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Study of 3-O-Methyldopa Quantification for Possible Integration into the Portuguese Newborn Screening Program

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Resumo

A deficiência da descarboxilase dos L-aminoácidos aromáticos (AADC) é uma doença genética rara que compromete a síntese de neurotransmissores, levando a sintomas neurológicos e de desenvolvimento severos. O diagnóstico precoce através do rastreio neonatal é essencial para um tratamento atempado e adequado. A quantificação de 3-O-metildopa (3-OMD), um metabolito que se acumula na deficiência de AADC, em amostras de sangue seco (DBS) é um método viável e minimamente invasivo para a deteção desta patologia. Esta trabalho avaliou a possibilidade de integrar a quantificação de 3-OMD no protocolo atualmente utilizado no Programa Português de Rastreio Neonatal utilizando o método de injeção de fluxo acoplada a espectrometria de massa em tandem (FIA-MS/MS).

Após a implementação e otimização do método, este foi validado em termos de sensibilidade, especificidade e precisão, não interferindo na deteção de outros biomarcadores. Para amostras com níveis elevados de 3-OMD, foi desenvolvido um teste de segundo nível através de cromatografia líquida acoplada a espectrometria de massa em tandem (LC-MS/MS) para resolver potenciais interferências. Embora não tenham sido identificados casos de deficiência de AADC nesta coorte, o método quantificou 3-OMD de forma consistente, detetando níveis elevados em pacientes em terapia com levodopa.

Estes resultados demonstram que a integração da quantificação de 3-OMD no fluxo existente de rastreio neonatal é viável, permitindo equacionar a adoção de uma abordagem promissora para a deteção precoce da deficiência de AADC.

Palavras-chave: Deficiência de AADC; 3-O-metildopa; Amostras de sangue seco; Espectrometria de massa; Rastreio neonatal

Abstract

Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare genetic disorder that impairs neurotransmitter synthesis, leading to severe neurological and developmental symptoms. Early diagnosis through newborn screening is essential for timely and appropriate treatment. The quantification of 3-O-methyldopa (3-OMD), a metabolite that accumulates in AADC deficiency, in dried blood spot (DBS) samples is a viable and minimally invasive method for detecting this condition. This study assessed the feasibility of integrating 3-OMD quantification into the current protocol used in the Portuguese Newborn Screening Program using flow injection analysis coupled with tandem mass spectrometry (FIA-MS/MS).

After the method was implemented and optimized, it was validated in terms of sensitivity, specificity, and precision, without interfering with the detection of other biomarkers. For samples with elevated 3-OMD levels, a second-tier test using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed to resolve potential interferences. Although no cases of AADC deficiency were identified in this cohort, the method consistently quantified 3-OMD, detecting elevated levels in patients undergoing levodopa therapy.

These results demonstrate that integrating 3-OMD quantification into the existing newborn screening workflow is feasible, presenting a promising approach for the early detection of AADC deficiency.

Keywords: AADC deficiency; 3-O-methyldopa; Dried blood spots; Mass spectrometry; Newborn Screening

Index

1. Introduction **1**

1.1. AADC Deficiency 1

1.2. Metabolic Pathway 3

1.3. Diagnosis 4

1.4. Differential Diagnosis 7

1.5. Treatment 9

1.6. Gene Therapy 10

1.7. Newborn Screening 12

1.8. Newborn Screening Program in Portugal 13

2. Objectives **17**

3. Materials and Methods **18**

3.1. Samples 18

3.2. Calibration Materials 18

3.3. Implementation of 3-OMD quantification on DBS by Flow Injection Analysis Tandem Mass Spectrometry (FIA-MS/MS) 18

3.3.1. Method 18

3.3.2. Sample Preparation 19

3.3.3. FIA-MS/MS Equipment and Analytical Conditions 19

3.4. Implementation of Second-tier Testing by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) 20

3.4.1. Method 20

3.4.2. Sample Preparation 21

3.5. Data Analysis 22

4. Results **23**

4.1. Implementation of 3-OMD Quantification by FIA-MS/MS Using 3-OMD-D_3 on DBS 23

4.1.1. Method Validation 23

4.1.2. Intereference of 3-OMD Quantification in the Quantification of Other Metabolites 23

4.1.3. Quantification of 3-OMD Concentration in Newborns 24

4.1.4. Quality Control DBS Samples 25

4.1.5. CSF Samples (Healthy Subjetcts and Patient) 26
4.2. Comparison of 3-OMD Quantification Approaches: 3-OMD-D_3 vs. $^{13}C_6$-Phe and $^{13}C_6$-Tyr 27
4.3. 3-OMD Values for Premature Newborns 32
4.4. Second Tier Testing by LC-MS/MS 32
4.4.1. Method Validation 32
4.4.2. Quantification of 3-OMD Concentration in Second Tier Testing 34
5. Discussion 36
6. Conclusion 39
7. References 41

Figure Index

Figure 1. Metabolic pathway of dopamine and serotonin synthesis.....3

Figure 2. Calibration Curve for 3-OMD quantification by FIA-MS/MS.....23

Figure 3. Box-and-whiskers plot showing the 3-OMD concentrations in 2066 normal newborns.....25

Figure 4. Box-and-whisker plot 3-OMD concentrations in normal newborns, with individual data points from two patients on DOPA therapy.....25

Figure 5. 3-OMD concentration (CSF on filter paper) in healthy subjects and a patient undergoing DOPA treatment.....27

Figure 6. Correlation between 3-OMD levels quantified using 3-OMD-D_3 and $^{13}C_6$-Phe as an internal standard.....28

Figure 7. Correlation between 3-OMD levels quantified using 3-OMD-D_3 and $^{13}C_6$-Tyr as an internal standard.....28

Figure 8. Calibration curve for 3-OMD quantification by FIA-MS/MS, using $^{13}C_6$-Phe as the internal standard.....29

Figure 9. Calibration curve for 3-OMD quantification by FIA-MS/MS, using $^{13}C_6$-Tyr as the internal standard.....29

Figure 10. Box-and-whiskers plots of 3-OMD concentrations in DBS samples quantified using FIA-MS/MS with internal standards $^{13}C_6$- Phe and $^{13}C_6$-Tyr.....31

Figure 11. Box-and-whisker plot of 3-OMD concentrations ($^{13}C_6$-Phe internal standard) in normal newborns, with individual data points from two patients on DOPA therapy.....31

Figure 12. Box-and-whisker plot of 3-OMD concentrations ($^{13}C_6$-Tyr internal standard) in normal newborns, with individual data points from two patients on DOPA therapy.....32

Figure 13. Calibration curve for 3-OMD quantification by LC-MS/MS.....33

Figure 14. Chromatographic separation of endogenous 3-OMD (blue peak) and 3-OMD-D_3 (red peak) in a basal sample using LC-MS/MS.....33

Figure 15. Chromatographic separation of endogenous 3-OMD (blue peak) and 3-OMD-D_3 (red peak) in a 10 μM enriched sample using LC-MS/MS.....34

Figure 16. Comparison of 3-OMD concentrations in 82 samples analyzed by both FIA-MS/MS and LC-MS/MS methods.....35

Table Index

Table 1. Symptoms of AADC deficiency.....2

Table 2. Key diagnostic tests and additional potential tests for AADC deficiency.....7

Table 3. Principles of screening established by Wilson and Jungner (1968).....14

Table 4. Panel of diseases included in the Portuguese Newborn Screening Program.....15

Table 5. Findings from newborn screening in Portugal, 1979–2022.....16

Table 6. Mass spectrometry instrument parameters for 3-OMD quantification by FIA-MS/MS.....20

Table 7. 3-OMD fragmentation conditions for MRM detection.....20

Table 8. LC-MS/MS elution gradient for 3-OMD quantification.....21

Table 9. Validation parameters for 3-OMD quantification by FIA-MS/MS.....23

Table 10. 3-OMD normal newborn concentration.....24

Tabel 11. Quality control data for 3-OMD concentrations in DBS measured by FIA-MS/MS.....26

Table 12. Quality control data for 3-OMD concentrations in DBS measured by FIA-MS/MS, using $^{13}C_6$- Phe and $^{13}C_6$-Tyr as internal standard.....30

Table 13. 3-OMD normal newborn concentrations (using $^{13}C_6$-Phe as internal standard).....30

Table 14. 3-OMD normal newborn concentrations (using $^{13}C_6$-Tyr as internal standard).....30

Abbreviations

3

3-HMG – 3-Hydroxy-3-Methylglutaric Aciduria
3-MCC deficiency – 3-Methylcrotonylglycinuria
3-MT – 3-methoxytyramine
3-OMD/3OMD – 3-O-methyldopa

5

5HIAA – 5-hydroxyindoleacetic acid
5-OH-Trp – 5-hydroxytryptophan

A

AADC – Aromatic L-amino acid decarboxylase
AAV – Adeno-associated virus
ALDH – Aldehyde dehydrogenase

B

BH4 – Tetrahydrobiopterin

C

CAD – Collision Gas
CH – Congenital hypothyroidism
CID – Collision-induced dissociation
COMT – SAM-dependent catechol-O-methyltransferase
CP – Cerebral palsy
CPT I – Carnitine palmitoyltransferase I deficiency
CPT II/ CACT – Carnitine palmitoyltransferase II deficiency
CSF – cerebrospinal fluid
CUD – Primary carnitine deficiency
CUR – Curtain Gas
CV – Coefficient of variation

D

DBH – Dopamine- β -hydroxylase
DBS – Dried blood spots
DDC – Dopa decarboxylase
DOPAC – 3,4-dihydroxyphenylacetic acid

E

EEG – Electroencephalography
ESI-MS/MS – Electrospray ionization tandem mass spectrometry

F

FIA-MS/MS – Flow-injection analysis tandem mass spectrometry

G

GA I – Glutaric Aciduria type I
GS1 – Ion Source Gas 1
GS2 – Ion Source Gas 2
GTP – Guanosine triphosphate
GTPCH – GTP cyclohydrolase I

H

HPLC-MS/MS – High-performance liquid chromatography tandem mass
HVA – Homovanillic acid

I

IQR – Interquartile range
IS – IonSpray Voltage
IVA – Isovaleric Aciduria

L

LCHAD/TFP – Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
LC-MS/MS – Liquid chromatography tandem mass spectrometry
L-DOPA – L-3,4-dihydroxyphenylalanine
LOD – Limit of detection
LOQ – Limit of quantification

M

MADD – Multiple acyl-CoA dehydrogenase deficiency
MAO – Monoamine oxidase A
MATI/III Deficiency – Hypermethioninemia
MCAD – Medium-chain acyl-CoA dehydrogenase deficiency
MRI – Magnetic resonance imaging
MRM – Multiple reaction monitoring
MS/MS – Tandem mass spectrometry
MS1 – First quadrupole mass spectrometer
MS2 – Second quadrupole mass spectrometer
MSUD – Maple Syrup Urine Disease

N

NBS – Newborn screening
NMDs – Neuromuscular diseases

P
PA – Propionic Aciduria
PD – Parkinson's disease
PK – Pyridoxal kinase
PKU – Phenylketonuria
PLP – Pyridoxal 5'-phosphate
PNMT – Phenylethanolamine N-methyltransferase
PNPO – Pyridoxamine-phosphate oxidase
PNSP – Portuguese Newborn Screening Program
PTPS – 6-pyruvoyl-tetrahydropterin synthase

Q
QC – Quality control

R
R^2 – Correlation coefficient

S
SAM – S-adenosyl-methionine
SCHAD – Short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
SNpc – Substantia nigra pars compacta
SR – Sepiapterin reductase

T
TAM – PLP-dependent transaminases
TEM – Temperature
TH – Tyrosine hydroxylase
TPH – Tryptophan hydroxylase

V
Vit-C – Vitamin C
VLA – Vanillactic acid
VLCAD – Very long-chain acyl-CoA dehydrogenase deficiency
VMA – Vanillylmandelic acid
VPA – Vanilpyruvic acid
VTA – Ventral tegmental area

W
WHO – World Health Organization

1. Introduction

1.1. AADC Deficiency

Aromatic L-amino acid decarboxylase (AADC) deficiency (OMIM #608643) is a rare autosomal recessive neurometabolic disorder resulting from pathogenic homozygous or compound heterozygous variants in the dopa decarboxylase (DDC) gene (1). Human AADC, encoded by a singular copy on chromosome 7's short arm at 7p12.1–p12.3 of the DDC gene (NG_008742.1; NM_000790), plays a pivotal role in neurotransmitter biosynthesis, including serotonin and dopamine (the precursor for norepinephrine and epinephrine) (Figure 1). Consequently, its deficiency culminates in neuromuscular, neurological, and behavioral manifestations (2).

