



G. d'Annunzio" UNIVERSITY OF CHIETI - PESCARA



School of Advanced Studies "G. d'Annunzio"

PHD COURSE IN BIOMOLECULAR AND PHARMACEUTICAL SCIENCES

CURRICULUM IN MOLECULAR MEDICINE

XXXVIII CYCLE

Steroid Hormone Profiling in Newborn Screening: Clinical and Research Applications

Department of Medical, Oral and Biotechnological Sciences

Analytical Biochemistry - Proteomics - Endocrinology Unit

Scientific Disciplinary Sector BIOS 07/A

Ph.D. Student
Dr Maria Lucia Tommolini

Coordinator:

Prof Assunta Pandolfi

Tutor

Prof Luca Federici

Academic Years 2022/2025

Project Theme - DM 351/2022 PNRR

Development of new methodologies in the first and second tier tests, for the expansion to new pathologies in newborn screening programs.

This PhD project was produced while attending the PhD programme in Biomolecular and Pharmaceutical Sciences at the University of Chieti, Cycle XXXVIII, with the support of a scholar-ship financed by the Ministerial Decree no. 351 of 9 April 2022, based on the NRRP-funded by the European Union–NextGenerationEU.

Contents

PART ONE - Scientific Background and Research Results

Abstract	5
Aims.....	6
Chapter 1 – Introduction: Newborn Screening and Metabolomics Approaches.....	7
1.1 Italian Newborn Screening.....	8
1.2 Inborn Errors of Metabolism.....	9
1.3 Mass Spectrometry (MS/MS) in Newborn Screening.....	18
1.4 Contribution of Second-tier Tests to Reduce False Positives.....	20
1.5 Abruzzo Pilot Project and DGR n.782 24/11/2025.....	22
1.6 Analytical Strategies for Steroid Profiling.....	23
Chapter 2 – Congenital Adrenal Hyperplasia and Newborn Screening Strategies.....	30
2.1 Adrenal Steroidogenesis.....	31
2.2 Congenital Adrenal Hyperplasia: Pathophysiology and Clinical Variants.....	34
2.3 First-tier CAH Screening.....	37
2.4 Second-tier CAH Screening: Development and Validation.....	38
2.5 Plasma Steroid Determination by LC-MS/MS.....	45
2.6 CAH Screening Outcomes in Abruzzo region: Results from Recent Years.....	47
2.7 Potential Effects of Neonatal Nutrition on CAH Screening.....	48
2.8 Multidisciplinary Approach for CAH Diagnosis in Pediatric Age.....	52
2.9 Beyond False Negatives in NBS.....	57
Chapter 3 – Salivary Cortisol: Biological Relevance and Methodological Development for Its Quantification.....	65
3.1 Cortisol as a Biomarker in Stress Assessment.....	66
3.2 Methodological Development for Salivary Cortisol Quantification.....	70
3.3 Salivary Cortisol Applications.....	72

Discussion.....	76
Conclusions.....	80

PART TWO - Academic and Scientific Activities

List of Publications.....	81
Research and Clinical Activities.....	85
Research Activities Abroad.....	86
Teaching Activities.....	86
Third Mission Activities.....	86
Conferences and Scientific Dissemination.....	87
Professional Awards and Merits.....	90

PART ONE

Scientific Background and Research Results

Abstract

Newborn screening (NBS) represents a fundamental preventive strategy for the early identification of inherited metabolic diseases (IEMs), enabling timely interventions that reduce morbidity and mortality. In Italy, the progressive evolution of NBS, supported by the introduction of tandem mass spectrometry, has allowed the simultaneous detection of numerous metabolic disorders from a single dried blood spot sample. Among these conditions, congenital adrenal hyperplasia (CAH), primarily caused by 21-hydroxylase deficiency, represents a paradigmatic example for which a two-tier NBS (first-tier test and second-tier test) approach has demonstrated high sensitivity and specificity, effectively reducing false-positive results and unnecessary recalls.

The optimization of CAH screening cut-offs, taking into account clinical and environmental factors, may further decrease the number of second-tier tests, leading to improvements in screening efficiency as well as reductions in time costs. In this context, steroid profiling based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) plays a central role in enhancing diagnostic accuracy.

Beyond its clinical application in NBS, LC-MS/MS-based steroid profiling favors the investigation of hypothalamic–pituitary–adrenal (HPA) axis physiology and stress-related biomarkers. In particular, the quantification of salivary cortisol allows the assessment of stress responses and neurobehavioral adaptation in preterm and full-term infants including in response to environmental-based interventions during the first days of life.

Overall, this work highlights steroid characterization by LC-MS/MS as a key tool both for the early diagnosis of endocrine and metabolic disorders such as CAH and for the study of neonatal stress physiology, supporting integrated clinical, biochemical and neuroendocrine assessments in the neonatal period.

Aims

The overall goals of this work was to improve the effectiveness, diagnostic accuracy and sustainability of NBS for CAH through the implementation of LC-MS/MS-based steroid profiling, besides to extend its application to the investigation of HPA axis physiology and neonatal stress responses.

The specific aims of the study were:

- **To develop and validate an LC-MS/MS-based 2-TT for steroid profiling in CAH newborn screening**, in order to enhance diagnostic specificity compared with first-tier immunoassays, reducing false-positive results and unnecessary recalls and increase the positive predictive value of the screening program.
- **To optimize CAH screening cut-offs by considering clinical and environmental factors**, within a personalized medicine framework, with the aim of improving the identification of newborns truly at risk and reducing the unnecessary use of 2-TT, thereby improving screening efficiency and reducing turnaround time and costs.
- **To apply plasma steroid profiling by LC-MS/MS as a complementary diagnostic approach**, to improve the biochemical characterization of the different forms of CAH and the main differential diagnoses, allowing a more accurate distinction between classical, non-classical and atypical variants.
- **To develop an LC-MS/MS method for the quantification of salivary cortisol**, using a non-invasive and easily accessible biological matrix, to investigate HPA axis physiology and stress responses in the neonatal period, both under physiological conditions and in response to environmental and sensory interventions.

Chapter 1

Introduction: Newborn Screening and Metabolomics Approaches

Newborn screening (NBS) represents one of the most widespread and effective preventive strategies in public health, with the primary objective of identifying inborn errors of metabolism (IEMs) during the very first days of life, between 48 and 72 hours after birth. Early recognition of these disorders, many of which may lead to severe clinical outcomes or be potentially fatal if untreated, enables prompt clinical intervention and significantly improves prognosis by reducing morbidity and mortality. The foundations of modern newborn screening date back to the early 1960s, when Dr. Robert Guthrie introduced a pioneering assay for the detection of phenylketonuria (PKU). This method, later known as the Guthrie test, was based on the analysis of dried blood spots (DBS) collected on filter paper and relied on a bacterial inhibition principle. The assay allowed the indirect estimation of phenylalanine (Phe) concentrations, exploiting the ability of excess Phe to counteract the inhibitory effects of specific analogues on bacterial growth. In practice, blood samples obtained through heel prick were absorbed onto filter paper, from which small discs were punched and placed onto agar plates seeded with *Bacillus subtilis*. When elevated Phe levels were present, bacterial proliferation occurred despite the presence of β -2-thienylalanine, and the extent of growth was proportional to the Phe concentration in the blood sample ^[1].

This innovative and cost-effective approach rapidly proved suitable for large-scale application and marked a turning point in diagnostic medicine. More importantly, it contributed to establishing the fundamental concept that detecting diseases during the pre-symptomatic phase allows timely treatment and prevents irreversible damage in affected newborns. The first official NBS programs were implemented in 1963 in several U.S. states, including Massachusetts, Delaware, Vermont, and Oregon, subsequently promoting the gradual adoption of similar screening initiatives worldwide, albeit with considerable variability in timing and implementation.

Over the years, newborn screening has benefited from substantial technological progress, incorporating a wide range of analytical methodologies such as radioimmunoassays, colorimetric and fluorometric immunoassays, isoelectric focusing, and molecular genetic testing ^[2]. Among these advancements, liquid chromatography coupled with tandem mass

spectrometry (LC-MS/MS) has emerged as the cornerstone of contemporary NBS programs. Owing to its high analytical sensitivity and specificity, rapid turnaround time, robustness, and capability to perform high-throughput multiplex analyses, LC-MS/MS has dramatically expanded the spectrum of detectable disorders and represents the current gold standard in newborn screening laboratories.

1.1 Italian Newborn Screening

In Italy, NBS has progressively evolved as structured national public health program regulated by specific legislative measures. The first NBS activities were promoted by the Ministry of Health in 1978 and initially implemented on a regional basis. However, mandatory screening was formally established only in 1992 and was restricted to a small number of conditions, namely congenital hypothyroidism, PKU and cystic fibrosis.

A major expansion of the Italian NBS system became possible following the introduction of tandem mass spectrometry (MS/MS), which allows the simultaneous detection of a broad spectrum of inborn errors of metabolism (IEMs) from a single dried blood spot sample. In this context, Law No. 147 of 27 December 2013 represented a crucial step, allocating dedicated funding to support the experimental implementation of extended newborn screening across the entire national territory. This phase laid the groundwork for a more comprehensive and uniform screening strategy.

