to *Wassenberg et al.*, instrumental exams can be performed as well, but are not core diagnostic exams. In fact, brain MRI is normal in 75 % of patients and the abnormalities found are not specific for AADCD. Ictal EEG is useful to differentiate AADCD from epilepsy. ^{18}F.FDG-PET scan is not done routinely since it is an invasive diagnostic exam for its radioactivity. It is performed only in patients who underwent gene therapy to monitor the injection effectiveness.

4.8 Differential diagnosis

After all these considerations, we can affirm that biochemical, metabolic, and genetic tests are crucial to obtain the definitive diagnosis. However, physical evaluation and clinical presentation should always be considered and implemented in the diagnostic workflow. AADCD is a neurotransmitter disorder which can mimic other neurological diseases and, therefore, misdiagnosis and diagnostic delay are commons (Table 5).

Differential diagnoses include[d]:

- **Cerebral palsy:**

This term indicates a group of permanent neuromotor disorders, causing limitations that are attributed to non-progressive disturbances, that occur in infancy or early childhood, accompanied by developmental delay. This is the most frequent physical disability in young children, with a prevalence of 2-3 cases per 1,000 patients.

There are several forms of CP:

- *Spastic CP*: the most common form. It presents with stiffness, spastic muscles, abnormal movements, difficulty in controlling muscle movements, dysarthria, and dysphagia. These children fail to sit, crawl, and walk.
- *Athetoid or dyskinetic CP*: athetosis, dystonia, chorea, and movements are conducted slowly or rapidly, but in a very unpredictable way. Their pace might change due to emotional tension and these movements typically are absent during sleep.
- *Ataxic CP*: weakness, poor coordination, tremors, unsteady balance, and difficulty with fine or rapid movements.

- *Mixed CP*: patients who present a mixture of the various forms.

Some of these symptoms are very similar to those present in AADC-deficient patients, therefore a correct clinical, laboratory and instrumental evaluation is necessary. From a clinical perspective, both disorders might present with dystonia, developmental delay, hypotonia, seizure. However, typical for AADCD and uncommon for CP are oculogyric crisis, multiple autonomic disfunctions, diurnal variations of the symptoms. These are key distinguishing clinical symptoms, that, however, might not always be present. Therefore, it is always important for the differential diagnosis towards AADCD to run laboratory tests such as CSF neurotransmitter analysis, AADC enzyme plasma activity or, more easily, the new 3OMD screening on dried blood spot. Brain MRI may be also helpful to differentiate the two disorders. Brain MRI are a first-line investigation in patients who present with an undiagnosed motor disorder. In AADCD, only 25 % of patients show brain abnormalities, usually of the white matter or with a mild atrophy, while almost all children with CP show some degree of brain anomalies (usually malformations).

- **Epilepsy:**

Epilepsy is a chronic neurological disorder characterised by recurrent and unpredictable seizures due to abnormal excessive or synchronous neuronal activity in the brain. It is one of the most common neurological diseases in the world, affecting around 50 million people of all ages.

Epileptic seizures can be divided into:

- *Generalized seizures*: begin in bilateral, distributed neuronal networks and are comprised of absence, generalised tonic-clonic (GTC), myoclonic, and atonic subtypes.
- *Focal seizures*: originate in neuronal networks limited to part of one cerebral hemisphere and the clinical manifestation depends on the area of cortex involved.
- *Epileptic spasms*: are characterised by sudden extension or flexion of extremities that are held for several seconds and reoccur in clusters.

Paroxysmal events that often develop in patients with AADCD, such as oculogyric crises, tonic or dystonic posturing of the limbs, myoclonus, and chorea, can mimic epileptic seizures and, consequently, can be misdiagnosed. Since the clinical presentation is similar, the differential diagnosis relies only on laboratory and instrumental exams. EEG is the easiest and first test that should be performed. An EEG performed during a crisis shows abnormalities if the patient is epileptic, otherwise epilepsy should be excluded. It is important to remember that intercritical EEG is almost always normal, even if the patient is a known epileptic, therefore it is not necessary. It is essential to differentiate AADCD from epilepsy because the treatment is completely different. Since AADCD is so rare and often undiagnosed, patients might receive several antiepileptic treatments with no benefits and might be exposed to side effects. In conclusion, epilepsy and AADCD are mutually excluding in most cases, but in some they may coexist[5]. This is probably because the alterations present are so severe that they determine both disorders. After the EEG, if the suspicion for AADCD is strong, laboratory tests, such as 3OMD dried blood spot screening, AADC enzyme plasma activity or CSF analysis should be performed.

- **Neuromuscular disorders**:

Neuromuscular disorders are a broad group of genetic heterogeneous diseases affecting the muscle and/or the peripheral nervous system.

Types of neuromuscular disorders include:

- Amyotrophic lateral sclerosis (ALS)
- Charcot-Marie-Tooth disease
- Multiple sclerosis
- Muscular dystrophy
- Myasthenia gravis
- Myopathy
- Myositis, including polymyositis and dermatomyositis.
- Peripheral neuropathy
- Spinal muscular atrophy

Neuromuscular disorders vary in disease course and severity, depending on the affected body area, but typical symptoms include muscle weakness, abnormal or impaired ambulation, joint contractures, skeletal deformities, altered sensory perception (neuropathies) and respiratory failure. Myalgia, rhabdomyolysis, and fatigable weakness may occur. Since 50% of AADCD patients present with movement disorders (hypokinesia, chorea, dystonia, ballismus, dyskinesia, tremor, myoclonus), this disorder can mimic many neuromuscular disorders, such as congenital myasthenia gravis, leading to misdiagnosis. From the clinical point of view, AADCD and neuromuscular disorders share symptoms such as cognitive

alterations, hypotonia, akinesia, myoclonus, ptosis, dystonia. However, some symptoms are more specific to AADCD, such as oculogyric crises, dyskinesia, and autonomic disfunctions.

- **Mitochondrial disorders**:

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise because of dysfunction in the mitochondrial respiratory chain, with a prevalence of 20 per 100,000. Common clinical features of mitochondrial diseases include ptosis, external ophthalmoplegia, proximal myopathy and exercise intolerance, cardiomyopathy, sensorineural deafness, optic atrophy, pigmentary retinopathy, and diabetes mellitus. Overlapping symptoms between AADCD and mitochondrial disorders include hypotonia, hypertonia, seizure, ptosis and autonomic disfunction, while oculogyric crises and dyskinesia are typical of AADCD. Spasticity and abnormal neuroimaging are instead typical of mitochondrial disorders. However, it is important to remember that oculogyric crises might happen in mitochondrial disorders and up to 25% of AADC-deficient patients has abnormal brain-imaging. The classical tests such as CSF analysis, AADC enzyme plasma activity and 3OMD dried blood spot screening can be performed. However, performing molecular testing may be more reliable.