Since its initial description in 1990, in a case report involving monozygotic twins exhibiting severe hypotonia and developmental delay at 2 months of age (3), AADC deficiency has been documented in nearly 350 reported cases (4). While the precise global prevalence of this condition remains uncertain, estimated birth rates stand at approximately 1:32,000 in Taiwan, 1:42,000 to 1:190,000 in the United States,1:116,000 in the European Union, and 1:162,000 in Japan (4). The higher prevalence observed in East Asian populations can be attributed to the potential founder effect of the IVS6 +4 A>T mutation (5).

The symptoms associated with this disorder typically manifest within the first six months of life (6) and vary widely in severity. They range from severe, seen in most cases (6), to milder forms (7–11). Motor function can be categorized into three phenotypic categories: severe, with minimal or no developmental milestones achieved; moderate, with partial developmental progress; and mild, where patients can walk independently without assistive devices (12).

The most commonly reported symptoms include hypotonia, movement disorders (such as oculogyric crises and dystonia), developmental delay, and autonomic issues (like ptosis and excessive sweating). Neurological observations also include epileptic seizures, behavioral problems (such as irritability and excessive crying), and sleep disturbances (including insomnia and hypersomnia). Non-neurological symptoms frequently involve gastrointestinal disturbances, including diarrhea, constipation, and feeding challenges (such as swallowing difficulties and vomiting). Additionally, some patients experience intermittent hypoglycemia, as noted in previous research (2,6,12,13) (Table 1).

Table 1. Symptoms of AADC deficiency

Category	Symptoms
Core Findings	➢ Hypotonia ➢ Movement disorders (oculogyric crises and/or dystonia) ➢ Development delay ➢ Autonomic symptoms (ptosis and/or excessive sweating)
Other Neurological Findings	➢ Epileptic seizures ➢ Behavioral problems (irritability, excessive crying, dysphoria) ➢ Sleep disturbances (both insomnia and hypersomnia)
Non-neurological Findings	➢ Gastrointestinal problems (diarrhea, constipation, gastroesophageal reflux, and feeding difficulties) ➢ Hypoglycemia

Definitive genotype-phenotype correlations in AADC deficiency are difficult to establish. The homozygous IVS6+4A>T splice variant is strongly associated with severe disease manifestations (6), while the c.304G>A (p.Gly102Ser) pathogenic variant has shown a positive response to levodopa therapy in documented cases (14). However, beyond these specific examples, establishing consistent correlations between genotype, biochemical markers, and clinical phenotype remains elusive (6).

The prognosis for individuals with AADC deficiency is challenging, particularly regarding survival rates and age-related factors. An international cohort of 63 patients revealed that nearly half were younger than 6 years old and 71% were under 13 years old, also highlighting the prevalence of severe cases in younger patients. Additionally, a significant proportion of adult patients displayed a milder phenotype, with less severe motor impairment and no oculogyric crises. This indicates a higher risk of childhood mortality associated with severe disease manifestations. Complications such as pneumonia and life-threatening events during oculogyric crises further contribute to early mortality (12). In 2012, Hwu and colleagues (15) documented 20 living individuals with AADC deficiency and 10 deceased individuals who had passed away between the ages of 1 and 7 years old, all exhibiting a severe phenotype. These findings suggest an approximate one-third risk of childhood mortality within this patient cohort (15).

1.2. Metabolic Pathway

The schematic representation in Figure 1 illustrates the pivotal involvement of AADC in biosynthetic pathways crucial to neurotransmitters.

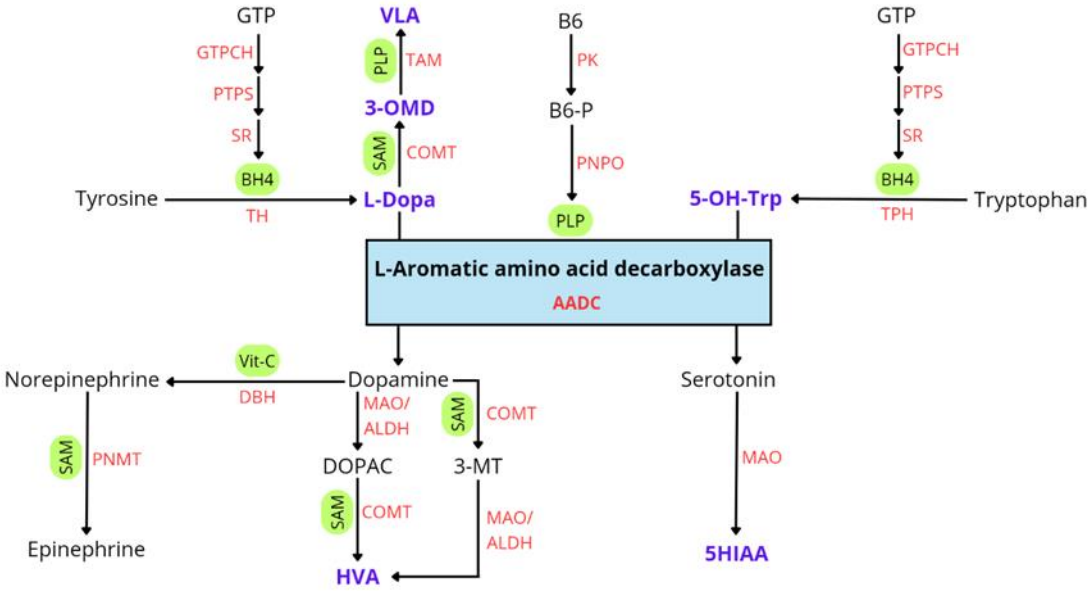

Figure 1. Metabolic pathway of dopamine and serotonin synthesis adapted from Himmelreich N. et al. (13). PLP: pyridoxal phosphate; TH: Tyrosine hydroxylase; TPH: tryptophan hydroxylase; 5-OH-Trp: 5-hydroxytryptophan; BH4: tetrahydrobiopterin; GTPCH: GTP cyclohydrolase I; PTPS: 6-pyruvoyl-tetrahydropterin synthase; SR: sepiapterin reductase; GTP: guanosine triphosphate; DBH: dopamine-β-hydroxylase; PNMT: phenylethanolamine N-methyltransferase; Vit-C: vitamin C; SAM: S-adenosyl-methionine; MAO: monoamine oxidase A; ALDH: aldehyde dehydrogenase; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; COMT: catechol-O-methyltransferase; 3-MT: 3-methoxytyramine; 5HIAA: 5-hydroxyindoleacetic acid; 3-OMD: 3-O-methyl-dopa; VLA: vanillactic acid; TAM: PLP-dependent transaminases.

AADC is pivotal in the final step of catecholamine and serotonin biosynthesis. This enzyme requires pyridoxal 5'-phosphate (PLP), the active form of vitamin B6, as a cofactor. PLP is synthesized from vitamin B6 through phosphorylation by pyridoxal kinase (PK) and subsequent oxidation by pyridoxamine-phosphate oxidase (PNPO). In the pathway, tyrosine is converted to L-DOPA (L-3,4-dihydroxyphenylalanine) by tyrosine hydroxylase (TH), and tryptophan is converted to 5-hydroxytryptophan (5-OH-Trp) by tryptophan hydroxylase (TPH), both reactions requiring tetrahydrobiopterin (BH4) as a cofactor. AADC then catalyzes the decarboxylation of L-DOPA to dopamine and 5-OH-Trp to serotonin.

The synthesis of BH4 involves a series of enzymatic reactions catalyzed by GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR), originating from guanosine triphosphate (GTP). Dopamine undergoes subsequent conversion to norepinephrine and, ultimately, epinephrine, mediated by dopamine-β-hydroxylase (DBH) and

phenylethanolamine N-methyltransferase (PNMT), respectively, relying on vitamin C (Vit-C) and S-adenosyl-methionine (SAM).

The degradation pathways involve monoamine oxidase A (MAO) and aldehyde dehydrogenase (ALDH) for dopamine, resulting in 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), the latter facilitated by SAM-dependent catechol-O-methyltransferase (COMT). Alternatively, 3-methoxytyramine (3-MT) can lead to HVA via COMT, MAO, and ALDH.

Serotonin degradation proceeds through MAO, culminating in 5-hydroxyindoleacetic acid (5HIAA). Furthermore, L-DOPA undergoes methylation to 3-OMD by COMT, followed by transamination to vanillactic acid (VLA) by PLP-dependent transaminases (TAM) (13).

1.3. Diagnosis

According to the 2017 consensus guidelines established by Wassenberg and colleagues (6), essential diagnostic procedures for AADC deficiency include cerebrospinal fluid (CSF) analysis, plasma AADC activity measurement, and genetic testing (6). The primary laboratory diagnostic approach involves analyzing neurotransmitter levels in the CSF, which requires performing a lumbar puncture. This invasive procedure is not always the first choice, especially in patients presenting with non-specific symptoms. However, in confirmed cases of AADC deficiency, CSF analysis typically reveals a distinct pattern: there is a notable decrease in the levels of neurotransmitter metabolites such as 5HIAA and HVA, alongside an increase in precursor compounds like L-Dopa, 5-OH-Trp, and 3-OMD. Notably, the metabolism of tetrahydrobiopterin (BH4) remains intact in AADC deficiency, resulting in normal levels of neopterin and biopterin in affected patients. This characteristic is crucial for differentiating AADC deficiency from BH4-related disorders, which typically exhibit abnormal pterin levels, thereby facilitating accurate diagnosis.

As expected, there are notable biochemical parallels between the CSF profiles of AADC deficiency and pyridox(am)ine 5-phosphate (PNPO) deficiency, where a secondary AADC dysfunction arises due to a deficiency of the enzyme's cofactor, pyridoxal phosphate (PLP). Both conditions exhibit reduced levels of key amine metabolites, such as 5-HIAA and HVA, alongside an accumulation of 3-OMD. However, PNPO deficiency is characterized by elevated glycine and threonine concentrations in the CSF, along with significantly reduced PLP levels (6). The low PLP impairs the function of PLP-dependent enzymes, such as threonine dehydratase and the glycine cleavage enzyme. Therefore, amino acid analysis can be useful for distinguishing between PNPO

deficiency and AADC deficiency (16,17). Furthermore, while PNPO deficiency typically presents as severe neonatal epileptic encephalopathy, the clinical picture of AADC deficiency differs (2,6,16).

AADC activity in plasma can be assessed using both 5-HTP and L-Dopa as substrates, with L-Dopa being the established standard method. This process involves enriching these natural AADC substrates and analyzing the reaction products—dopamine or serotonin—using HPLC (High-performance liquid chromatography tandem mass spectrometry) (18,19). Recently, Civallero et al. (20) developed an LC-MS/MS (Liquid chromatography tandem mass spectrometry) method that enhances the accuracy of identifying and quantifying reaction products, reducing the risk of false positives or negatives. This method also allows for the simultaneous evaluation of other metabolites related to AADC activity by incorporating their corresponding multiple reaction monitoring (MRM) conditions into the assay. This advancement makes it suitable for incorporation into IEM laboratories for the diagnostic confirmation of AADC deficiency (20).

In AADC deficiency patients, enzyme activity in plasma is typically reduced. Notably, in heterozygotes, enzyme activity is reduced compared to controls and is approximately 35–40% lower than expected based on one deficient allele. However, this level of activity is usually sufficient and typically does not result in clinical signs or symptoms (18).

Molecular genetic testing for AADC deficiency relies on the detection of compound heterozygous or homozygous pathogenic variants within the DDC gene (6). In a study of 348 patients, the Asian c.714+4A>T splice variant was identified as the most common DDC variant, with an allele frequency of 32.3%, appearing in 30 different genotypes. Of these patients, 48% were homozygotes across 41 different genotypes, while 181 were identified as compound heterozygotes (4).

A confirmed diagnosis should rely on positive findings from at least two of the three diagnostic approaches mentioned earlier. This approach is in line with established guidelines, which also specify that if genetic testing is performed initially, functional confirmation should involve assessing AADC enzyme activity in plasma and/or neurotransmitter metabolites in CSF (6).

Additional potential diagnostic tests for AADC deficiency involve assessing prolactin and catecholamine metabolite levels in blood, as well as measuring 3-OMD in dried blood spots (DBS) and evaluating organic acids in urine (6).

Prolactin levels can be increased in dopamine biosynthesis disorders because dopamine serves as an inhibitor of prolactin secretion (21). In a study of 37 untreated patients with AADC deficiency, serum prolactin concentrations were elevated in 34 patients, while three had normal levels (22).