The definitive institutional recognition of extended newborn screening was achieved with Law No. 167 of 19 August 2016, which included NBS for IEMs among the Essential Levels of Assistance (LEA), thereby guaranteeing equal access to screening services for all newborns in Italy. From a regulatory perspective, this legislation established that a disease may be included in the screening panel when there is robust scientific evidence demonstrating clinical benefit from early diagnosis and availability of effective therapeutic interventions. Importantly, the framework also allows access to innovative treatments that are still under advanced stages of clinical experimentation.

Subsequently, the Decree of the Ministry of Health issued on 13 October 2016 defined a panel of 49 disorders subject to mandatory screening and provided detailed guidance on organizational aspects of the NBS system. These included standardized procedures for sample collection, communication with families, informed consent, and coordination

among regional screening centers, with the aim of harmonizing practices nationwide. The decree also introduced a periodic review mechanism, scheduled every three years, to update the screening panel in accordance with advances in scientific knowledge and technological innovation. Further expansion of the program was encouraged by Law No. 145 of 2018, which opened the possibility of including additional disease groups, such as severe congenital immunodeficiencies, neuromuscular disorders, and lysosomal storage diseases.

At the regional level, the Abruzzo Region has played an active role in the implementation of newborn screening programs. Screening for congenital hypothyroidism and PKU has been operational since 1992, while cystic fibrosis screening was added in 2015. Extended newborn screening was officially activated in 2018 following the designation of the regional NBS reference center within the Laboratories of Analytical Biochemistry, Proteomics, Endocrinology, and Medical Genetics at the Center for Advanced Studies and Technology (CAST) of the "G. d'Annunzio" University of Chieti–Pescara. The regional laboratory performs MS/MS-based analyses for disorders such as aminoacidopathies, organic acidemias, urea cycle disorders, and fatty acid oxidation defects, alongside immunometric assays for congenital hypothyroidism, cystic fibrosis, biotinidase deficiency and galactosemia.

More recently, Abruzzo region has further strengthened the screening framework through a pilot project aimed at the experimental monitoring of additional rare diseases. These efforts highlight the dynamic nature of the Italian NBS system and its ongoing commitment to expanding preventive strategies through scientific innovation and evidence-based policy.

1.2 Inborn Errors of Metabolism

Conditions targeted by newborn screening arise from genetic alterations affecting enzymes or regulatory proteins that govern essential metabolic pathways. These disorders are predominantly inherited in an autosomal recessive manner, while a smaller proportion follow autosomal dominant or X-linked transmission. Functional impairment or dysregulation of metabolic proteins can disrupt biochemical homeostasis through multiple mechanisms. These include the pathological build-up of precursor molecules, which may exert toxic effects on tissues, the insufficient production of downstream metabolites required for normal cellular function, and the redirection of metabolic intermediates into atypical pathways, resulting in the formation of abnormal compounds with detrimental biological effects ^[3]. Although individually rare inborn errors of metabolism collectively

represent a significant health burden, with an estimated prevalence of 1:784 in the United Kingdom ^[4], 1:2,500 in Canada ^[5], and 1:2,555 in Italy ^[6]. Based on established classification frameworks, inborn errors of metabolism can be broadly categorized into disorders involving complex molecules or small molecules. The former are generally associated with a later clinical onset, as observed in lysosomal storage diseases, whereas the latter typically present with acute manifestations, including amino acid disorders, urea cycle defects, organic acidemias, and fatty acid oxidation disorders ^[7]. An alternative classification approach focuses on the biochemical consequences of the enzymatic defect, whereby the clinical phenotype arises from the toxic accumulation of upstream metabolites, the deficiency of essential downstream products, or impaired energy production ^[8]. Neurological issues, including delayed development, low muscle tone, and seizures, are common in most IEMs, often occurring alongside gastrointestinal problems such as vomiting, enlarged liver, diarrhea, and dehydration ^[9]. Current criteria for including IEMs in newborn screening programs include disease frequency, availability of reliable and cost-effective diagnostic tests, severity and risk if untreated, and the potential benefits of early diagnosis for patients and families ^[10], early detection before symptoms is crucial. Treatment availability, disease awareness, and screening costs may also be considered but are not strict requirements ^[9]. Beyond immediate neonatal emergency protocols, long-term treatments for IEMs can be grouped into several categories, as shown in **Figure 1**:

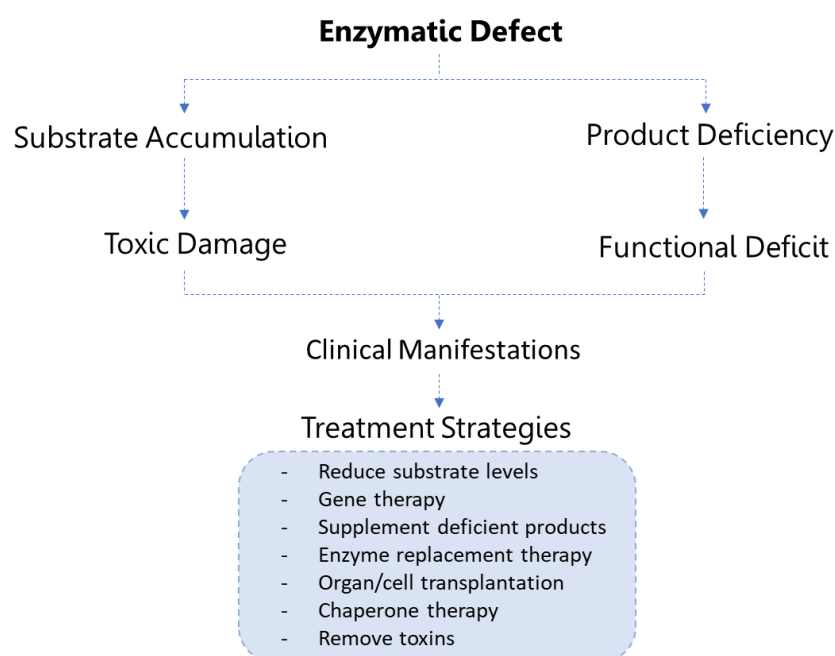


Figure 1. Overview of the primary treatment approaches for IEMs by mechanisms that have different targets.

The main IEMs included in extended newborn screening, according to Law 167/2016, Ministerial Decree 13 October 2016, and related regulations, can be grouped into the following categories, as also shown in **Table 1**:

- **Amino acid disorders (AAs)** involve enzyme defects in intermediate metabolic pathways that impair the breakdown of one or more amino acids ^[11]. Phenylketonuria (PKU) is caused by mutations in the PAH gene, which encodes phenylalanine hydroxylase, the enzyme converting phenylalanine (Phe) into tyrosine (Tyr). If untreated, toxic Phe levels can result in neurological damage or death, though milder forms such as benign hyperphenylalaninemia (HPA) and tetrahydrobiopterin deficiency have also been described ^[12,13]. Tyrosinemia type I results from a deficiency of fumarylacetoacetate hydrolase (FAH), the enzyme catalyzing the final step of Tyr degradation. This leads to elevated succinylacetone (SA) and clinical signs of liver failure, with renal and neurological complications, as well as long-term risk of hepatocellular carcinoma ^[14,15]. Tyrosinemia type II, due to tyrosine aminotransferase deficiency, causes high Tyr levels and is associated with ocular abnormalities, painful palmoplantar keratosis, and cognitive delay ^[16]. Maple syrup urine disease (MSUD) prevents the catabolism of leucine (Leu), isoleucine (Ile), and valine (Val), producing multi-organ symptoms including developmental delay, intellectual disability, seizures, coma, and potentially death ^[17]. Homocystinurias arise from remethylation defects or cystathionine beta-synthase (CBS) deficiency, resulting in high homocysteine (Hcy) and altered methionine (Met) levels, with neurological and hematological manifestations ^[18].
- **Urea cycle disorders (UCDs)** are inherited metabolic conditions that disrupt the body's ability to eliminate ammonia. Type 1 citrullinemia (*CTLN1*) is caused by mutations in the *ASS1* gene, which encodes argininosuccinate synthetase (*ASS*) ^[19], type 2 citrullinemia (*CTLN2*), on the other hand, is linked to deficiencies in citrin, a key mitochondrial transporter ^[20]: both conditions lead to elevated citrulline (Cit) levels, which can result in developmental delays, seizures, and vomiting. Argininemia occurs when arginase 1 (*ARG1*) is deficient, causing arginine (Arg) to accumulate, *ARG1* normally converts Arg into ornithine (Orn) and urea; clinically, this disorder presents with spastic paraparesis, progressive neurological and motor decline, growth and cognitive delays, seizures, and a high risk of early death ^[21]. Argininosuccinic aciduria is another type of UCD caused by mutations in the *ASL* gene, which encodes argininosuccinate lyase (*ASL*), this enzyme normally converts argininosuccinic acid (ASA) into arginine and fumarate. Elevated ASA levels lead to

symptoms such as lethargy, vomiting, respiratory alkalosis, seizures, coma, and, if untreated, death ^[22].