- **Hyperekplexia**:

Hereditary hyperekplexia (HPX), an inherited neuronal disorder caused by genetic defects leading to dysfunction of glycinergic inhibitory transmission, is characterized by the clinical core features of exaggerated startle responses to unexpected sensory (e.g., tactile, acoustic)

stimuli that typically does not habituate, and stiffness. HPX, a rare and underdiagnosed disorder, is manifest immediately after birth and commonly improves with age. Establishing the correct diagnosis early is essential so that proper management may be initiated to alleviate stiffness and reduce the risk of complications, such as potentially life-threatening apnoea during episodes of stiffness. Hyperekplexia can be an acquired feature of many disorders and it may also be observed in infants and children with complex genetic disorders associated with developmental delay/intellectual disability often resulting from an inborn error of metabolism or brain malformation.

	AADCD	Cerebral Palsy	Epilepsy	Neuromuscular disorders	Mitochondrial disorders
Symptoms:					
– **Akinesia**	+	-	-	+	-
– **Chorea**	+	-	-	+	-
– **Developmental delay**	+	+	-	-	-
– **Diurnal variation**	+	-	-	-	-
– **Dyskinesia**	+	-	-	-	-
– **Dystonia**	+	+	+	+	
– **Generalized tonic seizure**	-	-	+++	-	-
– **Hypertonia**		+	-	+	+
– **Hypotonia**	+	+	-	+	+
– **Multiple autonomic dysfunction**	+	-	-	-	
– **Myoclonus**	+	+/-	+	+	+/-
– **Oculogyric crisis**	+	-	-	-	-
– **Ptosis**	+	-	-	+	+
– **Spasticity**	-	+	-	+/-	+/-
– **Seizure**	+/-	+	+++		+/-
Laboratory exams (AADCD):					
– **CSF analysis**	+	-	-	-	-
– **AADC enzyme activity**	+	-	-	-	-
– **3OMD dried blood spot screening**	+	-	-	-	-
Instrumental exams:					
– **Ictal EEG**	- *	-	+	-	-
– **Brain MRI**	-	+	+/-	+/-	+
DDC gene mutations	+	-	-	-	-

Table 5: differential diagnosis

*NB: ictal EEG can be positive if there is an overlapping with an epileptic disease.

The aforementioned disorders have clinical aspects in common, but they always differ from AADCD in terms of laboratory and instrumental findings. However, there are some diseases that have a completely different clinical presentation but share the biochemical alterations as AADCD. If only biochemistry is considered, AADCD must be differentiated from conditions such as PNPO deficiency and tetrahydrobiopterin disorders.

In AADCD, CSF analysis shows an increase of 3OMD, L-DOPA, 5HTP and lower levels of 5-HIAA, HVA, MPHG. These biochemical alterations are present also in PNPO and tetrahydrobiopterin disorders (Figure 12). To further differentiate these disorders from a biochemical point of view, CSF pterins levels must be analysed: if low, they are indicative of tetrahydrobiopterin disorders, while normal levels are present in both AADCD and PNPO. These last two disorders can be easily differentiated through the assessment of AADC enzyme plasma activity: this test is altered in AADCD and normal in PNPO. However, these are just example of how to differentiate AADCD from other pathologies based only on laboratory data. In day-to-day practice, all the data available must be collected and integrated to make the diagnosis. For example, it is possible to distinguish PNPO and AADCD just on the basis of the clinical presentation, since PNPO presents as a severely neonatal epileptic encephalopathy, while AADCD is unlikely to present with seizures.