Serotonin levels in whole blood have consistently been found to be lower in all 15 patients studied, highlighting its potential as a marker for AADC deficiency. However, data on plasma levels of monoamines and their metabolites— for example L-Dopa, VLA, HVA, VMA, MHPG, norepinephrine, epinephrine, and 5-OH-Tpr—are limited to only a few cases. As a result, it remains challenging to determine the reliability of these tests for diagnosing AADC deficiency (6).
In AADC deficiency, impaired enzyme activity leads to the accumulation of L-Dopa, which is then converted to 3-OMD. The detection of 3-OMD, particularly using DBS, has proven to be a suitable method for screening AADC deficiency (23).
Increased urinary vanillactic acid (VLA) levels, detectable through urinary organic acid analysis, have been reported in AADC deficiency (2,24). VLA is formed by the reduction of vanilpyruvic acid (VPA) and is absent in normal urine, making even small amounts diagnostically significant (19,24). However, this elevation is often subtle and can be easily overlooked if not specifically tested for in a specialized laboratory (2), and normal levels do not exclude the diagnosis (6). Urinary levels of dopamine, HVA, and vanillylmandelic acid (VMA) are not dependable biomarkers for AADC deficiency, as they can vary widely—being normal, decreased, or increased. Consequently, these measurements cannot definitively diagnose or rule out the condition (6).
While magnetic resonance imaging (MRI) of the brain and electroencephalography (EEG) are not mandatory for AADC deficiency diagnosis, its inclusion should be contemplated in the assessment of patients with neurodevelopmental delays to potentially exclude conditions like cerebral palsy and to distinguish oculogyric crises from epileptic episodes, respectively (6).

To provide a clearer overview of these diagnostic approaches, Table 2 summarizes the key diagnostic tests and additional potential tests for AADC deficiency.

Table 2. Key diagnostic tests and additional potential tests for AADC deficiency

Key Diagnostic Tests	
Diagnositc Method	**AADC Deficiency Indicators**
Neurotransmitter metabolites in CSF	Low levels of 5-HIAA and HVA, along with elevated 3-OMD, L-Dopa, and 5-HTP, combined with normal pterin
AADC activity in plasma	Severely reduced AADC activity in plasma
Molecular diagnosis	Compound heterozygous or homozygous pathogenic variants in the DDC gene
Other Diagnostic Tests	
Diagnostic Method	**AADC Deficiency Indicators**
Prolactin in blood	Elevated prolactin levels suggest AADC deficiency, but normal levels do not exclude the condition
Neurotransmitter metabolites in blood	Whole blood serotonin is consistently low in AADC deficiency, but plasma levels of monoamines and their metabolites (for example L-Dopa, VLA, HVA, VMA, MHPG, norepinephrine, epinephrine, and 5-HTP) are unreliable for diagnosis and do not replace CSF measurements.
3-OMD in DBS	Elevated levels of 3-OMD
Neurotransmitter metabolites in urine	Urinary dopamine, HVA, and VMA are inconsistent markers, but elevated VLA suggests AADC deficiency
MRI	There is no specific MRI pattern for AADC deficiency, but MRI is needed to rule out other conditions in neurodevelopmental delay cases.
EEG	EEG helps differentiate oculogyric crises from seizures and assists in evaluating neurodevelopmental delay in suspected AADC deficiency.

MRI: Magnetic resonance imaging; EEG: Electroencephalography

1.4. Differential Diagnosis

The symptoms associated with AADC deficiency often overlap with those seen in a range of conditions, including cerebral palsy, seizure disorders, neuromuscular, juvenile parkinsonism and other deficiencies in monoamine neurotransmitters. This overlap can complicate clinical recognition, particularly in milder cases of AADC deficiency.

Cerebral palsy (CP) encompasses a range of persistent conditions affecting movement and posture development, resulting in physical activity limitations. These conditions stem from non-progressive disruptions during fetal or infant brain development. Many childhood symptoms of CP resemble those seen in individuals with monoamine neurotransmitter disorders like AADC deficiency, including hypotonia, dystonia, and developmental delays. However, AADC deficiency

is distinguished by unique features such as oculogyric crises and diurnal variation, where motor symptoms often worsen in the evening and improve with sleep, although the latter may not always be present (25).

Epilepsy, characterized by irregular brain activity leading to seizures or abnormal behaviors, sensations, and occasional loss of consciousness, carries neurological, cognitive, psychological, and social ramifications. Paroxysmal episodes, frequently experienced by individuals with AADC deficiency, such as oculogyric crises, tonic or dystonic limb postures, and chorea, may be misinterpreted as seizures, resulting in misdiagnosis. Therefore, distinguishing epileptic seizures from non-epileptic paroxysmal episodes through EEG analysis is vital to prevent misdiagnosing AADC deficiency as epilepsy and to ensure appropriate treatment for patients (6,26).

Neuromuscular diseases (NMDs) are a diverse and heterogeneous group of disorders characterized by dysfunction in the peripheral nervous system, specifically involving abnormalities in the anterior horn cells, peripheral nerves, neuromuscular junctions, or muscles (27,28). While hypotonia, hypokinesia, and ptosis can resemble symptoms of AADC deficiency, the condition is characterized by distinctive clinical features such as dystonia and oculogyric crises, which help differentiate it from NMDs (28,29)

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopamine-producing neurons in the substantia nigra pars compacta (SNpc), leading to a reduction in dopamine levels in the motor areas of the putamen (30). PD is primarily identified by four key symptoms: resting tremor, muscle rigidity, bradykinesia or akinesia (slowness or absence of movement), and loss of postural reflexes (31). Additionally, patients may experience other signs such as dysphagia, dystonia, autonomic dysfunction, sensory disturbances, and cognitive or neurobehavioral issues (32). When the clinical onset is before age 21 years, PD is defined as juvenile (33), which can result from various underlying causes, including genetic ones, infections, immune-mediated conditions, structural brain abnormalities, and exposure to toxins or certain medications (32). On the other hand, AADC deficiency is a distinct neurodevelopmental disorder resulting from a single-gene mutation, typically presenting with symptoms within the first six months of life, and notably, it does not involve neurodegeneration (6).

Deficiencies in monoamine neurotransmitters, such as those caused by tyrosine hydroxylase deficiency (which affects dopamine synthesis) or sepiapterin reductase deficiency and other BH4 deficiencies (which impact both dopamine and serotonin production), can present with symptoms

similar to AADC deficiency. However, these disorders can be differentiated from AADC deficiency through CSF analysis, which reveals a lack of elevated 3-OMD in these conditions (29).

1.5. Treatment

First line treatments for AADC deficiency encompass dopamine agonists (e.g., pramipexole or ropinirole), MAO inhibitors (e.g., tranylcypromine or selegiline), and pyridoxine/pyridoxal phosphate (PLP) therapy (6).

Dopamine agonists act by directly activating postsynaptic dopamine receptors, resembling elevated dopamine levels in the nervous system. These medications have demonstrated beneficial effects, including improvements in head control, alleviation of hypotonia, reduction of oculogyric crises, enhancements in voluntary movements, and relief from autonomic symptoms. Conversely, potential adverse effects may include irritability, weight loss, exacerbation of failure to thrive, instances of vomiting, and varying degrees of dyskinesia (6).

MAO inhibitors work by blocking the degradation of dopamine and serotonin, thus enhancing the levels of monoamines in the system. These medications have shown to be effective in alleviating at least one symptom, such as hypotonia, while not necessarily impacting others. Reports of side effects were not frequent (6).

Pyridoxal phosphate (PLP), the active form of vitamin B6, serves as a crucial cofactor for the AADC enzyme. Pyridoxine, a more readily available and cost-effective form of vitamin B6, is often used in clinical practice for treating AADC deficiency. Therapeutic responses to pyridoxine monotherapy have been mixed; among reported cases, only one patient with a mild phenotype, carrying two heterozygous mutations (c.97G>C and c.1385G>C) in the DDC gene – located at the entrance of the catalytic site, altering the binding of the substrate – exhibited a clear favourable response (7). Some patients have shown improvement when pyridoxine is administered alongside other medications, suggesting potential benefits of combination therapy (6). Nonetheless, adverse effects such as gastrointestinal discomfort, sleep disturbances, and severe motor restlessness have been documented, especially at higher doses and when used concurrently with L-Dopa. Prolonged high-dose usage can also lead to reversible polyneuropathy, underscoring the importance of careful dosing and monitoring during treatment (6).

To alleviate specific symptoms linked to AADC deficiency, anticholinergic medications may be considered to address autonomic symptoms, dystonia, and oculogyric crises. Moreover, melatonin can be utilized to enhance sleep patterns in patient (6).

5-OH-Trp should not be used to treat this condition, as it is the substrate for AADC to form serotonin, it's use becomes counterproductive (6). L-Dopa supplementation can also be counterintuitive, as in AADC deficiency patients its levels are already elevated. Research shows L-Dopa should only be administrated as first line treatment for patients with L-Dopa binding-site variants (e.g. p.[G102S], p.[R347Q], p.[R160W]) (6,14,34,35). Centrally acting dopamine antagonists, typically employed for their antiemetic and antipsychotic effects, are not recommended for use in this pathology as they possess the risk of exacerbating symptoms associated with dopamine deficiency (6).

Unfortunately, most patients with AADC deficiency experience limited benefit from these available medical therapies (2,34) .

1.6. Gene Therapy

Rare genetic diseases, which arise from mutations in one or more genes, often lead to severe health challenges, including reduced quality of life and premature death. Despite the profound impact of these conditions, therapeutic options remain limited, with approved treatments available for only about 5% of such diseases (36). This significant gap highlights the urgent need for innovative approaches like gene therapy. By restoring normal cellular function through the introduction of functional copies of defective genes, gene therapy offers a promising solution to cure these diseases (37), potentially transforming the landscape of treatment for countless patients.

The approach to gene therapy for AADC deficiency involves delivering a functional copy of the DDC gene into the basal ganglia region of the brain using an adeno-associated viral vector (38–41). Recent research has documented clinical enhancements in symptoms and motor function subsequent to gene introduction (38–41).

As part of a phase 1 clinical trial, Hwu and colleagues utilized an adeno-associated virus (AAV) type 2 vector to bilaterally introduce the AADC gene into the putamen of four Taiwanese children, aged 4 to 6 years, diagnosed with AADC deficiency and sharing the IVS6+4 A>T mutation. The AADC vector administration employed a stereotactic approach. Following gene transfer, patients experienced weight gain and demonstrated gradual improvements in motor scores. One patient

achieved the ability to stand 16 months post-procedure, while the remaining three patients achieved supported sitting within 6 to 15 months. The primary adverse event noted in this study was transient dyskinesia (15). Later, a phase 1/2 trial reported data from an additional ten patients, with a median age of 2.71 years. This more recent study demonstrated clinical benefits for all patients, particularly the younger ones, evident in improvements across various independent motor assessments, PET imaging, neurochemical concentrations in CSF, and symptom relief (38).

Kojima et al. conducted an open-label phase 1/2 study in Japan, where gene delivery into the putamen was performed on six patients aged 4 to 19 years, using an AAV vector expressing AADC similar to the one described in the Taiwanese study (15,38). These patients exhibited diverse genetic backgrounds and varying degrees of severity. Five patients displayed a severe phenotype, characterized by the absence of voluntary movement or speech, while one patient had a moderate phenotype and could walk with support. Over a period of up to 2 years post-gene therapy, significant improvements in motor function were observed in all patients. Among those with the severe phenotype, three patients attained the ability to stand with support, and one patient could walk with the assistance of a walker. Moreover, the patient with the moderate phenotype demonstrated the capacity to run and ride a bicycle, along with cognitive function improvement. Older patients (aged >8 years) also demonstrated progress, although the therapy appeared to be more effective in younger patients (39).

Pearson et al. demonstrated the effective and safe administration of a viral vector carrying AADC (AAV2-hAADC) into two specific regions of the midbrain— the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA)— in children diagnosed with AADC deficiency. In their investigation, all seven subjects (aged 4 to 9 years) exhibited measurable clinical improvements post-gene delivery, characterized by the complete resolution of oculogyric crises in six out of the seven patients. Among the five subjects who were monitored for an extended 18-month period following the procedure, four achieved the capability to sit independently, while two attained the ability to walk with 2-hand support (40).

François-Heude et al. recently documented the initial compassionate use of intraputaminal delivery of eladocagene exuparvovec in two children aged over 10 years with a severe form of AADC deficiency, marking the first such experience in Europe. In line with previous research, both patients exhibited improvements in motor and non-motor functions, leading to an overall enhancement in their quality of life. This study reinforces the consistent safety record of

intraputaminal DDC gene delivery, suggesting its potential as a viable option for adolescents and older individuals suffering from severe AADC deficiency (41).