- **Organic acidemias (OAs)** are inherited metabolic disorders where amino acid breakdown is impaired, causing toxic acids to accumulate. Methylmalonic acidemia (MMA) and propionic acidemia (PA) are severe conditions caused by deficiencies in methylmalonyl-CoA mutase and propionyl-CoA carboxylase, respectively ^[23], they lead to high propionyl-carnitine (C3), abnormal acyl-carnitine ratios, and toxic levels of methylmalonic, methylcitric and propionic acids. Glutaric acidemia type I (GA I) results from reduced glutaryl-CoA dehydrogenase (GCDH) activity, causing glutaryl-carnitine (C5DC), glutaric, and 3-hydroxyglutaric acid accumulation from lysine, hydroxylysine, and tryptophan metabolism ^[24]. Isovaleric acidemia (IVA) is due to isovaleryl-CoA dehydrogenase (IVD) deficiency, leading to isovaleric acid, 3-hydroxyisovaleric acid, isovaleryl-carnitine (C5), and isovalerylglycine accumulation, symptoms include vomiting, lethargy, and, if untreated, coma or death ^[25]. 3-Hydroxy-3-methylglutaric aciduria (HMG) arises from defective 3-hydroxy-3-methylglutaryl-CoA lyase (HL), causing incomplete leucine breakdown, elevated C5OH\C6DC, and severe symptoms including vomiting, diarrhea, hypotonia, seizures, and CNS problems ^[26]. Short/branched-chain acyl-CoA dehydrogenase deficiency (SBCADD) affects isoleucine metabolism, increasing C5 levels and primarily impacting the CNS ^[27]. Malonic aciduria, caused by malonyl-CoA decarboxylase (MCD) deficiency, leads to high malonyl-carnitine (C3DC) and neurological, digestive, metabolic, and cardiac problems ^[28]. Multiple carboxylase deficiencies result from defects in biotin-dependent carboxylases, causing elevated C5OH, metabolic acidosis, hyperammonemia, hypotonia, seizures, feeding issues, and coma ^[29].
- **Fatty acid β -oxidation disorders (FAODs)** include a diverse group of conditions caused by defects in mitochondrial β -oxidation or in the transport of fatty acids via the carnitine shuttle pathway ^[30]. Carnitine transporter deficiency (CTD) arises from a lack of the organic cation transporter type 2 (OCTN2), which normally transports free carnitine (C0) across cell membranes, this deficiency results in low C0 levels and is associated with episodes of hypoketotic hypoglycemia, hepatomegaly, elevated liver enzymes, and hyperammonemia ^[31]. Carnitine palmitoyltransferase 1A deficiency (CPT1AD) impairs the mitochondrial import of long-chain fatty acids, causing hypoketotic hypoglycemia, hepatomegaly, and muscle weakness, blood analysis shows increased C0 levels and reduced palmitoyl- and stearyl-carnitine

(C16 and C18) ^[32]. Carnitine-acylcarnitine translocase deficiency (CACTD) results from a malfunctioning carnitine shuttle. This leads to elevated levels of C16, C18, oleoyl-carnitine (C18:1), and linoleoyl-carnitine (C18:2), which contribute to hyperammonemia, cardiac arrhythmias, lethargy, hypoketotic hypoglycemia, hepatomegaly, and rhabdomyolysis ^[33]. CPT2 deficiency (CPT2D) shows similar acyl-carnitine profiles. Very-long-chain acyl-CoA dehydrogenase deficiency (VLCADD) can present with severe cardiomyopathy and early neonatal death. Biochemical markers include elevated myristoyl-carnitine (C14), tetradecenoyl-carnitine (C14:1), and tetradecadienoyl-carnitine (C14:2) ^[30]. Long-chain hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and trifunctional protein deficiency (TFPD) both affect the mitochondrial trifunctional protein (TFP), patients display increased 3-hydroxyoctadecanoyl-carnitine (C18:OH), 3-hydroxyoctadecenoyl-carnitine (C18:1OH), 3-hydroxyhexadecanoyl-carnitine (C16OH), and 3-hydroxyhexadecenoyl-carnitine (C16:1OH), along with clinical signs including hypoglycemia, hepatomegaly, encephalopathy, and often cardiomyopathy ^[34]. Medium-chain acyl-CoA dehydrogenase deficiency (MCADD) is the most frequent FAOD, it is marked by elevated octanoyl-carnitine (C8), hexanoyl-carnitine (C6), decanoyl-carnitine (C10), and decenoyl-carnitine (C10:1), causing hypoketotic hypoglycemia that can rapidly progress to lethargy, coma, and death ^[35]. Medium/short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (M/SCHADD) affects C4OH levels and presents with metabolic acidosis, severe hypoglycemia, hyperammonemia, and multi-system failure, aberrant acyl-carnitine profiles, ranging from butyryl-carnitine (C4) to C18, are a key diagnostic feature ^[36].

Disease	Acronym	Group	Group Name	Primary Marker	Marker Ratio	High Risk
Phenylketonuria	PKU	AA	Amino Acid Metabolism & Transport Disorders	Phe	Phe/Tyr	No
Benign hyperphenylalaninemia	HPA	AA		Phe	Phe/Tyr	No
Biopterin synthesis deficiency	BIOPT (BS)	AA		Phe	Phe/Tyr	No
Biopterin regeneration deficiency	BIOPT (REG)	AA		Phe	Phe/Tyr	No
Maple syrup urine disease	MSUD	AA		Val, XLeu	Val/Phe, (Leu+Ile)/Phe, (Leu+Ile)/Ala	Yes
Homocystinuria (CBS defect)	HCY	AA		Met high	Met/Phe	Yes
Homocystinuria (severe MTHFR defect)	MTHFR	AA		Met low	–	Yes
Glutaric acidemia type I	GA I	OA	Organic Acidemias	C5DC	C5DC/C5OH, C5DC/C8, C5DC/C16	Yes
Isovaleric acidemia	IVA	OA		C5	C5/C0, C5/C2, C5/C3	Yes
Beta-ketothiolase deficiency	BKT	OA		C5:1	C5OH/C8	Yes
3-Hydroxy-3-methylglutaric aciduria	HMG	OA		C5OH, C6DC	C5OH/C8, C5OH/C2, C5OH/C10	Yes
Propionic acidemia	PA	OA		C3	C3/C2, C3/C16	Yes
Methylmalonic acidemia (Mut)	MUT	OA		C3	C3/C2, C3/C16	Yes
Methylmalonic acidemia (Cbl-A)	Cbl A	OA		C3	C3/C2, C3/C16	Yes
Methylmalonic acidemia (Cbl-B)	Cbl B	OA		C3	C3/C2, C3/C16	Yes
Methylmalonic acidemia with homocystinuria (Cbl C)	Cbl C	OA		C3 high, Met low	C3/C2, C3/C16, C3/Met	Yes
Methylmalonic acidemia with homocystinuria (Cbl D)	Cbl D	OA		C3 high and/or Met low	C3/C2, C3/C16, C3/Met	Yes
2-Methylbutyryl-CoA dehydrogenase deficiency	2MBG	OA		C5	C5/C0, C5/C2, C5/C3	Yes
Malonic aciduria	MAL	OA		C3DC	–	Yes

Multiple carboxylase deficiency	MCD	OA		C5OH	C5OH/C8, C5OH/C2, C5OH/C10	Yes
Citrullinemia type I	CIT I	UCD	Urea Cycle Disorders	Cit	–	Yes
Citrullinemia type II (citrin deficiency)	CIT II	UCD		Cit	–	Yes
Tyrosinemia type I	TYR I	UCD		Tyr	Tyr/Cit	Yes
Tyrosinemia type II	TYR II	UCD		Tyr	Tyr/Cit	No
Argininosuccinic acidemia	ASA	UCD		ASA	ASA/Arg	Yes
Argininemia	ARG	UCD		Arg	–	Yes
Carnitine transport defect	CUD	FAO	Congenital Lipoprotein Metabolism Disorders	C0 low	–	Yes
Carnitine palmitoyltransferase I deficiency	CPT Ia	FAO		C0 high, C16 low, C18 low	C0/(C16+C18)	Yes
Carnitine-acylcarnitine translocase deficiency	CACT	FAO		C16, C18:2, C18:1, C18	(C16+C18:1)/C2	Yes
Carnitine palmitoyltransferase II deficiency	CPT II	FAO		C16, C18:2, C18:1, C18	(C16+C18:1)/C2	Yes
Very-long-chain acyl-CoA dehydrogenase deficiency	VLCAD	FAO		C14, C14:1, C14:2	C14:1/C2	Yes
Mitochondrial trifunctional protein deficiency	TFP	FAO		C18OH, C16OH, C16:1OH, C18:1OH	C18OH/C18, C16OH/C16	Yes
Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency	LCHAD	FAO		C18OH, C16OH, C16:1OH, C18:1OH	C18OH/C18, C16OH/C16	Yes
Medium-chain acyl-CoA dehydrogenase deficiency	MCAD	FAO		C6, C8, C10, C10:1	C8/C10, C8/C2	Yes
Medium/short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency	M/SCHAD	FAO		C4OH	–	Yes
Glutaric acidemia type II / MADD	GA II / MADD	FAO		C4–C18		Yes

Table 1. Inherited metabolic disorders included in newborn screening using tandem mass spectrometry. (References: Law 167/2016, Ministerial Decree of 13 October 2016 and related

regulations, and SIMMESN – Società italiana per lo studio delle Malattie Metaboliche Ereditarie e Screening Neonatale, 2018 consensus document).