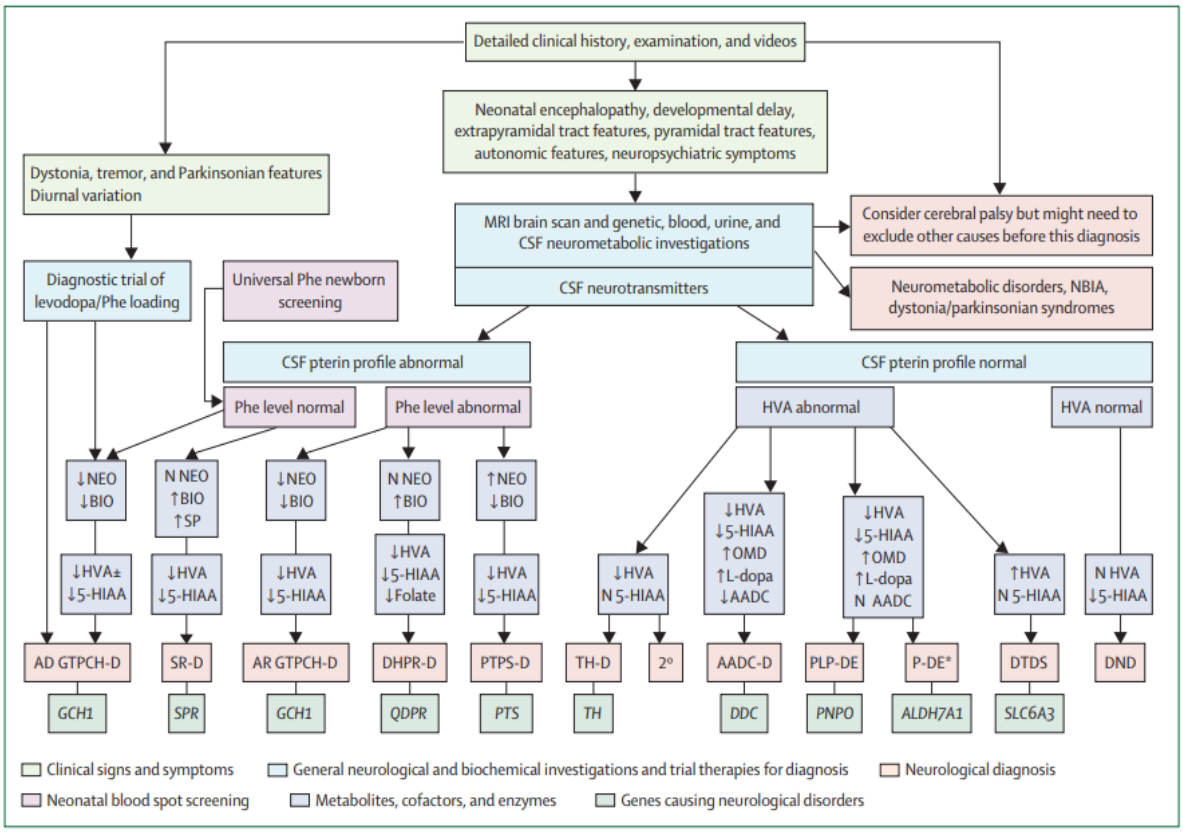

*Figure 12 Algorithm for the investigation and diagnosis of monoamine neurotransmitter disorders based on clinical presentation Appropriate neurometabolic investigations in blood, urine, and CSF could include the following: in blood, measurement of glucose, lactate, ammonia, aminoacids, biotinidase, thyroid function tests, acylcarnitine, carnitine profi le, white-cell enzymes, and very-long-chain fatty acids; in urine, measurement of aminoacids, organic acids, oligosaccharides, purines, and pyrimidines; in CSF, measurement of glucose, lactate, aminoacids, and neurotransmitter profi le. Skin and muscle biopsy may be undertaken for specialist metabolic investigations. NBIA=neurodegeneration with brain iron accumulation. Phe=phenylalanine. HVA=homovanillic acid. NEO=neopterin. BIO=biopterin. N=normal. SP=sepiapterin. 5-HIAA=5-hydroxyindoleacetic acid. OMD=3-ortho-methyldopa. L-dopa=levodihydroxyphenylalanine. AADC=aromatic L-amino acid decarboxylase. AD GTPCH-D=autosomal dominant GTP cyclohydrolase 1 defi ciency. SR-D=sepiapterin reductase defi ciency. AR GTPCH-D=autosomal recessive GTP cyclohydrolase 1 defi ciency. DHPR-D=dihydropteridine reductase defi ciency. PTPS-D=6-pyruvoyltetrahydropterin synthase defi ciency. TH-D=tyrosine hydroxylase defi ciency. 2o =secondary monoamine neurotransmitter defects. AADC-D=aromatic L-aminoacid decarboxylase defi ciency. PLP-DE=pyridoxal-phosphate-dependent epilepsy. P-DE=pyridoxine-dependent epilepsy. DTDS=dopamine transporter defi ciency syndrome. DND=dopa non-responsive dystonia. *Measurement of urine α-aminoadipic semialdehyde can assist diagnosis and diff erentiation of P-DE from PLP-DE. (Kurian et al, 2011[9])*

4.9 Therapeutic approach

AADCD is caused by a reduced or even absent enzyme activity in the brain. The AADC enzyme is responsible for the production of dopamine from L-DOPA and serotonin from 5HTP (5 hydroxytryptophan).

Therefore, the typical neurometabolic presentation in these patients' CSF is:

- High levels of 5HTP and L-DOPA and 3OMD (which derives from the methylation of L-DOPA done by COMT);
- Low levels of 5HIAA, HVA and MHPG;
- Normal levels of pterins

In peripheral blood, higher levels of 3OMD can be detected, while in urine samples VLA levels can be increased and VMA levels can be either normal, increased or reduced. The diagnosis can be done either through CSF analysis, AADC enzyme plasma activity assay or, better, molecular testing. More recently, the detection of 3OMD levels on dried blood spot has emerged as a valuable diagnostic tool[3].

Since AADCD is rare and patients may often remain undiagnosed, the administration of a proper therapy is generally delayed. Furthermore, there is no specific and validated aetiological treatment so far. AADCD is currently managed through symptomatic therapy, with a purpose of either balancing neurotransmitters levels or replacing their activity. The first treatment was done in 1992 by Hyland[14], the first who identified this disorder just few years before[13], who administered pyridoxine and bromocriptine with additional tranylcyrpromine, dezamphetamine and imipriamine. His approach was empirical, but these classes of medications would have been the most used in these patients during the following

years. An attempt to collect and categorize all the treatments was made in 2017 by *Wassenberg and colleagues*, whose guidelines are still followed up to today.

As first line treatment, they suggest using:

- Dopamine Agonists;
- MAO inhibitors;
- Pyridoxine or Pyridoxal Phosphate

- **<u>Dopamine Agonists</u>:**

The rational for their use is that they activate post-synaptic dopamine receptors directly, thus replacing dopamine activity. They can be divided into ergot-derived and non-ergot derived. Ergot derived DAs can be divided into two categories as well, depending on the presence of strong serotoninergic activity (5HT2b-R) or not. Ergot derived DAs with strong serotoninergic agonist activity are pergolide and cabergoline: they are strongly associated with cardiac valvulopathy and other severe fibrous complications[45], therefore should not be used in AADCD. Ergot derived DAs without serotoninergic effect (e.g., bromocriptine) have a lower risk of fibrosis, but have been reported some cases of pulmonary, retroperitoneal, and pericardial/cardiac fibrosis, although dose related[46]. Non-ergot derived DAs, such as pramipexole, ropinirole, rotigotine (transdermal patches) and apomorphine (subcutaneous), have a very low incidence of fibrotic complications[47], therefore they are the suggested and preferred type of Dopamine Agonists in these patients. DAs tended to obtain positive responses, especially from the motor, muscular and autonomic standpoint. In some patients

they did not work, in others they produced side effects such as irritability, weight loss, worsening of failure to thrive, vomiting and mild to severe dyskinesia.

- **MAO inhibitors:**

They are administered with the purpose of increasing dopamine and serotonin levels since their main action is to prevent substrate breakdown by interfering with the activity of degrading enzymes. MAO inhibitors used in AADCD were mostly tranylcypromine, selegiline and phelnezine. Some studies showed an improvement of at least one standpoint (e.g., hypotonia), some showed no improvement[48], or only temporary improvement[33] and even a worsening of dystonic crises[28,33]. Therefore, from a biochemical point of view MAOi are valid drugs, but AADC-deficient patient did not actually benefit from them clinically. It is important to notice that both DAs and MAOi are often administered in association with other molecules, therefore it is quite difficult to assess their true effectiveness and to relate the response to one single drug.

- **Pyridoxine or Pyridoxal Phosphate:**

Both are forms of vitamin B6. In particular, pyridoxal phospate (PLP) is the active form of pyridoxine and is the actual cofactor of AADC. Their rationale of use is to sustain the activity of the enzyme, ensuring at least the cofactor is present, since its deficiency can impact negatively AADC activity. Between PLP and pyridoxine, the latter is preferred because is more easily available and cheaper. Only one study used PLP[17], and 8 studies used pyridoxine in monotherapy, but just one patient with a milder phenotype had actual benefits[15]. Usually,

pyridoxine is administered in combination with other drugs, and it is difficult to evaluate its effectiveness. Side effects were reported such as gastrointestinal and sleeping problems, and a negative interaction between high doses of pyridoxine and L-DOPA that led to motor restlessness. Eventually, pyridoxine and PLP can cause reversible polyneuropathy at high doses. At the moment, both molecules, but especially pyridoxine, are suggested as first line therapy for AADCD, but close attention must be given to potential side effects.