In 2022, the European Medicines Agency (EMA) granted marketing approval to PTC Therapeutics, Inc. for Eladocagene exuparvovec (Upstaza®), permitting its intraputaminal administration to patients aged 18 months and older. This authorization applies to individuals with a clinically and genetically confirmed diagnosis of AADC deficiency, exhibiting a severe phenotype (42).

1.7. Newborn Screening

Despite AADC deficiency symptoms typically appearing in the first few months of life, the mean age of diagnosis is 3.5 years (6). This significant temporal gap highlights the challenges and frequent delays associated with diagnosing this condition. Notably, newborn screening for this condition can identify patients before the onset of symptoms (6).

The measurement of 3-OMD, a metabolite that accumulates in AADC deficiency, in dried blood spot (DBS) samples has emerged as a viable, rapid, and minimally invasive method to pre-symptomatically detect this condition. It can be integrated into existing newborn screening programs using tandem mass spectrometry.

Chen et al. demonstrated the stability of 3-OMD in DBS and developed a highly sensitive, reliable, and quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify this metabolite (16). This approach was integrated into the newborn screening (NBS) program for inborn errors, administered by the National Taiwan University Hospital Newborn Screening Center. From September 2013 to December 2015, a total of 127 987 newborns were screened for AADC deficiency. Among those screened, several newborns presented with elevated 3-OMD concentrations. All screen-positive cases were referred for confirmatory testing, which included medical history reviews, physical and neurological examinations, DDC gene sequencing, and repeat measurements of DBS 3-OMD concentration and serum prolactin levels. Ultimately, 4 newborns were confirmed to have AADC deficiency, all of whom carried pathogenic DDC variants. The study reported a positive predictive rate of 100% and a false-positive rate of zero, with no known false negatives (43).

Brennenstuhl and colleague presented a novel electrospray ionization tandem mass spectrometry (ESI-MS/MS) method to quantify 3-OMD concentrations in DBS that can be easily incorporated into existing NBS high-throughput workflows. Between July and August 2019, this

method was successfully tested in 38 888 unaffected newborns. Additionally, they also demonstrated in this study that 3-OMD concentrations decrease with age (44).

Burlina et al. introduced a swift technique for determining 3-OMD levels in DBS through flow-injection analysis tandem mass spectrometry (FIA-MS/MS). From April 2020 to November 2020, the Regional Center for Expanded Newborn Screening at the University Hospital of Padua examined 21 867 samples, without detecting any affected neonates. However, one newborn exhibited elevated 3-OMD levels attributed to maternal L-Dopa treatment (45).

Chen and colleagues demonstrated an efficient and streamlined approach for determining 3-OMD levels in DBS samples, which seamlessly integrated into Taiwan's expanded newborn screening program without disrupting the existing workflow. Employing a two-tier algorithm, a total of 157 371 newborns were screened for AADC deficiency between February 2020 and December 2022 using flow-injection analysis FIA-MS/MS with $^{13}C_6$-phenylalanine as an internal standard. Among them, 114 samples showed an elevated 3-OMD/$^{13}C_6$-Phe ratio. Subsequently, these samples underwent a second-tier HPLC method, and 8 newborns with elevated 3-OMD concentrations underwent further confirmatory tests. From these cases, 6 were identified to have DDC variants and were confirmed to have AADC deficiency (46).

Recently, Reischl-Hajiabadi et al. assessed screening for AADC deficiency by measuring 3-OMD levels in DBS using tandem mass spectrometry (MS/MS) as part of a prospective, multicenter, pilot NBS study in Germany. Over the period from January 2021 to June 2023, a total of 766 660 neonates participated in the study. Among them, 204 underwent second-tier analysis by HPLC-MS/MS, and 13 with positive NBS results were recalled for confirmatory diagnostics. AADC deficiency was confirmed in one newborn, and another newborn who died before confirmatory investigations was highly suspected to have the condition. The remaining 11 cases were confirmed as false positives, attributed to maternal intake of L-Dopa, prematurity, and other unresolved factors. Given the low number of false-positive results and the very high sensitivity and specificity observed, this multicenter study reaffirms 3-OMD as a dependable screening marker for AADC deficiency (47).

1.8. Newborn Screening Program in Portugal

In the 1960's, Robert Guthrie published a simple and efficient method for mass screening of hospital nursery infants for elevation in blood phenylalanine (associated with phenylketonuria), in

DBS on filter paper, and in 1965 most newborn infants were being screened for PKU in the United States of America (48,49).

By this time, the World Health Organization (WHO) recognized the growing importance of screening and felt the need to formalize a set of principles to guide the effective identification of diseases in populations. This led to the publication of 'The Principles and Practice of Screening for Disease' by Wilson and Jungner in 1968, which laid out key criteria for establishing screening programs. These principles, which remain influential today, are summarized in Table 3 (50):

Table 3. Principles of screening established by Wilson and Jungner (1968) (50)

Principle No.	Descriprion
1	The condition sought should be an important health problem
2	There should be an accepted treatment for patients with recognized disease
3	Facilities for diagnosis and treatment should be available
4	There should be a recognizable latent or early symptomatic stage
5	There should be a suitable test or examination
6	The test should be acceptable to the population
7	The natural history of the condition, including development from latent to declared disease, should be adequately understood
8	There should be an agreed policy on whom to treat as patients
9	The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole
10	Case-finding should be a continuing process and not a "once and for all" project

The Portuguese newborn screening program (PNSP) is a non-mandatory public health initiative with a coverage rate of over 99.9%, aimed at screening all newborns for rare diseases. Its primary goal is to prevent the progression of screened pathologies through pre-symptomatic diagnosis and prompt guidance for the early implementation of appropriate therapy. It started in 1979 with the screening for phenylketonuria (PKU), expanding in 1981 to include congenital hypothyroidism (CH) (51).

Newborn DBS samples are collected in hospitals and healthcare centers all over the country, between the 3rd and 6th days of life, and transported to National Institute Dr. Ricardo Jorge located in Porto where, to this date, they are systematically screened for 28 pathologies: Congenital Hypothyroidism, Cystic Fibrosis, Sickle Cell Disease, Spinal Muscular Atrophy (pilot study), and 24 Inherited Metabolic Diseases (Table 4) (52).

Table 4. Panel of diseases included in the Portuguese Newborn Screening Program

I.	**Congenital Hypothyroidism**
II.	**Cystic Fibrosis**
III.	**Sickle Cell Disease**
IV.	**Spinal Muscular Atrophy (pilot study)**
V.	**Inherited Metabolic Diseases (24 pathologies)**
Aminoacidopathies	Phenylketonuria / Hyperphenylalaninemia
	Tyrosinemia type I
	Tyrosinemia type II/III
	Maple Syrup Urine Disease (MSUD)
	Classic Homocystinuria
	Hypermethioninemia (MATI/III deficiency)
Urea cycle disorders	Citrullinemia type I
	Argininosuccinic aciduria
	Hyperargininemia
Organic acidurias	Propionic Aciduria (PA)
	Methylmalonic Aciduria (deficiency in methylmalonyl CoA mutase, deficiency of cobalamins synthesis)
	Isovaleric Aciduria (IVA)
	3-Hydroxy-3-Methylglutaric Aciduria (3-HMG)
	Glutaric Aciduria type I (GA I)
	3-Methylcrotonylglycinuria (3-MCC deficiency)/multiple carboxylase deficiency
	Malonic Aciduria
Mitochondrial Fatty Acid β-Oxidation Disorders	Short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (SCHAD)
	Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)
	Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD/TFP)
	Very long-chain acyl-CoA dehydrogenase deficiency (VLCAD)
	Carnitine palmitoyltransferase I deficiency (CPT I)
	Carnitine palmitoyltransferase II deficiency (CPT II / CACT)
	Multiple acyl-CoA dehydrogenase deficiency (MADD / Glutaric aciduria type II)
	Primary carnitine deficiency (CUD)

In some cases (inadequate samples due to insufficient blood for analysis or degradation during sample packaging and transportation and detection of slightly altered biomarker values) a second sample is requested for analysis repetiton. Moreover, in the case of great premature newborns (gestational age <32 weeks and/or birth weight <1.500g), extra samples are also required. From 1979 to 2022, a total of 4 138 786 newborns were screened. Diagnoses were made for 929 newborns with Inherited Metabolic Disorders, 1473 cases of Congenital Hypothyroidism, 80 cases of Cystic Fibrosis, 59 cases of Sickle Cell Disease, and 1 case of Spinal Muscular Atrophy (part of a pilot study) were identified (Table 5) (52).

Table 5. Findings from newborn screening in Portugal, 1979–2022 (52)

Disease	No. of Cases Identified	Incidence
Inherited Metabolic Disorders	929	1: 2 258
Congenital Hypothyroidism	1473	1: 2 788
Cystic Fibrosis	80	1:9 740
Sickle Cell Disease	59	1:1 713
Spinal Muscular Atrophy (pilot study)	1	1:15 373
Total	**2542**	**1: 644**

2. Objectives

The primary objective of this study is to assess the feasibility of incorporating 3-OMD quantification by FIA-MS/MS into the existing MS/MS workflow of the Portuguese Newborn Screening Program (PNSP). The specific objectives are:

- Validate the 3-OMD quantification method using 3-OMD-D_3 as an internal standard.
- Ensure that the incorporation of 3-OMD as a new biomarker does not interfere with the quantification of other biomarkers routinely measured in the MS/MS workflow, such as amino acids and acylcarnitines.
- Establish 3-OMD concentrations in a representative sample of normal newborns.
- Evaluate if the FIA-MS/MS method using $^{13}C_6$- Phe and $^{13}C_6$-Tyr as internal standards is suitable for screening purposes.
- Develop, implement, and validate a second-tier testing method using LC-MS/MS.

3. Materials and Methods

3.1. Samples

The samples used in this study were provided by the National Institute of Health Dr. Ricardo Jorge, following approval from the Ethics Committee. The primary samples consisted of anonymized DBS collected on filter paper. A total of 26 507 deidentified newborn samples were analyzed through first-tier testing utilizing FIA-MS/MS. Samples exhibiting initial high concentrations of 3-OMD were subsequently subjected to second-tier testing with Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Additionally, 3 quality control samples from Chromsystems were analyzed at different concentration levels (2.01 μM, 5.99 μM and 17.8 μM) to further validate the method's accuracy. To demonstrate the method's capacity to quantify 3-OMD at clinically relevant levels, 2 deidentified DBS samples from adult patients undergoing DOPA therapy were also analyzed, alongside with deidentified CSF samples spotted on filter paper from normal individuals and a Parkinson's disease patient undergoing DOPA treatment.

3.2. Calibration Materials

A six-point calibration curve was prepared by spiking human whole blood with pure standards of 3-OMD. The 3-OMD used was monohydrated powder, which was dissolved in 10 mL of water to create the stock solution. This stock solution was further diluted in methanol to achieve final concentrations of 0, 0.5, 1, 3, 10, and 30 μM. Fifty microliters (50 μL) of these spiked samples were then spotted on sample collection cards, dried at room temperature, and stored at -20°C until analysis.

3.3. Implementation of 3-OMD quantification on DBS by Flow Injection Analysis Tandem Mass Spectrometry (FIA-MS/MS)

3.3.1. Method

Tandem mass spectrometry (MS/MS) was developed over 20 years ago (53) and is most commonly performed using a triple quadrupole mass spectrometer. This instrument includes an ionization source, three mass filters (quadrupoles) in tandem, and a detector. The process involves ionizing the sample, selecting specific ions in the first quadrupole (MS1), fragmenting these ions in the collision cell (second quadrupole) using collision-induced dissociation (CID), and then selecting the resulting fragment ions in the third quadrupole (MS2). The system can operate

in multiple modes, such as product ion scan, precursor ion scan, and multiple reaction monitoring (MRM), to detect and quantify target compounds accurately and efficiently (54). FIA-MS/MS is tailored for liquid analysis, encompassing both raw and processed sample extracts. This technique permits direct injection into the ion source by bypassing the column compartment, facilitating the rapid measurement of multiple analytes with excellent analytical performance (55).