Other IEMs may enter the differential diagnosis with some of those described above, as they share primary biomarkers, as reported in **Table 2**. In tyrosinemia type III, mild elevations in tyrosine may be observed. Elevated methionine levels can result from deficiencies in glycine N-methyltransferase, methionine adenosyltransferase, or S-adenosylhomocysteine hydrolase. Increases in C5OH may indicate 3-methylglutaconic acidurias, 3-methylcrotonyl-CoA carboxylase deficiency, or 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency. Additionally, C4 levels can be elevated in isobutyryl-CoA dehydrogenase and short-chain acyl-CoA dehydrogenase deficiencies. Interestingly, four IMDs are monitored using immunofluorometric assays (**Tables 3 and 4**): (1) galactosemia, characterized by impaired galactose metabolism, particularly in the classic form due to reduced galactose-1-phosphate uridylyltransferase (GALT) activity ^[37], (2) biotinidase deficiency, a biotin-responsive neurocutaneous disorder ^[38], (3) congenital hypothyroidism, leading to psychomotor developmental delays caused by thyroid hormone deficiency ^[39], and (4) cystic fibrosis, caused by defects in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, characterized by elevated immunoreactive trypsinogen (IRT) levels and affecting both pulmonary and digestive systems ^[40].

Disease	Acronym	Shared Marker	Marker Ratio
Tyrosinemia type III	TYR III	Tyr	Tyr/Cit
Glycine N-methyltransferase deficiency	GNMT	Met	–
Methionine adenosyltransferase deficiency	MAT	Met	–
S-adenosylhomocysteine hydrolase deficiency	SAHH	Met	–
3-Methylglutaconic acidurias	3MGCA	C5OH	C5OH/C8
3-Methylcrotonyl-CoA carboxylase deficiency	3MCC	C5OH	C5OH/C8, C5OH/C0
2-Methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency	2M3HBA	C5:1, C5OH	C5OH/C8
Isobutyryl-CoA dehydrogenase deficiency	IBG	C4	C4/C2, C4/C3, C4/C8
Short-chain acyl-CoA dehydrogenase deficiency	SCAD	C4	C4/C2, C4/C3, C4/C8

Table 2. Inherited metabolic disorders that may enter the differential diagnosis of the conditions screened in newborns by tandem mass spectrometry (listed in Table 1), due to shared primary biomarkers. (References: Law 167/2016, Ministerial Decree 13 October 2016 and related regulations, and SIMMESN 2018 consensus document).

Disease	Acronym	Disease Group	High-Risk Condition	Screening Marker
Galactosemia	GALT	Carbohydrate metabolism and transport disorders	Yes	GALT activity
Biotinidase deficiency	BTD	Amino acid metabolism and transport disorders	No	Biotinidase enzyme activity

Table 3. Inherited metabolic disorders screened using methods other than tandem mass spectrometry. (References: Law 167/2016, Ministerial Decree 13 October 2016 and related regulations, and SIMMESN 2018 consensus document).

Disease	Acronym	Disease Group	Screening Marker
Congenital Hypothyroidism	CH	Endocrine disorders	Thyroid-stimulating hormone (TSH)
Cystic Fibrosis	CF	Multisystem (pulmonary and digestive) disorder	IRT

Table 4. Institutional newborn screening programs. (References: Framework Law No. 104, Art. 6, 5 February 1992; Regional Law No. 102, 1997; Regional Resolution No. 119, 10 December 2015).

1.3 Mass Spectrometry (MS/MS) in Newborn Screening

Tandem mass spectrometry (MS/MS) was introduced in the 1990s for detecting IEMs and quickly became recognized as a safe, reliable, and straightforward screening method. During the same decade, MS/MS-based protocols for newborn screening were established in the USA, allowing the detection of over 30 metabolic disorders involving amino acids, fatty acids, and other organic acids. Over time, the number of metabolites measurable in a single run increased, leading to the widespread adoption of MS/MS in newborn screening programs [41].

Mass spectrometry (MS) is an analytical method that separates ions in the gas phase according to their mass-to-charge ratio (m/z). It is extensively employed for identifying unknown compounds, quantifying specific analytes and investigating molecular characteristics. Coupling MS with other techniques allows detailed qualitative and quantitative analysis of complex mixtures of organic and inorganic substances, as well as structural insights through isotopic pattern characterization. MS is highly valued for its selectivity and sensitivity, which enable it to distinguish analytes in complex matrices while producing strong signals even at low concentrations, reflecting efficient ionization and transmission. In a mass spectrometer, ions are accelerated by an electric field and directed into mass analyzers that differentiate them based on m/z . Mass resolution, the ability to separate ions with very close molecular masses, is a key parameter. For instance, triple quadrupole instruments have limited resolution and cannot separate isobaric ions, whereas high-resolution systems can determine exact masses with precision up to four decimal places. Mass spectrometers operate under high vacuum and typically consist of three main components: (i) an ionization source to generate ions, (ii) one or more mass analyzers to

separate ions by m/z , and (iii) a detector to convert the ion beam into an electrical signal. Samples may be introduced via hyphenated systems, such as chromatography, or by direct infusion without prior separation, as in flow injection analysis (FIA).

Mass spectrometers used for newborn screening (NBS) share a common configuration ^[42,43] (**Figure 2**):

- Electrospray ionization (ESI) serves as a “soft” ionization method, charging analytes without disrupting their structure. Volatile samples enter the ESI via a stainless-steel capillary, where high voltage produces an aerosol of charged droplets. A coaxial nitrogen flow aids nebulization and directs the spray into the instrument. Solvent evaporation reduces droplet size, increasing charge density until Coulomb explosion releases ions, which pass through a heated cone for complete desolvation.
- First quadrupole separates gas-phase ions by their m/z values. Four parallel rods are charged with DC and RF voltages, stabilizing only ions of selected m/z , while others collide with the rods and are filtered out. The quadrupole’s resolution depends on RF and rod length.
- Collision cell fragments selected “precursor ions” via collision-induced dissociation (CID) with helium, generating predictable “fragment ions” for analysis.
- Second quadrupole separates these fragment ions by m/z . Using two mass analyzers in series defines tandem mass spectrometry (MS/MS). When a precursor ion is selected in the first quadrupole and its fragment detected in the second, this is called selected reaction monitoring (SRM); monitoring multiple precursor-product pairs is multiple reaction monitoring (MRM).
- Photomultiplier detector converts ion impacts on a phosphorus screen into amplified electrical signals.

In NBS, triple quadrupole MS/MS instruments operate in MRM mode, allowing semi-quantitative measurement of multiple analytes in ~1.2 minutes per run ^[44]. This enables detection of over 40 inborn errors of metabolism from neonatal dried blood spots (DBS) with minimal sample preparation and no prior chromatography. Fast run-to-run analysis supports high-throughput screening, though defining cut-offs to reduce false positives/negatives and performing confirmatory diagnostics remain challenges. Additionally, triple quadrupole MS lacks the resolution to distinguish isobaric compounds

[45], but coupling MS/MS with chromatographic methods can overcome this limitation, requiring collaboration among analytical chemists, biochemists and clinicians.

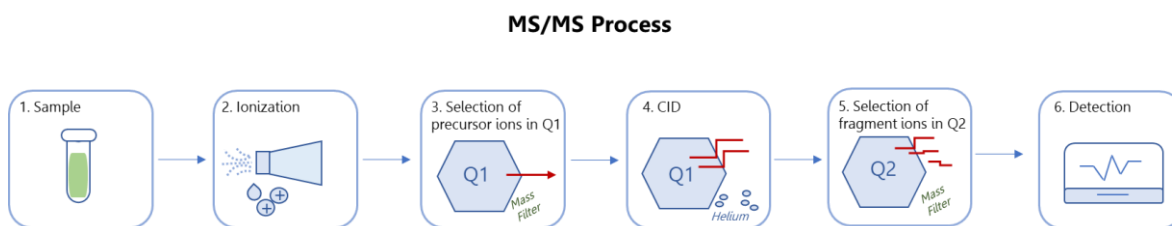


Figure 2. Schematic overview of tandem mass spectrometry in a triple quadrupole instrument operating in multiple reaction monitoring (MRM) mode, illustrating ion generation, precursor ion selection in the first quadrupole (Q1), fragmentation by collision-induced dissociation (CID), product ion selection in the second quadrupole (Q2), and signal detection.

1.4 Contribution of Second-tier Tests to Reduce False Positives

To reduce false-positive results and limit unnecessary newborn recalls, post-screening strategies increasingly include second-tier tests (2-TTs) within newborn screening (NBS) programs. Initial screening is typically performed on dried blood spot (DBS) samples collected 48–72 hours after birth using flow injection analysis coupled to tandem mass spectrometry (FIA-MS/MS). Simultaneous evaluation of more than 70 metabolic parameters requires rapid detection and careful interpretation of abnormal results within 48 hours of sample receipt. In some cases, primary biomarkers are sufficiently specific to raise immediate diagnostic suspicion and prompt urgent clinical action due to the risk of acute metabolic decompensation. In other situations, abnormalities may be reassessed by repeating the first-tier analysis on the same DBS (retest) or monitored over time, often leading to newborn recall for additional DBS collection. When retesting is not feasible because of limited or inadequate sample material, a new DBS must be requested, increasing parental anxiety. As a positive screening result does not represent a definitive diagnosis, retesting, follow-up, and second-tier testing, when available, are integral components of the NBS workflow (Figure 3). 2-TTs are generally performed using analytical approaches different from FIA-MS/MS to improve sensitivity and specificity. These methods target the same or metabolically related analytes on the original DBS sample. The main goals of second-tier testing are: (i) accurate quantification of altered primary biomarkers, (ii) measurement of secondary, metabolically linked markers, (iii) reduction of false positives and (iv) differential diagnosis among disorders sharing common biomarkers or affected by

interfering conditions. Mass spectrometry, particularly when coupled with liquid chromatography (LC-MS/MS), plays a central role in 2-TTs. LC-MS/MS enables accurate and reproducible quantification using calibration curves and benefits from chromatographic separation, which minimizes ion suppression and analytical interferences compared with FIA-based approaches. The decision to apply second-tier tests is guided by predefined cut-offs, which should be locally established by each NBS center to ensure analytical reliability. Implementation of 2-TTs has been shown to increase the positive predictive value (PPV) while reducing the false-positive rate (FPR) ^[46]. Age-adjusted cut-offs may further aid interpretation of longitudinal biomarker changes, such as acylcarnitines. Overall, NBS performance can be enhanced by optimizing cut-offs, incorporating informative biomarker ratios (e.g., Phe/Tyr for phenylketonuria), and applying 2-TTs strategies ^[47], thereby reducing unnecessary recalls and mitigating psychological and economic burdens on families and healthcare systems.