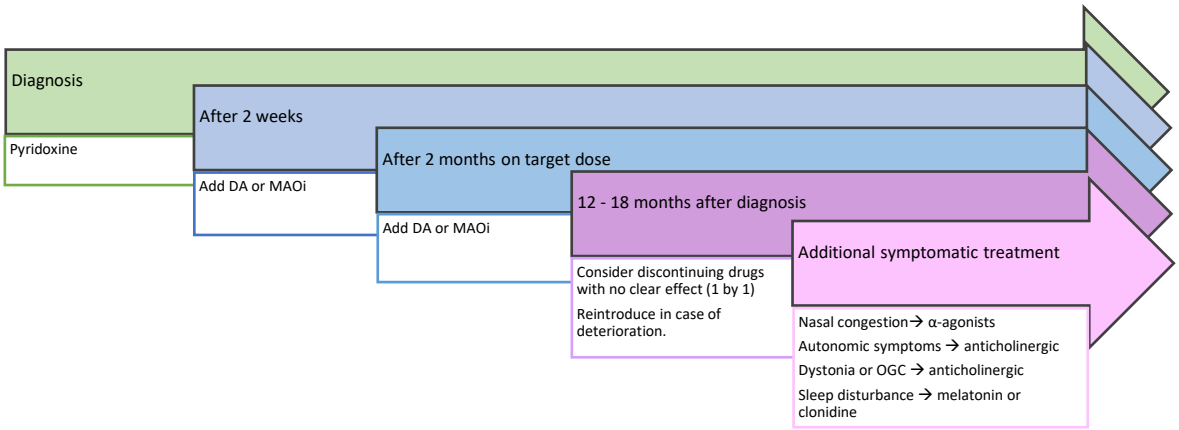

Figure 13 Algorithm for first line treatment

A possible algoritm treatment algortimn is showed in Figure 13. The first line treatment to start at diagnosis is pyridoxine, followed after two weeks by a Dopamine Agonist (DA) or a MAO inhibitor (MAOi). After two months of tretament at target dose, another medication is introduced, either a DA o a MAOi. The order of introduction a DA or a MAOi is interchangeble. Effect and tolerabilty affect dose escalation: if an agent as too many side effects and/or is not effective, consider replacing it with another drug form the same class of medication before passing to the next step. However, if side effects are

unbearable, stop the administration. After a year of stable treatment, the patient shoul be reassesed and drugs without clear effects on treatment should be discontinued, but one at time and gradually. Drugs should be immediately reintroduced in case of deterioration. Frequents assessments are always necessary.

DAs, MAOi and vitamin B6 in her different forms are the first line treatment and are meant to treat the most prominent symptoms, such as hypotonia, dystonia, oculogyric crises, autonomic disorders. However, these drugs are beneficial in a limited number of cases. Therefore, they are administered in association among themselves or with other additional drugs, including:

- **<u>Anticholinergic drugs</u>:**

Anticholinergic drugs (e.g., trihexyphenidyl, bentropine, biperiden) are the typical treatment for other movement disorders, especially parkinsonisms[49] and dystonia[50]. Since AADCD is also caused by a lack of dopamine too, this disorder can be considered as a form of child parkinsonism and, therefore, anticholinergic drugs might have a positive effect. Although their exact mechanism of action is not known, it seems they might influence the imbalance between dopaminergic and cholinergic pathways, enforcing the former and weakening the latter. In AADCD, they can be useful for treating autonomic symptoms (e.g., ptosis, miosis, nasal congestion, excessive sweating, temperature instability, etc.), dystonia and oculogyric crises. Currently they are considered as adjuvants and can be associated with the on-going therapy in those patients who might need a better control of autonomic symptoms, dystonia, and oculogyric crises.

- **Melatonin:**

It can be considered to treat sleep disturbances AADC-deficient patients experience. The rationale of its use is that, since serotonin levels are impaired, melatonin (which is a serotonin product) is impaired as well and its supplementation can be beneficial. However, there is very limited evidence of melatonin use in AADCD in the literature[28,31,51].

- **Benzodiazepines:**

There is a very limited evidence of use of BDZ in AADCD[5,22,52]. BDZ have many effects including myorelaxation and sedation. AADC-deficient patients tend to be hypotonic, and severe patient might present also with hypotonia involving respiratory muscles, thus requiring some form of mechanical ventilation. BDZ can worsen hypotonia, due to their effect on muscle tone, and might create breathing problems, not only by weakening respiratory muscles, but also with their effect on the bulbar respiratory centres, reducing respiratory drive. Plus, they might worsen dysphagia in these patients. However, these drugs can be useful for treating dystonia and oculogyric crises, which can last for hours and endanger patients' life. Therefore, these drugs cannot be administered continuously, but intermittently, and are indicated for sustained oculogyric and dystonic crises. Drugs used in AADCD patients and with benefits were clobazam and diazepam[22,52].

- **Other less important symptomatic drugs** include:

α-adrenoreceptor nose drops, used to control nasal congestion, which can be very annoying for these patients. The problem with these drugs is that all the molecules that interact with

autonomic receptors tend to reduce their effectiveness over time, due to tachyphylaxis. To restore nose drops effect and to reduce the necessary dosage, topic steroids can be used for a limited period of time. Clodine, imidazoline and α2-agonists can be used for irritability and sleep disturbances[53]. Botulinum toxins can be used to treat dystonia.

- **L-DOPA:**

A particular treatment in patients with AADCD is L-DOPA[8,22,33,54], either alone or with carbidopa. AADCD is characterized by elevated levels of L-DOPA; therefore, its administration seems counterintuitive in these subjects, and it is in most cases. However, there is a subset of AADCD patients that benefits from L-DOPA: those whose pathogenic variants affect the L-DOPA binding site (e.g., G102S; R347Q; R160W). This is a perfect example of how molecular characterization has strict implications in therapy (precision medicine). Exclusively in these patients, L-DOPA is considered a first line of treatment, alongside folinic acid supplementation because of possible depletion. Subsequently, it is recommended to determine CSF 5-MTHF levels before and during L-DOPA therapy.

- **Folinic acid:**

Except for L-DOPA treatment, which can actually deplete it, folinic acid usually is not deficient. Its deficiency might happen if the methylation of L-DOPA to 3OMD is excessive, with subsequent depletion of SAM and 5-MTHF. Administration of folinic acid led to moderate improvement in patients who received it[17,51,55], with few or no side effects.

Therefore, supplementation with folinic acid (and not folic acid) can be considered in all AADCD patients, but it has a pivotal role in those with low CSF 5MTHF levels.

- **5-hydroxytryptophan**:

It is the precursor of serotonin; therefore, its levels are higher in AADCD. Its administration is as counterintuitive as L-DOPA. However, while L-DOPA proved itself to be beneficial for a specific subset of patients, none of the subjects treated with 5-HTP showed a positive response[27,29,33,55]. Hence, its administration is not recommended.

- **SSRI:**

The rationale is to increase serotonin availability in synapses by blocking its reuptake. SSRIs (such as paroxetine, ergotamine, and fluoxetine) were used in four patients with no benefits and many side effects, such as: worsening of oculogyric crises and hypotonia, lethargy and dystonic reactions[23,27,28,33].

- **Anti-epileptic drugs:**

Often, AADCD is misdiagnosed as epilepsy, therefore these patients receive inappropriate AED treatment with no benefits but relevant side effects, hence the importance of correct diagnosis and the need for an ictal EEG for differential diagnosis. Very few AADC-deficient subjects happen to suffer both from AADCD and epilepsy: this is the only subset of patients who require AED[5].