3.3.2. Sample Preparation

The FIA-MS/MS method employed for the first-tier quantification of 3-OMD is an adaptation of the protocol currently used in the laboratory (56). The specific use of 3-OMD-D_3 as an internal standard, however, is based on the protocol described by Brennenstuhl et al (44). Briefly, a 3.2 mm DBS sample is punched into a 96-well plate, and 100 µL of methanol (MS/MS grade) containing deuterated internal standards for amino acids, acylcarnitines (NSKAB mixture, Cambridge Isotope Laboratories), and 3-OMD-D_3 (CDN Isotopes, order no. D-6782) at a concentration of 0.1 µM is used as the extraction solution. The samples are allowed to elute for 25 minutes with gentle shaking at room temperature. The extracts are then transferred to a new well plate and dried under nitrogen flow. Subsequently, 60 µL of 3N HCl in butanol is added, and the plate is sealed and incubated for 15 minutes at 70°C. After incubation, the excess HCl-butanol is evaporated under nitrogen flow. The samples are then reconstituted with 240 µL of acetonitrile/water (1:1, MS/MS grade) containing 0.01% formic acid.
For the quantification of 3-OMD using $^{13}C_6$- Phe and $^{13}C_6$-Tyr as internal standards, the sample preparation followed the same steps, except that no 3-OMD-D_3 was added to the extraction solution.

3.3.3. FIA-MS/MS Equipment and Analytical Conditions

The 3-OMD measurements were conducted on Sciex Qtrap4500 and 4000Qtrap mass spectrometers. The analysis was performed in MRM mode, with the conditions for mass spectrometry detailed in Table 6. The ion fragmentation parameters for 3-OMD and 3-OMD-D_3 are provided in Table 7.

Table 6. Mass spectrometry instrument parameters for 3-OMD quantification by FIA-MS/MS

Parameter	Setting
Curtain Gas (CUR)	30.0
Collision Gas (CAD)	Medium
IonSpray Voltage (IS)	4500.0
Temperature (TEM) (°C)	250.0
Ion Source Gas 1 (GS1)	20.0
Ion Source Gas 2 (GS2)	15.0
Interface Heater	On

Table 7. 3-OMD fragmentation conditions for MRM detection

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)
3-OMD	268.1	166.1	110.0	10.0	26.0	8.0
3-OMD-D_3	271.1	169.1	110.0	10.0	26.0	8.0

3.4. Implementation of Second-tier Testing by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

For residual neonatal DBS specimens with first-tier 3-OMD concentrations above the defined cut-off, 3-OMD was measured in DBS using LC-MS/MS.

3.4.1. Method

Despite having a longer analytical time, Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) is more suitable for second-tier testing as it significantly reduces false-positive rates compared to FIA-MS/MS. This is because LC-MS/MS provides superior separation of analytes, improving the detection of low-abundance compounds and reducing interference from other substances. The chromatographic separation provided by LC ensures that compounds of interest are better isolated before ionization, reducing the likelihood of unintended degradation and improving the reliability of the analysis. Furthermore, LC effectively resolves isobaric interferences, where MRM conditions may not be specific to the analyte of interest. By separating the analyte from interfering agents before MS/MS analysis, LC-MS/MS enhances the specificity and accuracy of the measurements, making it a more effective method for second-tier testing in NBS (57,58).

3.4.2. Sample Preparation

The sample preparation followed the same procedure used in the FIA-MS/MS method. The 3-OMD measurements were performed using HPLC Agilent 1100 series coupled with MS/MS 4000Qtrap from Sciex, and separation was carried out on a Column Penomex Gemini 3u C6-phenyl 110A. A 5 µL aliquot of the reconstituted sample was injected into the LC system using an autosampler, and the mobile phase consisted of acetonitrile/water (1:1) containing 0.01% formic acid. The fragmentation conditions for the analysis were identical to those used for the FIA-MS/MS method, as detailed in Table 7. The gradient elution program is detailed in Table 8. Quantification was performed by dividing the intensity of 3-OMD by the intensity of 3-OMD-D_3, at the used concentration.

Table 8. LC-MS/MS elution gradient for 3-OMD quantification

Total Time (min)	Flow Rate (µL/min)	A (%)	B (%)
0.00	200	40.0	60.0
2.00	200	10.0	90.0
14.0	200	0.0	100.0
17.0	200	85.0	15.0
21.0	200	85.0	15.0

A (%): H_2O; B(%): Acetonitrile

3.5. Data Analysis

Microsoft Excel (version 2408) was employed to calculate descriptive statistics, including means, standard deviations, percentiles, precision, accuracy, limits of detection (LOD), and limits of quantification (LOQ). The LOD and LOQ were determined using the standard error of the regression and the slope of the calibration curve. Precision, expressed as the coefficient of variation (CV), was calculated from repeated measurements of calibration standards at different concentrations, reflecting the consistency of the method. Accuracy, expressed as the percentage of relative error, was determined by comparing the measured values to known reference values to assess how close the experimental results were to expected outcomes. IBM SPSS Statistics (version 29.0.1.0) was used for hypothesis testing, including independent t-tests, and graphical representations of the data were created using GraphPad Prism (version 6) and Microsoft Excel (version 2408).

4. Results

4.1. Implementation of 3-OMD Quantification by FIA-MS/MS Using 3-OMD-D_3 on DBS

4.1.1. Method Validation

The method demonstrated linearity over the range of 0 to 30 µM, with a correlation coefficient (R^2) of 0.999 (Figure 2). Precision, expressed as the coefficient of variation (CV), was 5.7%. Accuracy, expressed as % relative error, was 1.3%. The LOD and LOQ were determined to be 0.95 µM and 2.87 µM, respectively (Table 9).

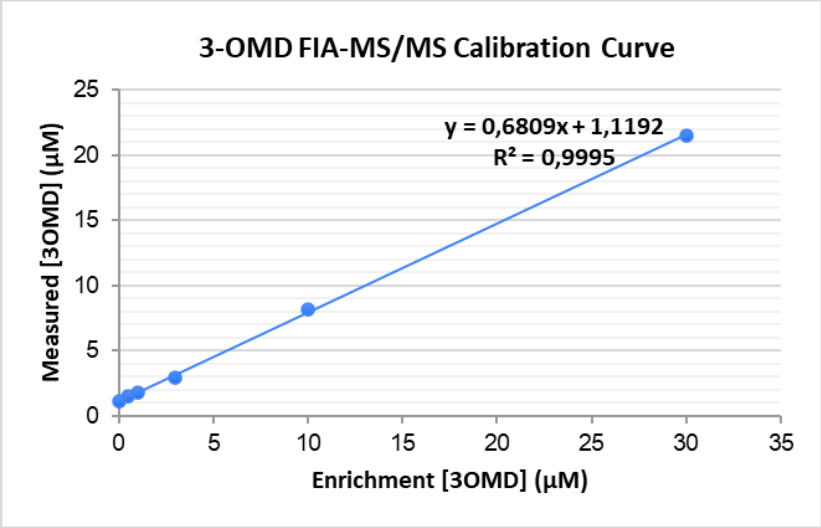

Figure 2. Calibration curve for 3-OMD quantification by FIA-MS/MS

Table 9. Validation parameters for 3-OMD quantification by FIA-MS/MS

Parameters	3-OMD
Median R^2	0.999
Precision (CV)	5.7%
Accuracy (% Relative Error)	1.3%
LOD	0.95 µM
LOQ	2.87 µM

4.1.2. Intereference of 3-OMD Quantification in the Quantification of Other Metabolites

Incorporating 3-OMD quantification into the NBS workflow aimed to enhance the screening process by adding the quantification of a new metabolite relevant for diagnosing AADC deficiency. The integration involved analyzing all existing biomarkers using both the original

method and the updated method with 3-OMD. To determine if the new protocol affected the quantification of the other biomarkers, an independent t-test was conducted to compare results from both approaches. The analysis produced a p-value greater than 0.05, indicating no statistically significant differences in the metabolite's quantification. This finding confirms that the incorporation of 3-OMD does not interfere with the quantification of other biomarkers in the NBS process. The successful integration of 3-OMD demonstrates that the updated method maintains the accuracy of the existing markers while providing additional diagnostic value.

4.1.3. Quantification of 3-OMD Concentration in Newborns

The newly implemented method was integrated into the routine NBS process, and 3-OMD was quantified as an additional parameter in 2066 DBS samples. The average concentration of 3-OMD was 1.9 μM and the standard deviation was 0.4. Percentile values at the 2.5th, 5th, 95th, and 99.5th percentiles are detailed in Table 10. A box-and-whiskers plot illustrating the distribution of 3-OMD concentration in these samples is presented in Figure 3.

In addition to the normal newborn samples, 2 samples from patients undergoing DOPA treatment were also analyzed using the same method. The results are presented in Figure 4, where the concentrations of 3-OMD in normal newborns are shown as a box-and-whiskers plot, with the data points for the DOPA-treated patients indicated separately. The findings demonstrate that 3-OMD concentrations in these patients, which were measured at 8.0 μM and 35.6 μM, are significantly elevated compared to those in normal newborns. These results demonstrate the method's capability to measure 3-OMD at clinically relevant levels.

Table 10. 3-OMD concentrations in normal newborns

Percentile	3-OMD (μM)
2.5	1.2
5	1.4
95	2.6
99.5	3.3

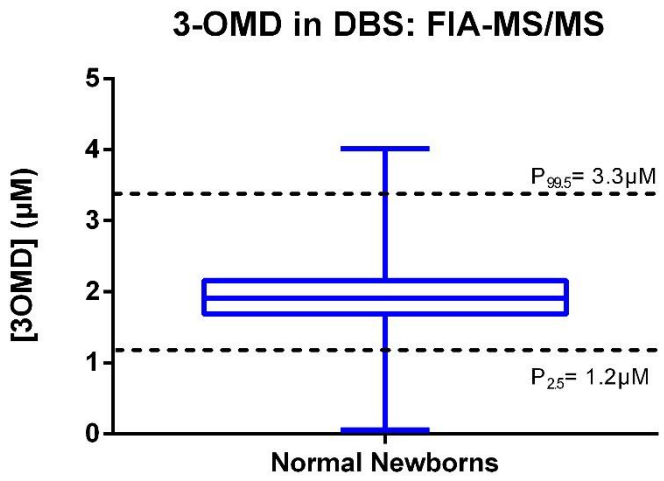

Figure 3. Box-and-whiskers plot showing the 3-OMD concentrations in 2066 normal newborns. The plot illustrates the median, interquartile range (IQR), and range of the data. 2.5th and 99.5th Percentiles are shown as dashed lines.

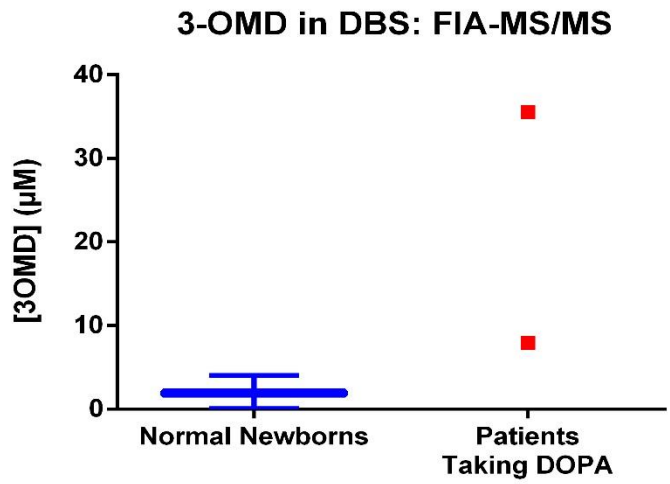

Figure 4. Box-and-whiskers plot of 3-OMD concentrations in normal newborns (blue), showing the distribution of values across the population. Individual data points representing 3-OMD concentrations from 2 patients undergoing DOPA therapy (red) are also displayed.

4.1.4. Quality Control DBS Samples

To validate the accuracy of the 3-OMD quantification method, quality control (QC) samples from Chromsystems were analyzed. The QC samples, specifically the MassCheck® Amino Acids, Acylcarnitines EXTENDED Dried Blood Spot Controls (Order No. 0295, 0296, 0297, Lot 3823), were evaluated at three different levels: Level I, Level II, and Level III. These control materials from Chromsystems are based on human whole blood.

The manufacturer provided the expected concentrations and ranges for 3-OMD for each QC level: Level I (Order No. 0295) with an expected concentration of 2.01 μM and a range of 1.41–2.61 μM; Level II (Order No. 0296) with an expected concentration of 5.99 μM and a range of 4.19–7.79 μM; and Level III (Order No. 0297) with an expected concentration of 17.8 μM and a range of 12.5–23.1 μM.