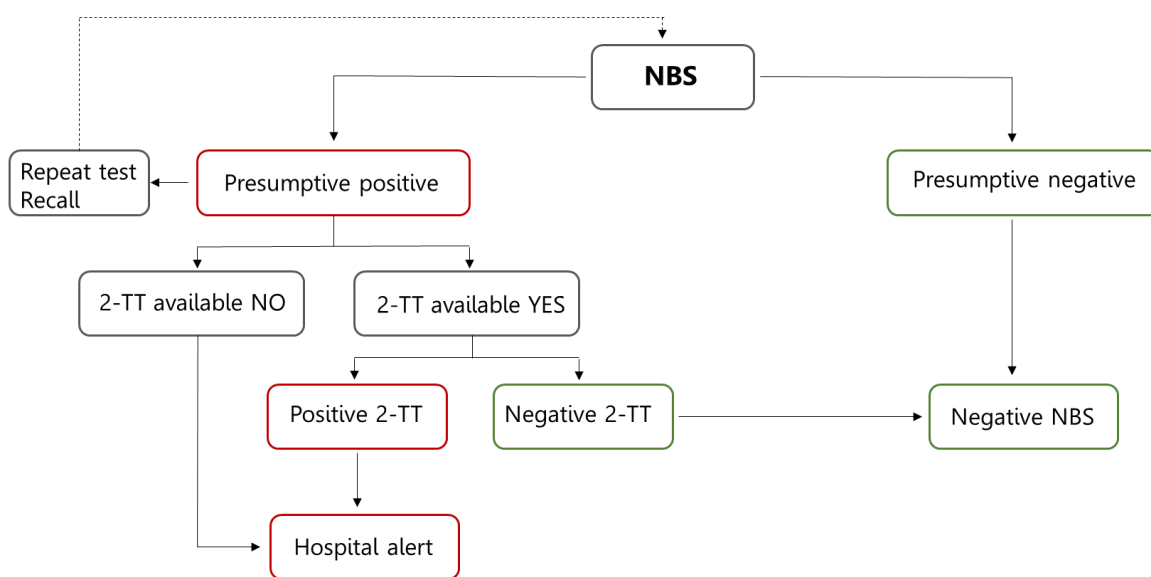


Figure 3. Workflow of NBS. Presumptive positive results may require retesting, recall or 2-TT, or clinical referral, whereas presumptive negative results correspond to a negative NBS outcome.

1.5 Abruzzo Pilot Project and DGR n.782 24/11/2025

In December 2022, a pilot project was approved in the Abruzzo Region to experimentally monitor additional rare diseases in newborns. The conditions included in this initiative are Severe Combined Immunodeficiency (SCID) due to Adenosine Deaminase Deficiency (ADA), aromatic L-amino acid Decarboxylase Deficiency (AADCD), Congenital Adrenal Hyperplasia (CAH), Spinal Muscular Atrophy (SMA), and three Lysosomal Storage Disorders (LSDs) in particular Mucopolysaccharidosis type I (MPS-I), Fabry disease (FD) and Gaucher disease (GD).

Before sample collection, parents receive detailed information about the project and provide informed consent, authorizing the use of their child's dried blood spot (DBS) - collected for the mandatory NBS of 40 diseases - for the extended pilot screening. A copy of the signed consent form is retained in the newborn's medical record. No additional blood collection is required, the analyses for the pilot conditions are performed on the same DBS sample used for routine screening.

Screening analyses for ADA-SCID and AADCD are incorporated into the existing tandem mass spectrometry (MS/MS) panel, which also detects aminoacidopathies, organic acidemias, UCDs, and fatty acid oxidation defects. 17- α -hydroxyprogesterone marker for CAH is measured via immunofluorimetric assays, SMA is assessed through a molecular analysis to detect SMN1 gene copies, and LSDs are analyzed through MS/MS-based enzymatic activity assays (α -galactosidase for Fabry, Glucocerebrosidase for Gaucher, α -L-iduronidase for MPS-I).

For AADCD, AGS, and LSDs, second-tier tests (2-TTs) are performed on the same DBS sample used for the first-tier screening before initiating any recall procedures. Specifically, for AADCD 3-O-methyldopa (3-OMD) levels are quantified by LC-MS/MS; for CAH 4-androstene-3,17-dione (A4), cortisol (CORT), 17-OHP, 11-deoxycortisol (11-DC), 21-deoxycortisol (21-DC) are measured by LC-MS/MS, also establishing (17-OHP + 4-A)/cortisol ratio; for LSDs (Fabry disease and Gaucher disease) lysosphingolipids are assessed by LC-MS/MS on the same sample.

Following the pilot project, Regional Resolution (DGR) n. 782 of 24 November 2025 formally integrated the additional disorders into the Abruzzo neonatal screening program, establishing a permanent regional panel of 56 diseases. This measure transfers responsibility from the University "G. d'Annunzio" to the Regional Health Service (ASLs), ensuring that the extended panel, including ADA-SCID, AADCD, CAH, SMA, MPS-I, Fabry

disease and Gaucher diseases, is guaranteed to all newborns without additional costs, marking a decisive step toward comprehensive, equitable neonatal care.

1.6 Analytical Strategies for Steroid Profiling

Laboratory testing is essential in clinical practice, particularly in endocrinology, where steroid quantification remains one of the most challenging routine analyses. Immunoassays (IA) are antibody-based bioanalytical techniques historically used for steroid quantification in clinical endocrinology, with formats including radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and fluoroimmunoassay (FIA). IA are popular due to practical advantages, such as small sample volumes (serum, urine, saliva), simple preparation, availability of commercial kits, automation, low costs, and ease of use even in small laboratories. When quality control and established reference ranges are applied, IA provide reliable results for commonly measured steroids. Nevertheless, IA have intrinsic limitations, in fact, specificity can be compromised by cross-reactivity with structurally similar steroids, potentially causing inaccurate quantification, particularly in complex biological matrices or under certain clinical conditions. Sensitivity is restricted to the linear range of the calibration curve, and inter-laboratory standardization is challenging. Steroid measurements may also be influenced by variations in binding proteins, steroid precursors, and metabolites, affecting hormones such as cortisol, testosterone, and estradiol, especially at low concentrations or during treatments affecting steroidogenesis. Moreover, IA are inherently single-analyte methods, making the development of assays for less commonly measured steroids laborious and expensive. These limitations highlight the need for analytical approaches that combine higher specificity with multiplexing capabilities ^[48].

MS-based methods allow high-resolution chromatographic separation, particularly for steroids with similar chemical structures, and provide rapid, reproducible results with excellent purification. Over the past 15 years, metabolomics has enabled comprehensive steroid profiling, overcoming many IA limitations. The multiplexing capability of MS-based approaches is particularly valuable in complex adrenal disorders, such as adrenal tumors and congenital adrenal hyperplasia, making it a powerful diagnostic tool. Gas chromatography coupled to mass spectrometry (GC-MS), often using isotopically labeled internal standards, has long been considered the gold standard for multiple steroid determinations. However, GC-MS is time-consuming due to extensive sample preparation, including derivatization to increase volatility and stability, limiting its routine clinical use.

Derivatization methods, tailored to functional groups such as oxo- and hydroxy-groups, are critical for accurate steroid quantification. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has become the preferred method in clinical laboratories due to its reduced sample preparation requirements, faster analysis times, and ability to simultaneously measure multiple steroids. Ultra-high-performance LC (UHPLC) further increases throughput, allowing results within hours. Nevertheless, LC-MS/MS analyses are affected by matrix effects, ion suppression, and potential isobaric interferences, which must be carefully managed to ensure accuracy. Reference intervals for LC-MS/MS are still being established, whereas IA have well-accepted ranges due to decades of clinical use. Imaging approaches, such as matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI), offer spatial visualization of steroid distribution within tissues. These techniques detect and localize endogenous and exogenous analytes in tissue sections, using chemical derivatization and laser ionization to enhance sensitivity. MALDI-MSI is particularly useful for studying adrenal steroid distribution, including cortisol, aldosterone, and 18-hydroxycortisol. While promising for research and clinical pathology, this approach is limited to tissue samples, requires specialized expertise, and is not applicable to routine analysis of biological fluids ^[48].