	Class	Drug	Mechanism	Dose recommendation
First line treatment agents	*Vitamin B6*	Pyridoxine	Cofactor, optimizes residual activity	100 – 200 mg
		Pyridoxal 5-Phosohate	Cofactor, optimizes residual activity	100 – 200 mg
	Dopamine Agonists	Pramipexole	Non-ergot derived	Start 0.005 – 0.010 mg/kg/die; max 0.075 mg/kg or 3.3 mg/die
		Ropinirole	Non-ergot derived	Start 0.25 mg/die; max 0.3 mg/kg/die or 24 mg/die
		Rotigotine patch	Non-ergot derived	Start 2 mg/die; max 8 mg/die
		Bromocriptine	Non-ergot derived	Start 0.1 mg/kg/die; max 30 mg/die
		Pergolide or carbergoline	Ergot derived	Do not use because of the higher risk of fibrotic complications
	Mao-inhibitors	Selegiline	Prevents monoamine breakdown	Start 0.1 mg/kg/die; max 0.3 mg/kg/die or 10 mg/die
		Tranylcypromine	Prevents monoamine breakdown	Start 0.1 mg/kg/die; max o.5 mg/kg/die or 30 mg/die
Additional symptomatic treatment	*Anticolinergic drugs*	Trihexyohenidyl	Restores neurotransmitter imbalance	Effective dose highly variable (6-60 mg/die); max 60 mg/die
		Benztropine	Restores neurotransmitter imbalance	Start 1 mg/die; max 4 mg/die
	α-adrenergic agonists, nose drops	Oxymetazoline or xylometazoline nose drops	Local vasoconstriction	Dose based on age, try the lowest available dose for chronic use
	Melatonin	-	Regulates sleep onset and day/night cycling	Start 3 mg/die; max 5-8 mg/die
	α2-agonist, centrally acting	Clonidine	Antihypertensive drug that can lead to sedation, useful for irritability and sleep disturbances	Start 0.1 mg/die; max 3 mg/die
Therapy for a specific patients' subset	*L-DOPA without carbidopa*	-	Only for patients with L-DOPA binding site variants	Start 0.5 – 1 mg/kg/die; max 15 mg/jg/die
	Folinic acid	-	In patients with low 5-MTHF in CSF, due to methylation of excessive amounts of L-DOPA in AADCD that may cause depletion of methyl donors.	1-2 mg/kg/die; max 20 mg/die

Table 6: symptomatic therapy overview[1]

- **<u>Drugs to avoid</u>:**

Although the literature or clinical data available on this topic are lacking, some drugs must be avoided in AADCD. This is the case for centrally acting dopamine antagonists, that might be used for their properties as antiemetic (AADCD patients tend to be often nauseated) and antipsychotic (AADCD patients due to their neurotransmitter imbalance have behavioural problem that might present as psychosis[38,39]), because they can potentially worsen the symptoms of dopamine deficiency. Haloperidol[48], metoclopramide and levomepromazine[56] are all contraindicated in these patients. As antiemetics, 5HT3R serotonin antagonists (e.g., ondansetron, granisetron) should be avoided as well, from a biochemical and pathophysiological standpoint. If vomit is particularly severe and supportive therapy to avoid dehydration and hypoglycaemia is not sufficient, medical therapy can be administered. Low doses of domperidone are suggested since it is a dopamine antagonist that does not cross the blood brain barrier and it should be well tolerated. Nevertheless, before introducing any treatment, it is always crucial to evaluate how these molecules can interfere with neurotransmitters.

- **<u>Non-medical treatment</u>:**

Alongside medical treatment there is an important series of non-medical treatments that may reveal beneficial. AADCD patients, especially children, need constant care and a multidisciplinary approach is particularly important.

Since patients presents with various degree of hypotonia, physiotherapy sessions to improve and maintain muscle strength, tone and control must be done regularly. For subjects with severe compromission of respiratory muscle, it is even more important, to avoid

stagnation of secretions and subsequent pneumonia. For patients with limb spasticity, physiotherapy is needed to ensure proper posture and paucity of movement, alongside possible orthopaedic intervention. Speech therapy is crucial. Severely affected individuals tend to emit verses rather than words, and do not speak. This is due to both cognitive and muscular impairment. A speech therapist may help improve communication skills and dysphagia as well, possibly enhancing feeding. In fact, feeding and nutritional assessment is particularly important. Patients with the severe form suffer from dysphagia and have feeding difficulties since birth. Therefore, they might need artificial feeding, through nasogastric tube or even gastrostomy. They need to be closely monitored form a nutritional point of view to ensure they grow properly and receive all the nutrients they need. Occupational therapy is useful to develop, recover, or maintain the meaningful activities, or occupations, of individuals, groups, or communities. In this case, occupational therapy is necessary to improve cognitive development and increase these patients' autonomy and help them participate to social situations. Eventually, neuropsychological treatment and support are essential to address the behavioural problems, that might burst into psychotic presentations, as well as possible autistic features and dysphoria[8,19,39].

4.10 Gene therapy

As previously mentioned, all these treatments, either medical or non-medical, are meant to treat the symptoms, but often with poor control and outcome (Table 7). This reinforces the necessity to develop an aetiological treatment.

Article, Year	Patient No.	M/F ratio	Response to Pyridoxine	Response to Bromocriptine	Additional treatments	Response to additional treatments
Hyland et al., 1992[14]	2	M	No clinical response	Slight improvement in oculogyric crises	Tranylcypromine Dexamphetamine Imipramine	Tranylcypromine: Improved spontaneous movement and improved muscle tone Dexamphetamine and Imipramine had no clinical response
Maller et al., 1997[25]	1	M	No clinical response	Partial improvement in muscle tone and head control	Tranylcypromine	Improvement in muscle tone, spontaneous movement and head control
Korenke et al., 1997[22]	1	F	Decrease oculogyric crises and improvement in muscle tone	Improvement in hypokinesia and hypotonia	Levodopa Selegiline	Levodopa: decreased extrapyramidal movement Selegiline: temporarily suppressed oculogyric crises, improve muscle tone and bowel function
Abeling et al., 1998[26]	1	F	NA	NA	NA	NA
Swoboda et al., 1999[27]	2	1:1	No clinical response	Decreased the frequency of oculogyric episodes and rigidity	L-dopa 5-hydroxytryptophan (5-HTP) Pergolide Tranylcypromine Trihexyphenidyl Buspirone Oxymetazoline hydrochloride Pseudoephedrine hydrochloride Sertraline hydrochloride Midodrine hydrochloride	L-dopa: No clinical response 5-HTTP: induced lethargy and worsened axial hypotonia Pergolide: complete resolution of dystonic spells and oculogyric crises Tranylcypromine: improved coordination and spontaneous movement in one patient Trihexyphenidyl: modestly improved tone, limb rigidity, and excessive sweating Buspirone: reduced limb rigidity and irritability initially, then, led to tardive dyskinesia Others: no significant clinical response
Fiumara et al., 2002[29]	2	M	Partial clinical improvement initially then	Partial clinical improvement initially then	Selegiline L-dopa 5-HTTP	Selegiline, L-dopa and 5-HTTP: Slight clinical improvement initially then