To assess the method's accuracy, the % Relative Error was calculated by dividing the difference between the measured value and the expected value by the expected value, and then multiplying by 100. The measured 3-OMD concentrations for these QC samples, along with the % Relative Errors, are summarized in Table 11. The results indicate that the measured concentrations were within the expected ranges for all QC levels, with % Relative Errors of 0.50%, 0.50%, and 2.81% for Levels I, II, and III, respectively. The mean % Relative Error across all levels was 1.3%, demonstrating the method's accuracy across different concentration levels.

Table 11. Quality control data for 3-OMD concentrations in DBS measured by FIA-MS/MS

QC Level	QC Sample Order No.	Expected Concentration (μM)	Range (μM)	Mean Measured Concentration (μM)	% Relative Error (%)
Level I	0295	2.01	1.41–2.61	2.02	0.50
Level II	0296	5.99	4.19–7.79	5.96	0.50
Level III	0297	17.8	12.5–23.1	17.3	2.8

4.1.5. CSF Samples (Healthy Subjetcts and Patient)

The FIA-MS/MS analysis of CSF samples spotted on filter paper, using 3-OMD-D_3 as the internal standard, included data from five samples from healthy subjects and one sample from a Parkinson's patient undergoing DOPA treatment. The mean concentration of 3-OMD in the healthy samples was 0.3 μM while the concentration in the Parkinson's patient sample was 0.9 μM (Figure 5). At the time of this analysis, DBS samples from patients undergoing DOPA therapy were not available, so CSF samples were used as an alternative matrix to evaluate the method's sensitivity. Although CSF differs from DBS, the objective was to test whether the method could detect 3-OMD concentrations at clinically relevant levels in a biological matrix. The successful quantification of 3-OMD in both healthy and Parkinson's patient samples confirmed the method's suitability for measuring this biomarker, regardless of the sample type. These results further support the method's ability to accurately measure 3-OMD at clinically relevant levels.

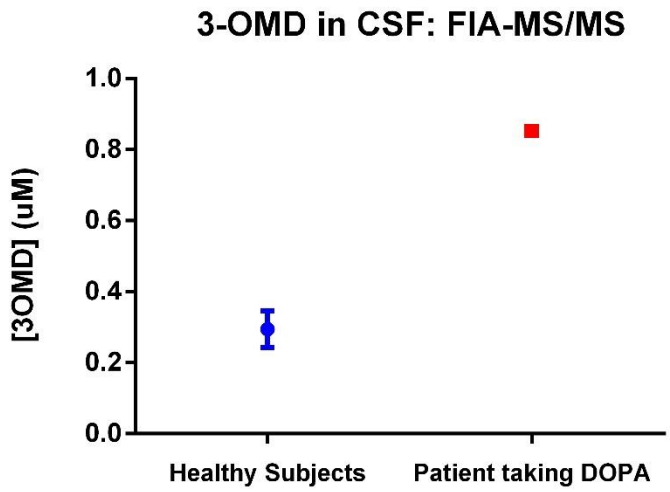

Figure 5. 3-OMD concentration (CSF on filter paper) in healthy subjects (blue) and a patient undergoing DOPA treatment (red).

4.2. Comparison of 3-OMD Quantification Approaches: 3-OMD-D_3 vs. $^{13}C_6$-Phe and $^{13}C_6$-Tyr

The use of $^{13}C_6$-Phe and $^{13}C_6$-Tyr as internal standards was inspired by methods described in published literature, which highlight the efficiency and robustness of these alternatives for the screening of AADC deficiency (45,46). A key advantage of using $^{13}C_6$-Phe and $^{13}C_6$-Tyr is that they are already employed in the screening for other metabolic disorders, thus eliminating the need for an additional deuterated standard and simplifying the workflow.

A total of 1000 DBS samples were analyzed by FIA-MS/MS using three different internal standards: 3-OMD-D_3, $^{13}C_6$-Phe and $^{13}C_6$-Tyr. Each sample was tested using all three standards to assess whether $^{13}C_6$-Phe and $^{13}C_6$-Tyr could serve as adequate alternatives for screening purposes. Figures 6 and 7 illustrate the correlation between the 3-OMD concentrations measured with 3-OMD-D_3 and those obtained using $^{13}C_6$-Phe and $^{13}C_6$-Tyr as internal standards, respectively. The results demonstrate a positive correlation between 3-OMD-D_3 and both $^{13}C_6$-Phe (Figure 6) and $^{13}C_6$-Tyr (Figure 7). The linear regression lines indicate that the alternative internal standards provide a reasonable approximation of 3-OMD levels, although the correlation is lower compared to that achieved with 3-OMD-D_3. This suggests that while $^{13}C_6$-Phe and $^{13}C_6$-Tyr may not be as precise as 3-OMD-D_3, they still offer sufficient accuracy for initial screening purposes. This may be particularly relevant for large-scale NBS, where cost and simplicity are important factors.

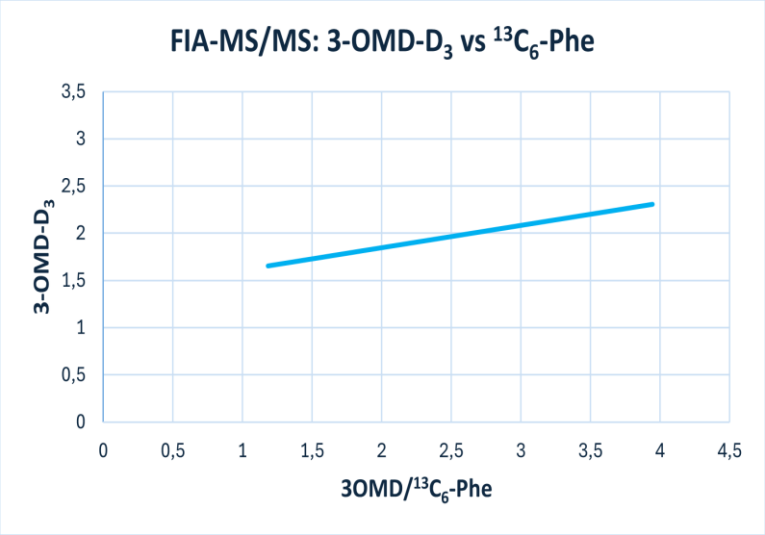

Figure 6. Correlation between 3-OMD levels quantified using 3-OMD-D_3 and $^{13}C_6$-Phe as an internal standard. The linear regression line illustrates the overall trend, indicating a positive correlation between the two methods.

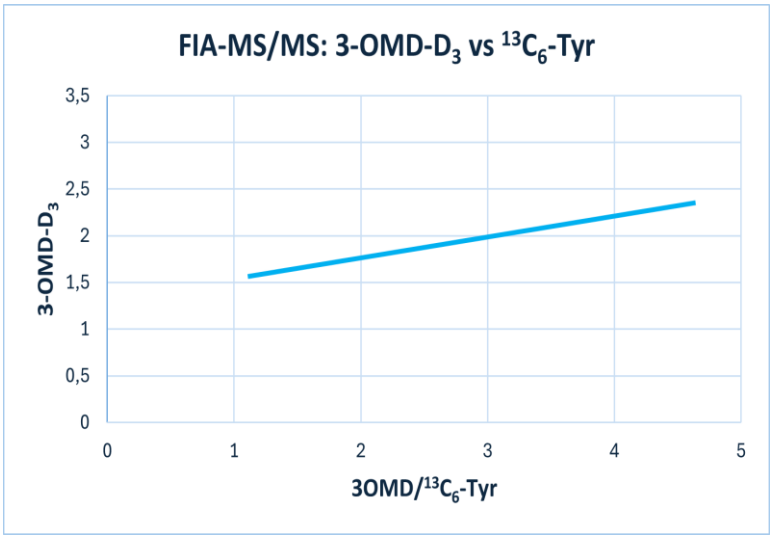

Figure 7. Correlation between 3-OMD levels quantified using 3-OMD-D_3 and $^{13}C_6$-Tyr as an internal standard. The linear regression line illustrates the overall trend, indicating a positive correlation between the two methods.

The calibration curve for $^{13}C_6$-Phe demonstrated a linear relationship up to 30 µM, with a high correlation coefficient (R^2 = 0.999) (Figure 8). However, the coefficient of variation (CV) for the $^{13}C_6$-Phe calibration was measured at 17.6%, which was significantly higher compared to the CV observed when using 3-OMD-D_3 as the internal standard. The LOD was established at 0.6 µM, and the LOQ was determined to be 1.9 µM.

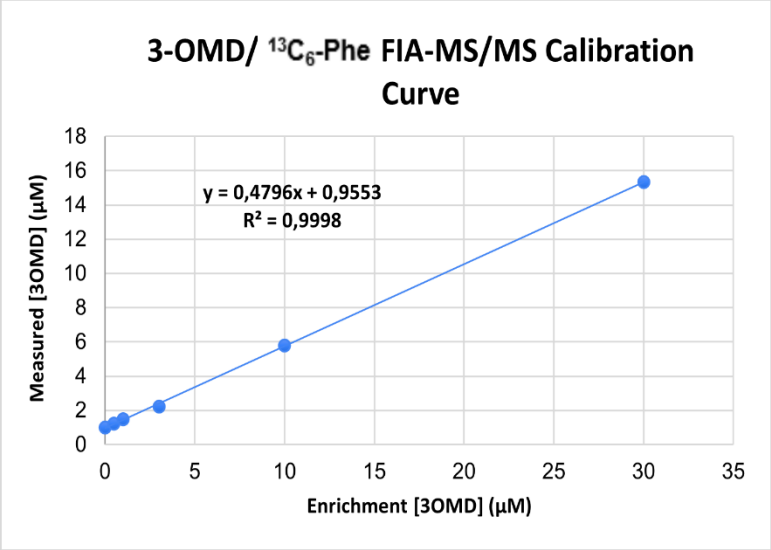

Figure 8. Calibration curve for 3-OMD quantification by FIA-MS/MS, using $^{13}C_6$-Phe as the internal standard.

Similarly, the calibration curve for $^{13}C_6$-Tyr also exhibited a strong linearity up to 30 µM, with an R^2 value of 0.999 (Figure 9). The CV for $^{13}C_6$-Tyr was calculated at 18.6%, which was higher than the CV observed with 3-OMD-D_3. The LOD was set at 0.5 µM, and the LOQ was found to be 1.5 µM.

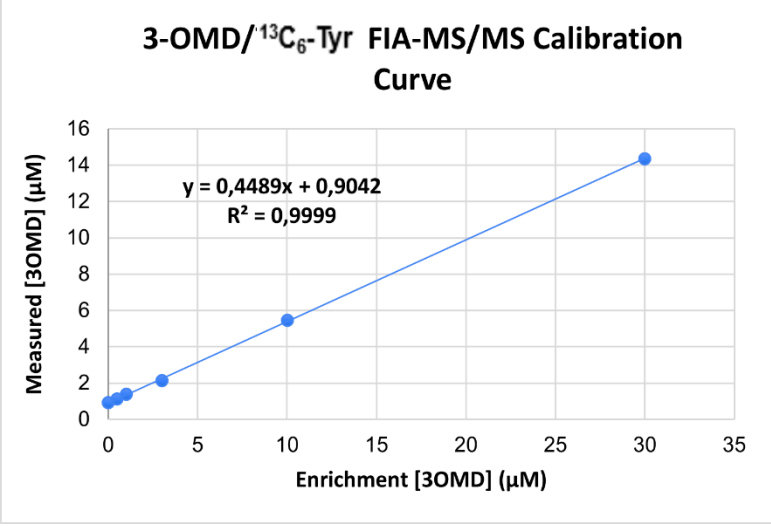

Figure 9. Calibration curve for 3-OMD quantification by FIA-MS/MS, using $^{13}C_6$-Tyr as the internal standard.

When analyzing the QC samples at three different concentration levels, the expected concentrations were 2.01 µM, 5.99 µM, and 17.8 µM, with respective acceptable ranges of 1.40-2.10 µM, 4.19-7.79 µM, and 12.5-23.1 µM. The measured 3-OMD concentrations using $^{13}C_6$-Phe

as the internal standard were 1.42 µM, 3.80 µM, and 8.65 µM. Similarly, the results obtained using $^{13}C_6$-Tyr were 1.31 µM, 3.28 µM, and 8.5 µM (Table 12). In both cases, the measured concentrations, particularly at the higher levels, fell outside the expected ranges, indicating less accuracy when using $^{13}C_6$-Phe and $^{13}C_6$-Tyr as internal standards, particularly at elevated concentrations.