While IA techniques suitable for routine measurement of commonly assessed steroids, MS-based methods represent the reference approach for accurate, specific and comprehensive steroid profiling, particularly in complex endocrine disorders (**Table 4**).

Category	Included methods	Advantages	Limitations
IA	RIA ELISA FIA	- Simple and rapid execution - Small sample volumes required - High level of automation - Low cost - Wide availability of commercial kits - Well-established clinical reference intervals	- Limited specificity due to antibody cross-reactivity - Interference from steroid precursors and metabolites - Influence of binding proteins - Single-analyte approach - Reduced accuracy at low hormone concentrations
MS-based methods	GC-MS LC-MS/MS UHPLC-MS/MS MALDI-MSI	- High analytical specificity and accuracy - Efficient separation of structurally similar steroids - Simultaneous measurement of multiple analytes (multiplexing) - High sensitivity, including at low concentrations - Reference approach for complex steroid profiling	- High instrumentation and maintenance costs - Requirement for highly specialized personnel - Matrix effects and ion suppression (LC-MS/MS) - More complex sample preparation compared with IA - MALDI-MSI limited to tissue samples and not suitable for routine analysis

Table 4. Comparison between Immunoassays (IA) and Mass Spectrometry MS-based methods for steroid quantification.

1. Dave Usha P, Das Bibhu R. Newborn screening — From 'Guthrie age to Genomic age'. *J Obstet Gynaecol India*. 2010 Jun;60(3):210–4. doi: 10.1007/s13224-010-0028-x. Epub 2010 Sep 9. PMID: PMC3394520.
2. Caggana M, Jones EA, Shahied SI, Tanksley S, Hermerath CA, Lubin IM. Newborn screening: from Guthrie to whole genome sequencing. *Public Health Rep*. 2013 Sep-Oct;128 Suppl 2(Suppl 2):14-9. doi: 10.1177/00333549131280S204. PMID: 23997299; PMCID: PMC3730001.
3. Ferreira CR, van Karnebeek CDM. Inborn errors of metabolism. *Handb Clin Neurol*. 2019;162:449-481. doi:10.1016/B978-0-444-64029-1.00022-9. PMID: 31324325.
4. Sanderson S, Green A, Preece MA, Burton H. The incidence of inherited metabolic disorders in the West Midlands, UK. *Arch Dis Child*. 2006 Nov;91(11):896-9. doi: 10.1136/adc.2005.091637. Epub 2006 May 11. PMID: 16690699; PMCID: PMC2082934.
5. Applegarth DA, Toone JR, Lowry RB. Incidence of inborn errors of metabolism in British Columbia, 1969-1996. *Pediatrics*. 2000 Jan;105(1):e10. doi: 10.1542/peds.105.1.e10. PMID: 10617747.
6. Dionisi-Vici C, Rizzo C, Burlina AB, Caruso U, Sabetta G, Uziel G, Abeni D. Inborn errors of metabolism in the Italian pediatric population: a national retrospective survey. *J Pediatr*. 2002 Mar;140(3):321-7. doi: 10.1067/mpd.2002.122394. PMID: 11953730.
7. Lanpher B, Brunetti-Pierri N, Lee B. Inborn errors of metabolism: the flux from Mendelian to complex diseases. *Nat Rev Genet*. 2006 Jun;7(6):449-60. doi: 10.1038/nrg1880. PMID: 16708072.
8. IJlsinger S, Das AM. Impact of selected inborn errors of metabolism on prenatal and neonatal development. *IUBMB Life*. 2010 Jun;62(6):403-13. doi: 10.1002/iub.336. PMID: 20503433.
9. Jeanmonod R, Asuka E, Jeanmonod D. Inborn Errors Of Metabolism. [Updated 2022 Jul 18]. In: StatPearls[Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-.
10. Giugliani R, Castillo Taucher S, Hafez S, Oliveira JB, Rico-Restrepo M, Rozenfeld P, Zarante I, Gonzaga- Jauregui C. Opportunities and challenges for newborn screening and early diagnosis of rare diseases in Latin America. *Front Genet*. 2022 Dec 8;13:1053559. doi: 10.3389/fgene.2022.1053559. PMID: 36568372; PMCID: PMC9773081.
11. Najafi R, Hashemipour M, Yaghini O, Najafi F, Rashidianfar A. Demographic and clinical characteristics of the children with aminoacidopathy in Isfahan Province, Central Iran in 2007-2015. *Indian J Endocrinol Metab*. 2016 Sep-Oct;20(5):679-683. doi: 10.4103/2230-8210.190556. PMID: 27730080; PMCID: PMC5040050.
12. van Spronsen FJ, Blau N, Harding C, Burlina A, Longo N, Bosch AM. Phenylketonuria. *Nat Rev Dis Primers*. 2021 May 20;7(1):36. doi: 10.1038/s41572-021-00267-0. PMID: 34017006; PMCID: PMC8591558.
13. Chen A, Pan Y, Chen J. Clinical, genetic, and experimental research of hyperphenylalaninemia. *Front Genet*. 2023 Jan 4;13:1051153. doi: 10.3389/fgene.2022.1051153. PMID: 36685931; PMCID: PMC9845280.
14. Sniderman King L, Trahms C, Scott CR. Tyrosinemia Type I. 2006 Jul 24 [Updated 2017 May 25]. In: AdamMP, Everman DB, Mirzaa GM, et al., editors. *GeneReviews®* [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2023.
15. Chinsky JM, Singh R, Ficicioglu C, van Karnebeek CDM, Grompe M, Mitchell G, Waisbren SE, Gucsavas-Calikoglu M, Wasserstein MP, Coakley K, Scott CR. Diagnosis and treatment of tyrosinemia type I: a US and Canadian consensus group review and recommendations. *Genet Med*. 2017 Dec;19(12). doi: 10.1038/gim.2017.101. Epub 2017 Aug 3. PMID: 28771246; PMCID: PMC5729346.
16. al-Hemidan AI, al-Hazzaa SA. Richner-Hanhart syndrome (tyrosinemia type II). Case report and literature review. *Ophthalmic Genet*. 1995 Mar;16(1):21-6. doi: 10.3109/13816819509057850. PMID: 7648039.

17. Wasim M, Awan FR, Khan HN, Tawab A, Iqbal M, Ayesha H. Aminoacidopathies: Prevalence, Etiology, Screening, and Treatment Options. *Biochem Genet.* 2018 Apr;56(1-2):7-21. doi: 10.1007/s10528-017-9825-6. Epub 2017 Nov 1. PMID: 29094226.
18. De Biase I, Gherasim C, La'ulu SL, Asamoah A, Longo N, Yuzyuk T. Laboratory evaluation of homocysteine remethylation disorders and classic homocystinuria: Long-term follow-up using a cohort of 123 patients. *Clin Chim Acta.* 2020 Oct;509:126-134. doi: 10.1016/j.cca.2020.06.014. Epub 2020 Jun 10. PMID: 32533987.
19. Quinonez SC, Lee KN. Citrullinemia Type I. 2004 Jul 7 [updated 2022 Aug 18]. In: Adam MP, Everman DB, Mirzaa GM, Pagon RA, Wallace SE, Bean LJH, Gripp KW, Amemiya A, editors. *GeneReviews®* [Internet]. Seattle (WA): University of Washington, Seattle; 1993–2023. PMID: 20301631.
20. Hayasaka K, Numakura C. Adult-onset type II citrullinemia: Current insights and therapy. *Appl Clin Genet.* 2018 Dec 12;11:163-170. doi: 10.2147/TACG.S162084. PMID: 30588060; PMCID: PMC6296197.
21. Bin Sawad A, Jackimiec J, Bechter M, Trucillo A, Lindsley K, Bhagat A, Uyei J, Diaz GA. Epidemiology, methods of diagnosis, and clinical management of patients with arginase 1 deficiency (ARG1-D): A systematic review. *Mol Genet Metab.* 2022 Sep-Oct;137(1-2):153-163. doi: 10.1016/j.ymgme.2022.08.005. Epub 2022 Aug 25. PMID: 36049366.
22. Nagamani SCS, Erez A, Lee B. Argininosuccinate Lyase Deficiency. 2011 Feb 3 [updated 2019 Mar 28]. In: Adam MP, Everman DB, Mirzaa GM, Pagon RA, Wallace SE, Bean LJH, Gripp KW, Amemiya A, editors. *GeneReviews®* [Internet]. Seattle (WA): University of Washington, Seattle; 1993–2023. PMID: 21290785.
23. Deodato F, Boenzi S, Santorelli FM, Dionisi-Vici C. Methylmalonic and propionic aciduria. *Am J Med Genet C Semin Med Genet.* 2006 May 15;142C(2):104-12. doi: 10.1002/ajmg.c.30090. PMID: 16602092.
24. Wajner M, Amaral AU, Leipnitz G, Seminotti B. Pathogenesis of brain damage in glutaric acidemia type I: Lessons from the genetic mice model. *Int J Dev Neurosci.* 2019 Nov;78:215-221. doi: 10.1016/j.jdevneu.2019.05.005. Epub 2019 May 21. PMID: 31125684.
25. Khan A, Zahid B, Khan S, Ahmad SA. Isovaleric Acidemia: A Rare Case of an Inborn Error of Metabolism. *Cureus.* 2020 Feb 29;12(2):e7150. doi: 10.7759/cureus.7150. PMID: 32257695; PMCID: PMC7108672.
26. Hao X, Wang J, Liu S, Chen Y, Zhang Y, Hao Y. 3-Hydroxy-3-methylglutaric aciduria with bilateral basal ganglia lesion: A case report. *Exp Ther Med.* 2016 Jun;11(6):2573-2576. doi: 10.3892/etm.2016.3243. Epub 2016 Apr 11. PMID: 27284350; PMCID: PMC4887778.
27. Kanavin OJ, Woldseth B, Jellum E, Tvedt B, Andresen BS, Stromme P. 2-methylbutyryl-CoA dehydrogenase deficiency associated with autism and mental retardation: a case report. *J Med Case Rep.* 2007 Sep 20;1:98. doi: 10.1186/1752-1947-1-98. PMID: 17883863; PMCID: PMC2045671.
28. Snanoudj S, Torre S, Sudrié-Arnaud B, Abily-Donval L, Goldenberg A, Salomons GS, Marret S, Bekri S, Tebani A. Heterogeneous Clinical Landscape in a Consanguineous Malonic Aciduria Family. *Int J Mol Sci.* 2021 Nov 23;22(23):12633. doi: 10.3390/ijms222312633. PMID: 34884438; PMCID: PMC8658006.
29. Maeda Y, Ito T, Ohmi H, Yokoi K, Nakajima Y, Ueta A, Kurono Y, Togari H, Sugiyama N. Determination of 3-hydroxyisovalerylcarnitine and other acylcarnitine levels using liquid chromatography-tandem mass spectrometry in serum and urine of a patient with multiple carboxylase deficiency. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2008 Jul 15;870(2):154-9. doi: 10.1016/j.jchromb.2007.11.037. Epub 2007 Dec 4. PMID: 18088573.
30. Merritt JL 2nd, Norris M, Kanungo S. Fatty acid oxidation disorders. *Ann Transl Med.* 2018 Dec;6(24):473. doi: 10.21037/atm.2018.10.57. PMID: 30740404; PMCID: PMC6331364.
31. Magoulas PL, El-Hattab AW. Systemic primary carnitine deficiency: an overview of clinical manifestations, diagnosis, and management. *Orphanet J Rare Dis.* 2012 Sep 18;7:68. doi: 10.1186/1750-1172-7-68. PMID: 22989098; PMCID: PMC3495906.