			deteriorate over time	deteriorated over time	Cabergoline	deteriorate over time. Cabergoline: no clinical response
Chang et al., 2004[30]	3	2:1	NP	NP	Levodopa/carbidopa	Marked clinical improvement initially but it deteriorated over time
Pons et al., 2004[31]	6	3:3	3/6 no clinical response, rest are favourable	3/6 slight improvement	Tranylcypromine Melatonin Pergolide	Tranylcypromine and pergolide: 3/6 favourable and rest no clinical response Melatonin: improvement in sleep pattern
Tay et al., 2007[32]	2	F	Partial clinical improvement	Slight clinical improvement	Selegiline	Improved muscle strength
Ito et al., 2008[5]	1	M	Partial improvement in vocalization and voluntary movement	Partial improvement in vocalization and voluntary movement	Valproic acid clobazam	Seizure reduction
Manegold et al., 2009[33]	9	6:3	4/9 with slight improvement	2/9 with slight improvement, other, deteriorated after discontinuation of therapy	Selegiline, tranylcypromine L-dopa Pergolide	Selegiline: used in 3/9 One patient improved temporarily, others deteriorated Tranylcypromine: used in 2/9, one deteriorate and one improved L-dopa: 6/9, three improved and three showed no clinical response Pergolide: 1/9, no clinical response
Lee et al., 2009[18]	8	4:4	No clinical response	No clinical response	Moclobemide Akineton	Moclobemide: 2/9, mild improvement in the duration of oculogyric crises and irritability Akineton: 3/9, no response
Brun et al., 2010[8]	78	41:31	15/55 good clinical response, rest are no clinical response	15/38 good clinical response, rest are no clinical response	Selegiline L-dopa Pergolide Tranylcypromide Trihexyphenidyl	Selegiline: 19/78 L-dopa: 10/78 Pergolide : 12/78 Tranylcypromide: 22/78 Trihexyphenidyl: 15/78 All had no clinical response
Hwu et al., 2012[34]	4	1:3	NA	NA	Gene therapy	Weight gain and improved motor function
Alfadhel and Kattan, 2013[35]	1	M	No clinical response	No clinical response	NP	NP

Table 7: therapy overview[35]. Abbreviations: M: male; F: female; NA: not available; NP: not prescribed.

In the most recent years, gene therapy has emerged as the next future weapon for hereditary disorders and chronic diseases in general. Many studies are being conducted in this field. So far, only five gene therapies have been approved for commercialization and are currently available, i.e., Luxturna, Zolgensma, the two chimeric antigen receptor T cell (CAR-T) therapies (Yescarta and Kymriah), and Strimvelis (the gammaretrovirus approved for adenosine deaminase-severe combined immunodeficiency [ADA-SCID] in Europe). Dozens of other treatments are under clinical trials and one of them targets AADCD[c].

Gene therapy is a therapeutic approach that aims to add, delete, or correct genetic material in order to change how a protein, or group of proteins, is produced by the cell. In this way, this approach aims to treat the deficiency underlying the associated human disease. There are two main types of gene therapy: gene addition and gene editing. Gene addition adds genetic material to a person's cells. Within gene editing, several different techniques can be used to achieve a particular therapeutic effect: gene inactivation (also called disruption, silencing, knockdown, or knockout) and gene correction. As a result, gene therapy can range from antisense oligonucleotide (e.g., for CMV) to viral vector.

Since AADCD is a hereditary monogenic disorder, gene therapy is mainly conducted as gene addiction and through viral vector: a copy of the mutated gene is inserted into the genome of a recombinant virus, which will act as a carrier and will insert the functioning gene copy into a selected and precise spot. Many types of vectors are possible, from adenovirus to retrovirus. Currently, AADCD is being treated with Adenovirus vectors of serotype 2 (AAV2).

Despite AADCD is a relatively newly identified pathology, the enzyme was a known target for gene therapy. In fact, the first gene therapy studies that targeted AADC were

originally conducted for treating Parkinson's disease[40,59]. Patients with PD lacks dopamine in the substantia nigra. Replacing AADC was one of the two genetic strategies alongside gene therapy to modulate GABAergic neuronal signalling through GAD replacement. In this alternative approach, AADC was directly delivered to the putamen of PD patients with the goal to increase dopamine production. The vector used was AAV2 and patients had a modest, but positive response[24]. This trial was conducted between 1980 and 1983. AADCD was identified only in 1990[13]: when this disorder was discovered, AADC enzyme had been a target for molecular therapy for at least ten years. However, gene therapy trials for AADCD did not start until the 2010s (Table 8).

In fact, the first gene therapy trial with AAV2-AADC was performed in Taiwan in 2012[34]. *Hwu and colleagues* selected four patients with confirmed diagnosis of AADCD: all of them had a severe presentation and were bedridden, unable to speak, lacked head control. They had oculogyric crises every 2 to 3 days, unstable body temperature, excessive sweating, and behavioural problems. These patients, aged 4-6 years, were all homozygous for IVS6+4A>T/IVS6+4A>T, except for one who had a compound heterozygosis (IVS6+4A>T/c.1297_1298insA). All of them received 1,6 x 10^{11} vg of AAV2/hAADC in total by direct bilateral stereotactic putamen injection on MRI guide. The injection was correctly performed in the right spot with no surgical complications: even though these patients lack noradrenaline and adrenaline they maintained stable blood pressure and heart rate during surgery. Then, they were followed for 24 months. Two out of four presented with transient dyskinesias that interfered with feeding and one experienced significant apnoeic events that lasted 10 months. However, all four of them showed improvements of both motor and cognitive skills, with a decrease of frequency of oculogyric crises, irritability, and temperature instability. After six months from the transfer, three out of four patients had a

PET scan with 6-F-DOPA and showed an increase in its uptake from baseline ranging between 45 - 86 %. CSF analysis were also performed and showed increased levels of HVA and 5-HIAA. However, L-DOPA and 3OMD concentrations remained quite high.

In 2017, the same group of physicians this time led by *Chien* performed another trial on ten children in Taipei, Taiwan[41]. These patients had a definitive diagnosis and clinical symptoms of AADCD, were older than 24 months or had skull bones suitable for stereotactic surgery. They received 1,81 x 10^{11} vg of AAV2/hAADC in total through stereotactic brain surgery and all patients tolerated both surgery and vector injections. They were assessed at baseline and every three months during the first year post-injection. Primary efficacy endpoints were a Peabody Developmental Motor Scales-2 above 10 points and increased levels of HVA and 5-HIAA in CSF to obtain within the first twelve months post-surgery. One patient died from influenza B encephalitis few months later. The other nine successfully met the primary efficacy endpoints in the first year, especially regarding PDMS-2 and HVA levels; however, 5-HIAA concentrations in CSF did not increase. Some adverse events were reported, with the most common being pyrexia and orofacial dyskinesia, occasionally requiring hospital admission. Transient post-gene therapy dyskinesia occurred in all patients but was resolved with risperidone.