Table 12. Quality control data for 3-OMD concentrations in DBS measured by FIA-MS/MS, using $^{13}C_6$-Phe and $^{13}C_6$-Tyr as internal standards

QC Level	QC Sample Order No.	Expected Concentration (µM)	Range (µM)	Mean Measured Concentration (µM) using $^{13}C_6$-Phe	Mean Measured Concentration (µM) using $^{13}C_6$-Tyr
Level I	0295	2.01	1.41-2.61	1.42	1.31
Level II	0296	5.99	4.19-7.79	3.80	3.28
Level III	0297	17.8	12.5-23.1	8.7	8.5

A total of 24 441 samples were quantified for 3-OMD using FIA-MS/MS with $^{13}C_6$-Phe and $^{13}C_6$-Tyr as internal standards. The average concentration of 3-OMD with $^{13}C_6$-Phe as the internal standard was 1.6 µM, with a standard deviation of 0.6. Using $^{13}C_6$-Tyr as the internal standard, the average concentration was 1.7 µM, the standard deviation was 0.7. Percentile values at the 2.5th, 5th, 95th, and 99.5th percentiles are detailed in Table 13 for $^{13}C_6$-Phe and Table 14 for $^{13}C_6$-Tyr. Box-and-whiskers plots illustrating the distribution of 3-OMD concentrations for each internal standard are presented in Figure 10.

Table 13. 3-OMD normal newborn concentrations (using $^{13}C_6$-Phe as internal standard)

Percentile	3-OMD ($^{13}C_6$-Phe) (µM)
2.5	0.8
5	0.9
95	2.6
99.5	3.7

Table 14. 3-OMD normal newborns concentrations (using $^{13}C_6$-Tyr as internal standard)

Percentile	3-OMD ($^{13}C_6$-Tyr) (µM)
2.5	0.8
5	0.9
95	3.0
99.5	4.4

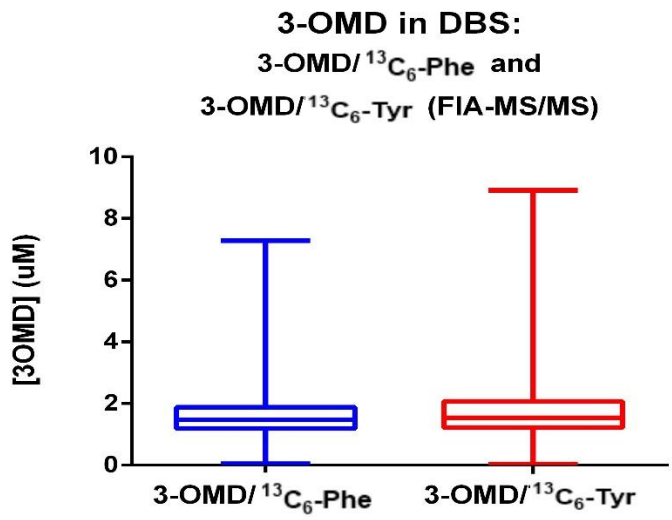

Figure 10. Box-and-whiskers plots of 3-OMD concentrations in DBS samples quantified using FIA-MS/MS with internal standards $^{13}C_6$-Phe and $^{13}C_6$-Tyr.

The FIA-MS/MS analysis of two DBS samples from patients undergoing DOPA therapy, utilizing $^{13}C_6$-Phe and $^{13}C_6$-Tyr as internal standards, revealed concentrations of 6.63 µM and 30.72 µM for $^{13}C_6$-Phe (Figure 11), and 6.32 µM and 28.28 µM for $^{13}C_6$-Tyr (Figure 12). These values are significantly elevated when compared to the concentrations observed in normal newborns. This indicates that both alternative internal standards are suitable for detecting 3-OMD at clinically relevant levels.

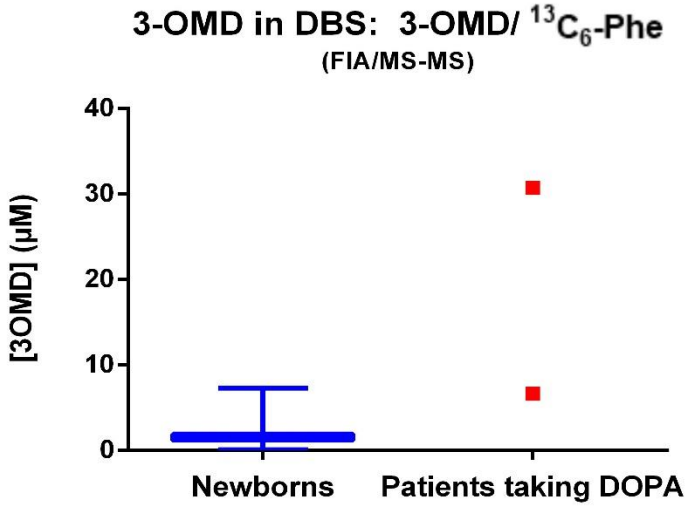

Figure 11. Box-and-whiskers plot displaying 3-OMD concentrations in 24 441 newborns (blue) alongside individual data points (red) for 3-OMD concentrations from two patients undergoing DOPA therapy, analyzed using $^{13}C_6$-Phe as the internal standard.

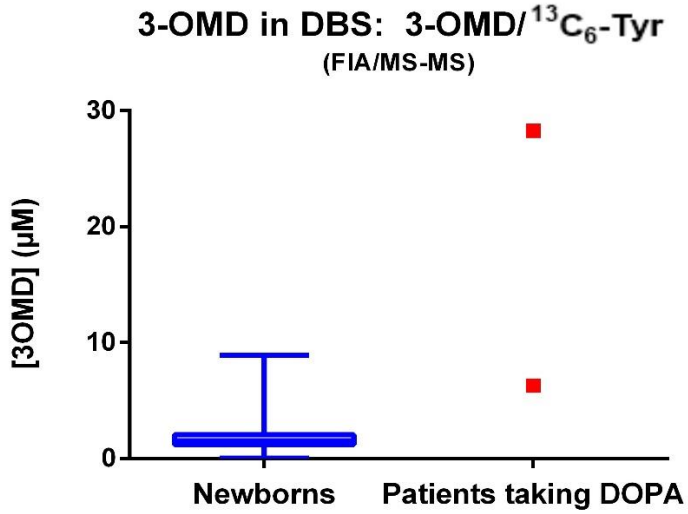

Figure 12. Box-and-whiskers plot displaying 3-OMD concentrations in 24 441 newborns (blue) alongside individual data points (red) for 3-OMD concentrations from two patients undergoing DOPA therapy, analyzed using $^{13}C_6$-Tyr as the internal standard.

4.3. 3-OMD Values for Premature Newborns

To assess whether premature newborns have elevated 3-OMD levels, 37 samples from premature infants were analyzed using FIA-MS/MS with $^{13}C_6$-Phe as the internal standard. The concentrations of 3-OMD in these premature newborns were within the normal range observed for full-term newborns.

The mean 3-OMD concentration in premature newborns was 1.4 µM, with a standard deviation of 0.6, and values ranging from 0.8µM to 2.8 µM.

These results indicate that 3-OMD levels in out cohort of premature newborns do not show the elevated concentrations reported in some literature for preterm infants (43,46,47).

4.4. Second Tier Testing by LC-MS/MS

4.4.1. Method Validation

The method demonstrated linearity over the range of 0 to 30 µM, with a correlation coefficient (R^2) of 0.999 (Figure 13). The LOD and LOQ were determined to be 0.70 µM and 2.13 µM, respectively. The chromatographic separation and detection of 3-OMD and 3-OMD-D_3 are illustrated in Figures 14 and 15.

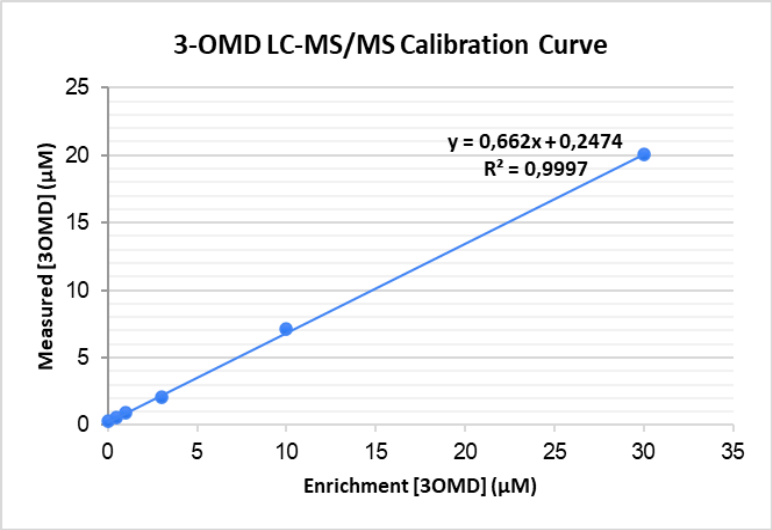

Figure 13. Calibration curve for 3-OMD quantification by LC-MS/MS

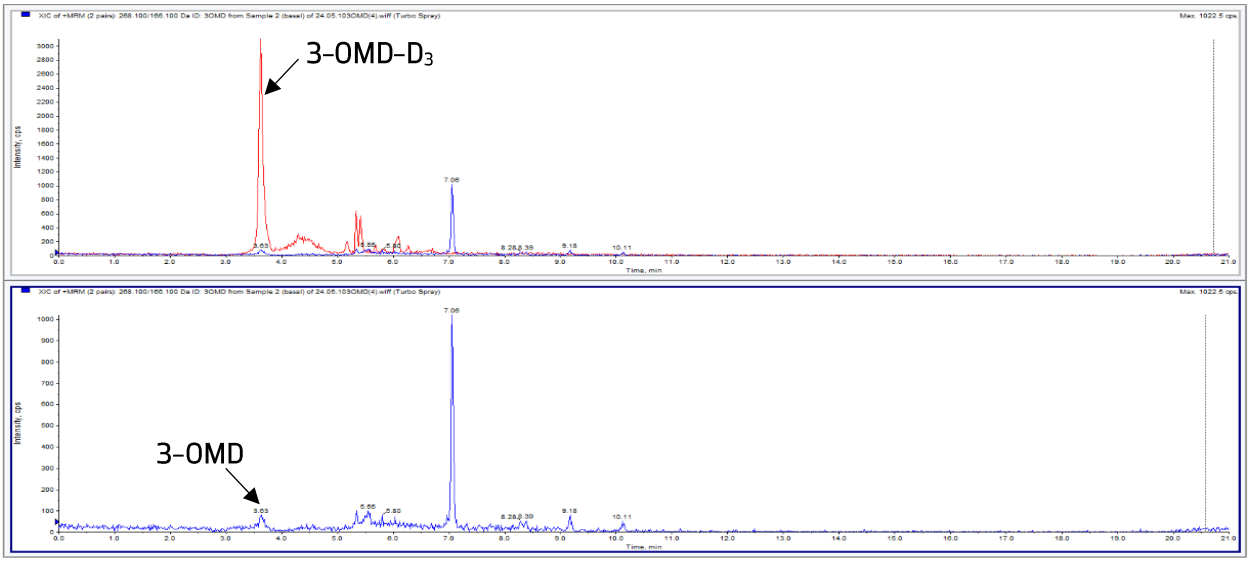

Figure 14. Chromatographic separation of endogenous 3-OMD (blue peak) and 3-OMD-D_3 (red peak) in a basal sample using LC-MS/MS. The red peak at 3.63 min represents the internal standard 3-OMD-D_3, while the blue peak at 3.63 min represents endogenous 3-OMD. The blue peak at 7.05 min is identified as an interferent.