32. Bennett MJ, Santani AB. Carnitine Palmitoyltransferase 1A Deficiency. 2005 Jul 27 [Updated 2016 Mar 17]. In: Adam MP, Everman DB, Mirzaa GM, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2023.
33. Stratakis CA, Raygada M. Carney Complex. 2003 Feb 5 [Updated 2018 Aug 16]. In: Adam MP, Everman DB, Mirzaa GM, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2023.
34. Prasun P, LoPiccolo MK, Ginevic I. Long-Chain Hydroxyacyl-CoA Dehydrogenase Deficiency / Trifunctional Protein Deficiency. 2022 Sep 1. In: Adam MP, Everman DB, Mirzaa GM, et al., editors.
35. Mason E, Hindmarch CCT, Dunham-Snary KJ. Medium-chain Acyl-CoA dehydrogenase deficiency: Pathogenesis, diagnosis, and treatment. *Endocrinol Diabetes Metab.* 2023 Jan;6(1):e385. doi: 10.1002/edm2.385. Epub 2022 Oct 27. PMID: 36300606; PMCID: PMC9836253.
36. Prasun P. Multiple Acyl-CoA Dehydrogenase Deficiency. 2020 Jun 18. In: Adam MP, Everman DB, Mirzaa GM, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2023.
37. Berry GT. Classic Galactosemia and Clinical Variant Galactosemia. 2000 Feb 4 [Updated 2021 Mar 11]. In: Adam MP, Everman DB, Mirzaa GM, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2023.
38. Wolf B. Biotinidase deficiency: "if you have to have an inherited metabolic disease, this is the one to have". *Genet Med.* 2012 Jun;14(6):565-75. doi: 10.1038/gim.2011.6. Epub 2012 Jan 5. PMID: 22241090.
39. Nagasaki K, Minamitani K, Nakamura A, Kobayashi H, Numakura C, Itoh M, Mushimoto Y, Fujikura K, Fukushima M, Tajima T. Guidelines for Newborn Screening of Congenital Hypothyroidism (2021 Revision). *Clin Pediatr Endocrinol.* 2023;32(1):26-51. doi: 10.1297/cpe.2022-0063. Epub 2022 Dec 4. PMID: 36761493; PMCID: PMC9887297.
40. Ramasli Gursoy T, Asfuroglu P, Sismanlar Eyuboglu T, Aslan AT, Yilmaz AI, Unal G, Kibar BS, Pekcan S, Hangul M, Kose M, Budakoglu II, Acican D. Evaluation of specificity and sensitivity of IRT/IRT protocol in the cystic fibrosis newborn screening program: 6-year experience of three tertiary centers. *Eur J Pediatr.* 2022 Dec 24. doi: 10.1007/s00431-022-04766-4. Epub ahead of print. PMID: 36565324.
41. Kononets V, Zharmakhanova G, Balmagambetova S, Syrlybayeva L, Berdesheva G, Zhussupova Z, Tautanova A, Kurmambayev Y. Tandem mass spectrometry in screening for inborn errors of metabolism: comprehensive bibliometric analysis. *Front Pediatr.* 2025 Feb 20;13:1463294. doi: 10.3389/fped.2025.1463294. PMID: 40051910; PMCID: PMC11882580.
42. Banerjee S, Mazumdar S. Electrospray ionization mass spectrometry: a technique to access the information beyond the molecular weight of the analyte. *Int J Anal Chem.* 2012;2012:282574. doi: 10.1155/2012/282574. Epub 2011 Dec 15. PMID: 22611397; PMCID: PMC3348530.
43. Thomas, Stefani N. (2019). Contemporary Practice in Clinical Chemistry || Mass spectrometry. , (), 171– 185. doi:10.1016/B978-0-12-815499-1.00010-7
44. He F, Yang R, Huang X, Tian Y, Pei X, Bohn MK, Zou L, Wang Y, Li H, Wang T, Gu M, Jiang T, Chen X, Zou H, Wei H, Tian W, Tang T, Adeli K, Wang Z. Reference Standards for Newborn Screening of Metabolic Disorders by Tandem Mass Spectrometry: A Nationwide Study on Millions of Chinese Neonatal Populations. *Front Mol Biosci.* 2021 Dec 16;8:719866. doi: 10.3389/fmolb.2021.719866. PMID: 34977148; PMCID: PMC8716770.
45. Sweetman L. Newborn screening by tandem mass spectrometry: gaining experience. *Clin Chem.* 2001 Nov;47(11):1937-8. PMID: 11673359.
46. Younesi S, Yazdani B, Taheri Amin MM, Saadati P, Jamali S, Modarresi MH, Savad S, Amidi S, Razavi H, Ghafouri- Fard S. Incorporation of second-tier tests and secondary biomarkers to improve positive predictive value (PPV) rate in newborn metabolic screening program. *J Clin Lab Anal.* 2022 Jul;36(7):e24471. doi: 10.1002/jcla.24471. Epub 2022 May 2. PMID: 35500172; PMCID: PMC9279966.

47. Malvagia S, Forni G, Ombrone D, la Marca G. Development of Strategies to Decrease False Positive Results in Newborn Screening. *Int J Neonatal Screen*. 2020 Nov 2;6(4):84. doi: 10.3390/ijns6040084. PMID: 33147868; PMCID: PMC7712114.

48. Rossi C, Cicalini I, Verrocchio S, Di Dalmazi G, Federici L, Bucci I. The Potential of Steroid Profiling by Mass Spectrometry in the Management of Adrenocortical Carcinoma. *Biomedicines*. 2020 Aug 28;8(9):314. doi: 10.3390/biomedicines8090314. PMID: 32872281; PMCID: PMC7555975.

Chapter 2

Congenital Adrenal Hyperplasia and Newborn Screening Strategies

Congenital adrenal hyperplasia (CAH) refers to a group of inherited autosomal recessive disorders of adrenal steroid biosynthesis, most frequently caused by 21-hydroxylase deficiency, which accounts for approximately 95% of cases. The enzymatic defect leads to impaired cortisol synthesis and, in more severe forms, reduced aldosterone production, resulting in chronic adrenocorticotrophic hormone stimulation and adrenal hyperplasia. Consequently, steroid precursors such as 17-hydroxyprogesterone (17-OHP) and adrenal androgens accumulate, while glucocorticoid and mineralocorticoid levels are decreased. These hormonal imbalances underlie a broad clinical spectrum, ranging from severe salt-wasting forms to milder simple virilizing and non-classical variants. The worldwide incidence of CAH is estimated at approximately 1 in 15,000 live births, with notable population-dependent variability, justifying its inclusion in neonatal screening programs. NBS in Abruzzo for CAH is primarily based on the measurement of 17-OHP in dried blood spots using immunoassay-based methods as a first-tier test. However, the limited specificity of immunoassays and the influence of physiological factors such as birth weight and gestational age contribute to elevated false-positive rates. To mitigate these limitations, gestational age and weight are adjusted cut-off values are applied and positive screening results are subsequently subjected to 2-TTs. 2-TT was developed and validated in our laboratory to address this need. More precisely, using an LC-MS/MS method we are able to quantify multiple adrenal steroids, including 17-OHP, 4-androstene-3,17-dione (A4), cortisol (CORT), 11-deoxycortisol (11-DC) and 21-deoxycortisol (21-DC), and to also evaluate (17-OHP + 4-A)/cortisol ratio. In addition to dried blood spot analysis, plasma steroid profiling represents a complementary diagnostic approach, allowing for accurate and simultaneous quantification of multiple steroids by LC-MS/MS in a single analytical run. In fact this method provides a more detailed biochemical characterization of adrenal steroidogenesis and further supports the differential diagnosis of CAH variants. Definitive diagnosis is achieved through molecular genetic analysis of the *CYP21A2* gene. These approaches offer a comprehensive framework for the early detection and precise characterization of CAH ^[1].