In 2019, the same techniques were used in a new cohort of patients by *Kojima et al.*[6]. This trial was held in Japan, enrolled six patients and differed from the Taiwanese because these patients had variable genetic backgrounds (Table 8). One subject had a moderate phenotype and four were older (aged 10-19 years) than the Taiwanese patients (aged 4-6 years). They received a total of 2 x 10^{11} vector genomes and to detect the expression of AADC and, later, a 6-[18F] fluoro-L-m-tyrosine-PET scan was performed and showed

persistently increased uptake in the broad areas of putamen. These patients were followed for a mean of 2 years, and they all showed a remarkable improvement in motor function. Three patients with the severe phenotype were able to stand with support, and one patient could walk with a walker, while the patient with the moderate phenotype could run and ride a bicycle. This patient with moderate phenotype also showed improvement in her mental function, being able to converse fluently and perform simple arithmetic. Dystonia disappeared and oculogyric crisis was markedly decreased in all patients. The patients exhibited transient choreic dyskinesia for a couple of months, but no adverse events caused by vector were observed. Younger patients showed a better response to therapy than older ones and the one with a possible partially conserved enzyme activity showed a better response than the Taiwanese ones.

Study	Patient No.	Gender	Age at GT	DDC gene mutations	Phenotype	Clinical results
Hwu et al. 2012[34]	1	F	4 y 3 m	IVS6+4A>T/IVS6+4A>T	Bedridden, no head control	Weight gain; gradual motor improvement and acquired motor milestones (head control, sitting with support); OCS reduced; improvement in autonomic dysfunctions and behaviour
	2	M	4 y 5 m	IVS6+4A>T/IVS6+4A>T	Bedridden, no head control	Weight gain; gradual motor improvement and acquired motor milestones (head control, sitting with support); OCS reduced; improvement in autonomic dysfunctions and behaviour
	3	F	4 y 6 m	IVS6+4A>T/IVS6+4A>T	Bedridden, no head control	Weight gain; gradual motor improvement and acquired motor milestones (head control, sitting with support); OCS reduced; improvement in autonomic dysfunctions and behaviour
	4	F	6 y 3 m	IVS6+4A>T/c.1297_1298insA	Bedridden, no head control	Weight gain; gradual motor improvement and acquired motor milestones (head control, sitting with support); OCS reduced; improvement in autonomic dysfunctions and behaviour
Chien et al 2017[41]	1	F	6 y 2 m	IVS6+4A>T/IVS6+4A>T	no head control	Partial head control
	2	M	7 y 8 m	IVS6+4A>T/IVS6+4A>T	no head control	Partial head control
	3	F	8 y 5 m	IVS6+4A>T/IVS6+4A>T	no head control	Partial head control
	4	M	2 y 5 m	IVS6+4A>T/1058T→C	no head control	Good head control, sit without support, stand with support
	5	M	2 y 6 m	IVS6+4A>T/IVS6+4A>T	no head control	Good head control, sit with support briefly
	6	F	6 y 5 m	IVS6+4A>T/1297dupA	no head control	Partial head control
	7	M	2 y 6 m	IVS6+4A>T/179T→C	no head control	Good head control, sit with support briefly
	8	F	2 y 10 m	IVS6+4A>T/286G→A	no head control	Good head control, sit with support briefly
	9	M	2 y 1 m	IVS6+4A>T/IVS6+4A>T	no head control	Good head control, sit with support briefly
	10	F	1 y 7 m	IVS6+4A>T/IVS6+4A>T	no head control	Good head control, sit with support briefly

(Continues)

Kojima et al. 2019[6]	1	M	15 y	329C4A / not detected	Severe, bedridden, gastrostomy, Laryngotracheal separation	Acquisition of voluntary movements, motor milestones (head control, sitting); stand with support; acquired hand movements, but difficulty in controlling their hands as well; improved respiration and swallowing; hypotonia slightly improved, but remained; OCG crises reduced markedly; mild cognitive, social and verbal improvement (able to smile, respond and utter sounds and better understanding the language); improvement in autonomic dysfunctions and behaviour
	2	F	12 y	329C4A/ not detected	Severe, bedridden, gastrostomy	Acquisition of voluntary movements, motor milestones (head control, sitting), stand with support; walk with a walker and then gradually became able to walk long distances; able to grasp objects and bring food to mouth; improved respiration and swallowing; able to eat orally and was weaned from tube feeding. hypotonia slightly improved, but remained; OGC reduced markedly; mild cognitive, social and verbal improvement (able to smile, respond and utter sounds and better understanding the language); improvement in autonomic dysfunctions and behaviour
	3	F	5 y	315G4C/385C4T	Moderate, walk with support, oral intake	Acquisition of voluntary movements, motor milestones (head control, sitting); improved respiration and swallowing; hypotonia slightly improved, but remained; she was able to walk independently 6 m. after the injection, ride a bicycle at 10 m post-operatory, and play on a swing 1y and 6 m after; OCG crises reduced markedly; marked improvement in her DQS; improvement in autonomic dysfunctions and behaviour
	4	M	19 y	1106A4G/ IVS6+4A4T	Severe, bedridden, nasogastric tube feeding, NIPPV	Acquisition of voluntary movements, motor milestones (head control, sitting); acquired hand movements, but difficulty in controlling their hands as well; improved respiration and swallowing; was weaned from NIPPV; hypotonia slightly improved, but remained, OCG crises reduced markedly; mild cognitive, social and verbal improvement (able to smile, respond and utter sounds and better understanding the language), improvement in autonomic dysfunctions and behaviour

(continues)

	5	M	10 y	IVS6+4A>T/IVS6+4A>T	Severe, bedridden, gastrostomy	Acquisition of voluntary movements, motor milestones (head control, sitting); stand with support, acquired hand movements, but difficulty in controlling their hands as well, improved respiration and swallowing; hypotonia slightly improved, but remained; OCG crises reduced markedly; mild cognitive, social and verbal improvement (able to smile, respond and utter sounds and better understanding the language); improvement in autonomic dysfunctions and behaviour
	6	M	4 y	236A4G/755A4G	Severe, bedridden, oral intake,	Acquisition of voluntary movements, motor milestones (head control, sitting); acquired hand movements, but difficulty in controlling their hands as well; improved respiration and swallowing; hypotonia slightly improved, but remained; OCG crises reduced markedly; mild cognitive, social, and verbal improvement (able to smile, respond and utter sounds and better understanding the language); improvement in autonomic dysfunctions and behaviour.

Table 8: comparison of gene therapy trials

In all these three studies the treatment vector (AAV2/hAADC) was made by inserting the human AADC gene with the CMV promoter into the serotype 2 adeno-associated vector. At the same time, other vectors were investigated, especially AAV serotype 9. One of the first studies regarding this new vector was led in 2013 by *Ciesielska* and consisted in the injection of human AADC into rats through it[42]. All adeno-associated viruses had been quite unaffected by immune issues, particularly AAV2, a neuron-specific serotype. However, this vector has a few limitations such as a relatively modest transduction efficiency and lacks non-neuronal transduction that might be desirable in few cases. This supported the need for a new vector. From this standpoint, AAV9 seems promising since it is neuron-specific, but can also transduce non-neuronal cells, such as astrocytes. This serotype also can cross the blood-brain barrier, thus opening the possibility of administrating gene therapy through vascular routes. Despite convenient, this type of delivery showed a flaw: the vector is neutralized by even modest titres of anti-AAV antibodies. In addition, the transduction of non-neuronal cells (e.g., antigen-presenting cells) can trigger an adaptive immune response in the brain. All these concerns were examined in this study.