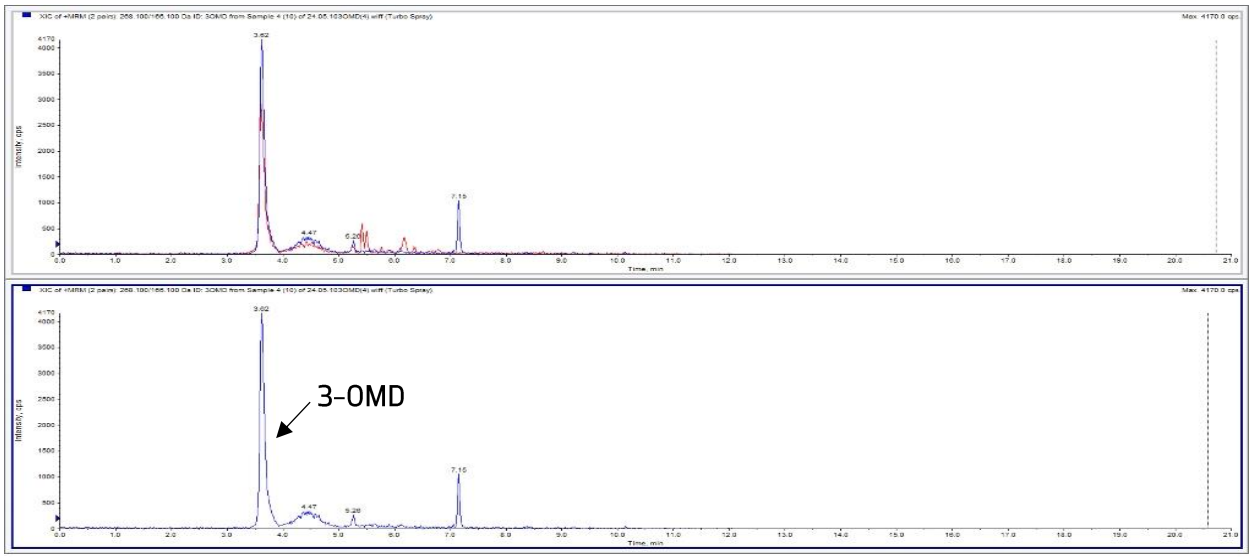

Figure 15. Chromatographic separation of endogenous 3-OMD (blue peak) and 3-OMD-D_3 (red peak) in a 10 µM enriched sample using LC-MS/MS. The red peak at 3.62 min represents the internal standard 3-OMD-D_3, while the blue peak at 3.62 min represents endogenous 3-OMD. The blue peak at 7.15 min is identified as an interferent.

4.4.2. Quantification of 3-OMD Concentration in Second Tier Testing

Of the 26 507 samples analyzed via FIA-MS/MS in initial testing, 82 samples exhibiting elevated 3-OMD concentrations (> 3.3 µM) were subsequently subjected to second-tier testing using LC-MS/MS. The mean concentration of 3-OMD in these samples was 0.8 µM, with a standard deviation of 0.3.

The results from the second-tier testing revealed that samples with high 3-OMD concentrations in the initial screening often showed normal values upon reanalysis (Figure 16). This indicates that chromatography separation in the second-tier testing effectively resolves true elevated 3-OMD levels from potential interferences.

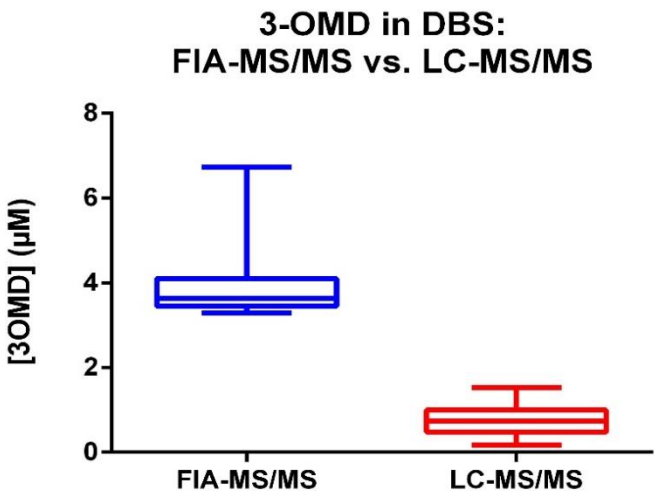

Figure 16. Comparison of 3-OMD concentrations in 82 samples analyzed by both FIA-MS/MS and LC-MS/MS methods. The blue box plot represents the 3-OMD concentrations measured using FIA-MS/MS, while the red box plot shows the results from 2nd tier LC-MS/MS analysis of the same samples. The LC-MS/MS results show consistently lower concentrations, reflecting the increased specificity of the method.

5. Discussion

AADC deficiency is a rare but severe neurotransmitter disorder for which early diagnosis is crucial, especially in light of the recent advancements in gene therapy. Studies have shown that gene therapy for AADC deficiency is significantly more effective when administered early in life, ideally before irreversible neurological damage occurs (23). This highlights the need for reliable screening methods capable of identifying affected newborns at a stage when interventions can be most beneficial. In this context, the quantification of 3-OMD in DBS has emerged as a promising marker for the early detection of AADC deficiency, providing an opportunity for timely diagnosis and treatment (43).

This study successfully validated the integration of 3-OMD quantification into the existing FIA-MS/MS method, ensuring the method met all necessary criteria for sensitivity, specificity, linearity, precision, and reproducibility. The validation results demonstrate that the method can reliably detect elevated 3-OMD levels, a hallmark of AADC deficiency, without compromising the quantification of other key biomarkers used in the PNSP.

The analysis of 2066 normal newborn samples enabled us to calculate key percentiles, including the 2.5th, 5th, 95th, and 99.5th percentiles, establishing a comprehensive baseline for 3-OMD levels in the population. This baseline is fundamental not only for understanding the normal distribution of 3-OMD in the population but also for identifying abnormal concentrations associated with AADC deficiency. In confirmed cases of AADC deficiency in newborn screening, literature reports 3-OMD concentrations ranging from 5.05 μM to 35.95 μM (23,44,46,47). Based on this data, we established a cut-off point of 3.3 μM (99.5th percentile) as the threshold for referring samples for second-tier LC-MS/MS testing, a critical step to minimize false positives while maximizing diagnostic accuracy. The decision to set the cut-off at the 99.5th percentile reflects a rigorous analysis of the dataset, considering both the necessity to avoid unnecessary second-tier testing in healthy individuals and the importance of early detection in affected individuals. This cut-off aligns well with the method's demonstrated linearity, ensuring that elevated 3-OMD concentrations can be accurately detected and quantified, thereby providing a robust framework for the identification of potential cases of AADC deficiency.

In addition to normal newborn samples, we analyzed quality control samples provided by a certified manufacturer at three different concentration levels. These samples allowed us to evaluate the accuracy and precision of the FIA-MS/MS method across a wide range of 3-OMD

concentrations. The results confirmed that the method was not only accurate but also highly precise.

We analyzed CSF samples spotted on filter paper from both normal subjects and a DOPA-treated patient. Despite the difference in matrix—CSF rather than blood—the method successfully identified significantly higher 3-OMD concentrations in the patient undergoing therapy. This result demonstrates that our FIA-MS/MS method, although developed for DBS, is still capable of detecting elevated 3-OMD levels in a different biological matrix, further reinforcing the method's versatility and reliability. Following this, the analysis of DBS samples from two patients undergoing DOPA therapy clearly demonstrated elevated 3-OMD concentrations, as expected due to the known impact of L-DOPA administration on 3-OMD levels. L-DOPA is metabolized into 3-OMD via a methylation process, so the elevated 3-OMD levels observed in these patients reflect the increased availability of L-DOPA.

To evaluate the practicality of using alternative internal standards, we conducted a comparison between FIA-MS/MS utilizing 3-OMD-D_3 and FIA-MS/MS using $^{13}C_6$-Phe and $^{13}C_6$-Tyr, since previous research has demonstrated their effectiveness in quantification within NBS workflows (45,46). This comparison was pivotal in determining whether $^{13}C_6$-Phe and $^{13}C_6$-Tyr could serve as viable substitutes for 3-OMD-D_3, potentially offering cost savings and streamlining the process by removing the need for an additional deuterated standard. Analysis of 24 441 newborn samples revealed that $^{13}C_6$-Phe and $^{13}C_6$-Tyr performed similarly to 3-OMD-D_3. While $^{13}C_6$-Phe and $^{13}C_6$-Tyr do not match the precision and accuracy of 3-OMD-D_3, they are sufficiently adequate for screening purposes.

The study also aimed to investigate differences in 3-OMD concentrations between premature and full-term newborns. Interestingly, our results indicated that 3-OMD levels in premature newborns were essentially the same as those in full-term newborns, contrary to some studies suggesting higher levels in preterm infants (43,46,47). This difference may be attributed to variations in cohort characteristics. Further research is needed to clarify these variations and determine whether they persist across larger populations or under different clinical conditions.

To enhance the accuracy of 3-OMD quantification and minimize false positives, LC-MS/MS was utilized as a second-tier test for DBS samples with initial 3-OMD concentrations exceeding the cutoff of 3.3 μM. The second-tier testing revealed that all samples flagged as elevated in the initial screening showed normal 3-OMD levels upon reanalysis. For samples that continued to show high 3-OMD levels, the next step would be to review the mother's medication history to

determine if any drugs could influence the 3-OMD metabolic pathway. This step is crucial, as previous studies have identified false positives in NBS due to maternal intake of certain medications like methyldopa and L-DOPA (44,45,47). If no such influence is identified, we would proceed with molecular diagnostics to detect potential pathogenic variants in the DDC gene. If no DDC variants are identified, PNPO deficiency should also be considered, as it can similarly elevate 3-OMD concentrations due to a secondary AADC dysfunction linked to a deficiency of the enzyme's cofactor, pyridoxal phosphate (PLP) (59). Moreover, after molecular diagnostic, functional confirmation should include assessing AADC enzyme activity in plasma and/or neurotransmitter metabolites in cerebrospinal fluid, according to guidelines (6). This comprehensive approach helps to refine the screening process and ensure accurate diagnosis.

While this study has successfully demonstrated the feasibility of incorporating 3-OMD quantification into the current MS/MS NBS workflow, there are some limitations that should be addressed. A key limitation lies in the comparison between 3-OMD-D_3 and the alternative internal standards $^{13}C_6$-Phe and $^{13}C_6$-Tyr. Although $^{13}C_6$-Phe and $^{13}C_6$-Tyr were found to be sufficient for large-scale screening purposes, their lower precision and accuracy relative to 3-OMD-D_3 underscores the need for caution adaptation, particularly with regard to minimizing false positives and maximizing detection accuracy.

As of this study, no cases of AADC deficiency have been detected in the cohort analyzed, so it has not yet been possible to calculate an accurate prevalence of the disease in Portugal. Continued analysis of a larger number of samples will be essential to determine the prevalence of AADC deficiency and further validate the reliability of 3-OMD quantification as part of the screening process. Expanding the dataset will also help confirm the method's reproducibility across the broader newborn population and ensure its robustness for the diversity of cases encountered in routine screenings.

Future efforts should include the testing of non-deidentified samples, as a part of a pilot study for AADC deficiency newborn screening. This will allow for further validation of the method's precision, reliability, and cost-effectiveness. Should this validation be achieved on a larger scale, it could then provide the necessary foundation for considering the inclusion of 3-OMD quantification in the routine screening process for AADC deficiency in Portugal.

6. Conclusion

This work aimed to assess the feasibility of incorporating 3-OMD quantification into the existing FIA-MS/MS workflow used in the Portuguese Newborn Screening Program, with the ultimate goal of enabling early detection of AADC deficiency. The results demonstrated that the FIA-MS/MS method for quantifying 3-OMD in DBS is reliable, sensitive, and specific, without interfering with the detection of other biomarkers in the screening panel. Through the analysis of over 2000 normal newborn samples, a cut-off of 3.3 μM was established, serving as a critical threshold for triggering second-tier testing by LC-MS/MS. Furthermore, the method was validated with an extended cohort of over 24 400 newborns using alternative internal standards, demonstrating its robustness and scalability across a large population.

In addition, the method was shown to perform accurately across various 3-OMD concentrations, as evidenced by its precise quantification in quality control samples. While no cases of AADC deficiency were detected in this cohort, the method proved effective in identifying elevated 3-OMD levels in patients undergoing DOPA therapy, demonstrating its utility across different biological matrices.

Second-tier testing results revealed that several samples initially flagged for high 3-OMD concentrations normalized upon reanalysis through LC-MS/MS. This underscores the value of chromatographic separation in second-tier testing, which effectively distinguishes true elevated levels from potential interferences, further enhancing the accuracy of the screening process.

The successful validation of 3-OMD quantification within the current FIA-MS/MS workflow paves the way for its potential inclusion in routine NBS. Incorporating 3-OMD could significantly improve the early diagnosis of AADC deficiency, enabling timely interventions, such as gene therapy, which appears to be most effective when administered early in life.

However, the absence of AADC deficiency cases in this cohort limits the ability to calculate the prevalence of the disease in Portugal. Continued analysis of larger sample sets will be essential to fully evaluate the method's effectiveness in identifying affected individuals. Future research should focus on increasing the sample size and conducting long-term follow-up studies to assess the reliability of 3-OMD as a screening biomarker. Moreover, evaluating potential sources of false positives, such as maternal medications, will be critical in refining the screening process.

In conclusion, the validation of 3-OMD quantification using FIA-MS/MS marks a significant step towards incorporating this biomarker into the Portuguese Newborn Screening Program. With continued research and refinement, this method could play a crucial role in detecting treatable

rare disorders like AADC deficiency, ultimately improving clinical outcomes through early diagnosis and intervention.

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