Ciesielska et al. injected AAV9/hAADC in rats' striatum and then analysed the tissues through immunohistochemistry. The samples showed an important inflammation response in the injection site that ultimately led to loss of neurons. This neurotoxicity was ascribed to a massive cell-mediated immune response. In addition, AAV9 seemed also to trigger a very strong humoral response, with an increase in the titre of circulating anti-AAV antibodies. Therefore, these data suggest that the use of AAV serotypes capable of transducing APC in the brain must be approached with some caution. It is also important to notice that this massive inflammatory response might be due to the insertion of the human AADC instead of the rat one: they share an 89% identity with significant sequence

divergence in several domains. On this basis, human AADC expressed in rat brain can be expected to be recognized as a foreign protein that elicits a full immune response with associated neuronal damage. This can impact the use of transgenes that are foreign to mammals, such as tetracycline transactivator[43], and must be carefully considered when the vector is designed.

AAV9 vector was also studied in mice by *Lee and colleagues*[19] who reported a positive response and showed a widespread expression of the deficient gene in neonatal mice. They did not experience any side effect, in contrast to what reported by Ciesielska. Despite what found by Ciesielska[42], AAV9 seems a promising and safe vector to the point of being approved for the treatment of SMA in 2020[c]. In 2015, *Lee et al*.[20] extended their research on AAV9 and confronted the best administration route in mice. They concluded that systemic administration is better than intracerebroventricular injection since it is an invasive and risky procedure: the distribution of the vector in the brain can be inhomogeneous, leading to areas which excessive gene expression. AVV9 is neuron-specific and can cross the blood-brain barrier, even though the expression levels of the DDC gene may not be as high as those with direct brain injection; however, the distribution is more homogeneous and predictable. Anyway, in the systemic administration route the promoter chose seems to have a higher impact on the gene transduction: the sinapsin I promoter appears to have a preferential neuronal expression (instead of the CMV promoter) and its vector, the AAVN-AADC, seems to produce a better survival, higher brain dopamine levels, and a lack of hyperactivity (that was indeed present in AAV9-AADC treated mice).

Gene therapy represents the future for many disorders and its development requires skills and important resources. Many obstacles are yet to be overcome.

Many challenges are still present and relevant, including how to[c]:

- infect target cells with sufficient vector to achieve the optimal therapeutic effect;
- overcome a high prevalence of anti-adenovirus immunity in the human population that may limit the efficacy of adenoviral vectors;
- understand differential expression of therapeutic proteins on target cells;
- produce viral particles on a large scale;
- formulate viral particles suitable for long-term storage;
- understand how a patient's own genetic background may affect the efficacy of gene therapy

Also, the ideal vector should[c]:

- have enough space to transport large therapeutic genes.
- have high transduction efficiency
- allow long-term and stable expression of the therapeutic gene.
- target the desired cell type
- direct the insertion of the therapeutic gene into a favourable part of the human genome, avoiding random insertion.
- not result in an immune response
- be suitable for large-scale manufacture.

Eventually, it should be considered that gene therapy is not immune to risks such as:

- immune response: antibodies can neutralize the vectors, thus making the injection less efficient; plus, if a cell-mediated response is triggered, neuronal loss may occur;
- insertional mutagenesis due to its location in unwanted locations: this happens with any vector that integrates its genome, especially with retrovirus, and can be very dangerous, with the possibility of inhibiting an oncosuppressor gene or activating an oncogene.
- Off-targeting and gene inactivation: undesired DNA changes occurring at non target sites in the genome and prevention of function of other important genes.
- Ectopic expression: the gene is transduced into other cells.
- Risks related to administration, especially to intracerebral injections, such as: infections, misplacement of the needle, cerebral lesions, etc.

5. CONCLUSIONS

AADCD is an extremely rare disorder, often remaining undiagnosed and with very poor quality of life and outcome. About 80 % of them presents with a severe phenotype: they are severely hypotonic, bedridden, cannot feed themselves and suffer from dysphagia. They also present with cognitive impairment and behavioural problems alongside possible psychotic traits. Therefore, they rely constantly onto their caregivers for their day-to-day life, and they need a long-term management. However, affected individuals may have a life expectancy comparable to general population and one AADCD patient was also able to conceive and give birth[44].

Of note, this disorder is not immune to complications. Hypotonia is one of the main features and is particularly concerning if it involves respiratory or pharyngeal muscles. It is not rare that AADCD patients need ventilatory support and present with dysphagia, which may lead to life threatening conditions, such as asphyxia and ab ingestis pneumonia. Oculogyric crises can also be fatal as well since they represent dystonic crises that might spread to the entire musculature, not only impairing breathing, but also leading to rhabdomyolysis. It is not rare for these episodes to last for hours (even four or more) and progress to a status dystonicus[57,58], a persistent muscle contraction that can cause rhabdomyolysis and subsequent renal failure. A dystonic crisis may be often triggered by infection or medications adjustments. The patient is admitted in intensive care, and requires sedation (usually through benzodiazepines), feeding and ventilatory support. AADC-deficient patients also tend to have a lower tolerance level towards stress. Therefore, events such as infections, (due to feeding and swallowing problems, but also to frequent hospitalizations) and surgery tend to have a higher mortality rate in these subjects. This is

probably due to the lack of adrenaline and noradrenaline with a subsequent weak sympathetic response, that might lead to hypotension, temperature instability and hypoglycaemia. Although the heart is structurally normal, a chronic lack of catecholamines might lead to cardiac complications (e.g., cardiac arrest) during illnesses and these stressful events.

In our study, we searched for *DDC* variants in a cohort of 1,200 children with undiagnosed neurodevelopmental conditions with the specific purpose of identifying possibly missed AADCD patients, in whom the adoption of an appropriate treatment could have been beneficial. As a second step, we aimed to develop an update diagnostic flowchart to enhance and simplify the diagnostic workflow in the AADCD, hopefully improving the diagnostic yield of borderline cases. In light of all the abovementioned considerations, the need for an early diagnosis and treatment is particularly relevant in AADCD patients. A correct diagnosis and early treatment can significantly improve their condition and help them in the achievement of developmental milestones, so that they can possibly become more independent. This may also lead to an improvement of their overall quality of life and reduce the incidence of complications.

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WEB RESOURCES:

a) https://www.ncbi.nlm.nih.gov/omim
b) https://pubmed.ncbi.nlm.nih.gov/
c) https://www.thegenehome.eu/hcp/types-of-gene-therapy
d) https://aadcinsights.eu/
e) http://intd-online.org/
f) https://gnomad.broadinstitute.org/
g) http://mendel.stanford.edu/SidowLab/downloads/gerp/
h) https://www. ncbi.nlm.nih.gov/snp/
i) http://exac.broadinstitute.org
j) https://www.ncbi.nlm.nih.gov/clinvar/
k) http://genetics.bwh.harvard.edu/ pph2/
l) https://cbcl.ics.uci.edu/public_data/DANN/
m) http://www.mutationtaster.org/
n) https://cadd.gs. washington.edu/snv

FIGURE INDEX:

TABLE INDEX:

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