2.1 Adrenal Steroidogenesis

Steroid hormones are a diverse family of biologically active molecules, largely produced by the adrenal cortex, but also by the gonads, placenta and, to a lesser extent, other tissues such as the brain. These hormones are derived from cholesterol, sharing the characteristic cyclopentane-perhydrophenanthrene nucleus, and are categorized according to their major physiological roles into glucocorticoids (GC), mineralocorticoids (MC) and sex steroids, with androgenic, estrogenic, or progestogenic activity ^[2]. In this context, the adrenal gland plays a central role for the steroid hormone production. The adrenal gland is composed of two distinct tissues occupying the same anatomical region. The adrenal cortex is a steroidogenic endocrine gland of mesodermal origin, closely associated embryologically and anatomically with the kidneys. In contrast, the adrenal medulla derives from the neural crest and represents a functional extension of the sympathetic nervous system ^[3].

The adrenal cortex is structurally and functionally divided into three layers, each specialized for the production of distinct steroid classes: zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR). ZG, the outermost layer, is primarily responsible for synthesizing mineralocorticoids, particularly aldosterone (ALDO), which regulates sodium balance, fluid homeostasis, and blood pressure. Aldosterone synthesis in ZG relies on aldosterone synthase (CYP11B2 or P450c11AS), while pregnenolone is converted into progesterone by 3 β -hydroxysteroid dehydrogenase type 2 (HSD3B2). The absence of 17 α -hydroxylase/17,20-lyase (CYP17A1 or P450c17) in ZG prevents the production of glucocorticoids and androgens. ZF, the thick intermediate layer, predominantly produces glucocorticoids, with cortisol (CORT) as the final product. Cortisol regulates metabolism, inflammation, and cardiovascular function, and also provides negative feedback on adrenocortical trophic hormone (ACTH) secretion. In ZF, pregnenolone undergoes 17-hydroxylation by CYP17A1 (P450c17), and subsequent enzymatic reactions via HSD3B2, 21-hydroxylase (CYP21A2 or P450c21), and 11 β -hydroxylase (CYP11B1 or P450c11) result in cortisol formation. A minor "mineralocorticoid-like" pathway in ZF produces 11-deoxycorticosterone (DOC) and corticosterone (B) under certain conditions. ZR, the innermost cortical layer adjacent to the adrenal medulla, synthesizes adrenal androgens, including dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), androstenedione (A4), and 11-oxygenated androgens such as 11 β -hydroxyandrostenedione (11OHA4) and 11-ketotestosterone (11KT). CYP17A1, together with the cofactor cytochrome b5 (CYB5A), catalyzes the conversion of pregnenolone to DHEA and 17-hydroxyprogesterone (17OHP) to androstenedione. DHEA can also be sulfated by sulfotransferase type 2A1 (SULT2A1), using 3'-phosphoadenosine-5'-phosphosulfate (PAPSS2) as a cofactor, controlling the pool of active androgens.

Additionally, testosterone (T) is synthesized in the ZR via aldo-ketoreductase type 1C3 (AKR1C3 or 17 β -hydroxysteroid dehydrogenase type 5, HSD17B5), and peripherally converted to dihydrotestosterone (DHT) by 5 α -reductase type 2 (SRD5A2). A recently described “backdoor pathway” allows DHT synthesis directly from androsterone, bypassing testosterone (**Figure 4**) [2].

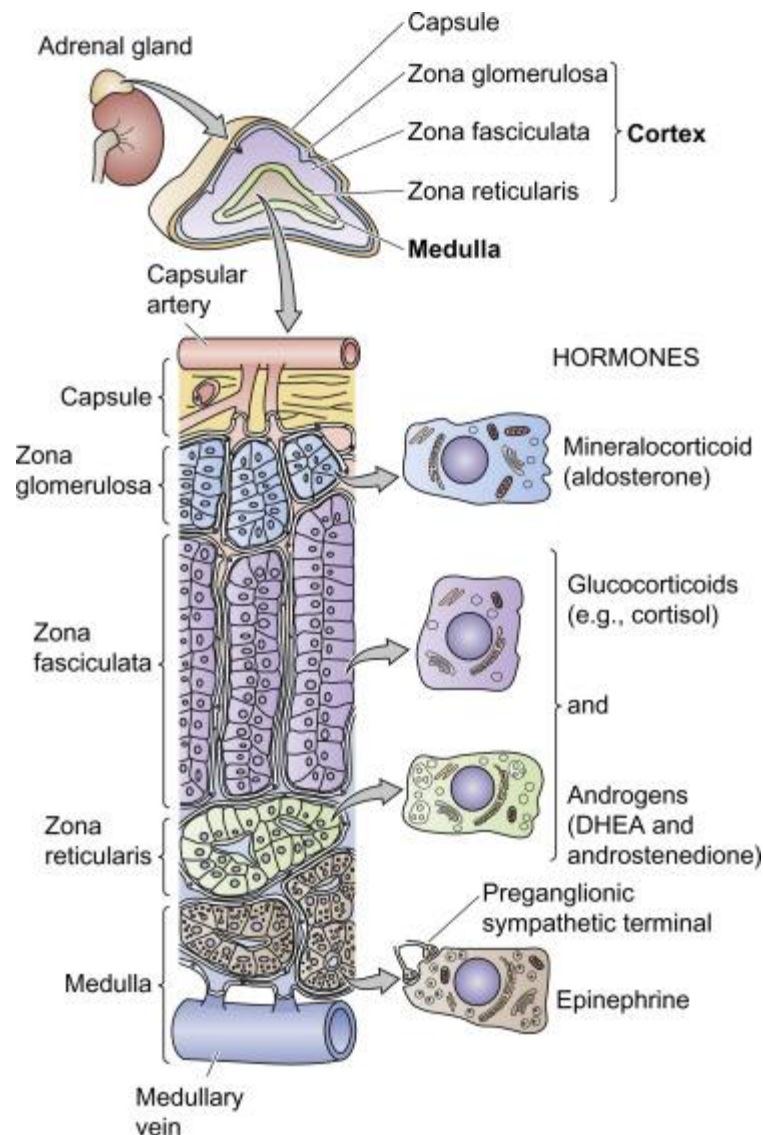


Figure 4. Structural and functional organization of the adrenal gland. The adrenal cortex comprises three concentric layers: ZG producing mineralocorticoids, ZF producing glucocorticoids and the ZR producing adrenal androgens. The medulla derived from neural crest cells. *Reproduced from Carlson et al., 2019.*

Steroidogenesis is tightly regulated both externally and internally. ACTH, produced by the anterior pituitary, mainly stimulates ZF and ZR, while angiotensin II (Ang II) and electrolyte levels selectively activate ZG. Internally, the expression and catalytic activity of specific enzymes ensure substrate flow toward the appropriate steroid product. The first step in all pathways involves cholesterol transport into mitochondria via steroidogenic acute

2.2 Congenital Adrenal Hyperplasia: Pathophysiology and Clinical Variants

CAH comprises a group of autosomal recessive disorders caused by defects in adrenal steroidogenesis that impair cortisol biosynthesis. The resulting loss of negative feedback on the hypothalamic–pituitary–adrenal (HPA) axis leads to chronic elevation of adrenocorticotrophic hormone (ACTH), adrenal hyperplasia, and accumulation of steroid precursors. The clinical manifestations of CAH are highly variable and depend on the specific enzymatic defect and the degree of residual enzyme activity. The historical origins of CAH can be traced to 1865, when the Neapolitan anatomist Luigi de Crecchio described the post-mortem findings of an individual with ambiguous genitalia and markedly enlarged adrenal glands. The autopsy revealed labioscrotal fusion, a curved phallus with hypospadias, bilateral undescended testes, and the simultaneous presence of male- and female-typical internal reproductive structures, including a uterus, fallopian tubes, ovaries, and vagina. Although the biochemical basis of the condition was unknown at the time, the total findings is now recognized as consistent with severe 21-hydroxylase deficiency. The individual reportedly lived as a male and likely died in adulthood from adrenal insufficiency during an acute gastrointestinal illness. More than a century later, in 1957, Decourt, Jayle, and Baulieu identified the non-classic, or mild, form of 21-hydroxylase deficiency, establishing that CAH encompasses a broad phenotypic spectrum rather than a single uniform disorder ^[4].

Among the various forms of CAH, 21-hydroxylase deficiency (21-OHD), caused by mutations in the *CYP21A2* gene, accounts for the vast majority of cases. Less frequent virilizing forms include deficiencies of 11 β -hydroxylase (*CYP11B1*) and 3 β -hydroxysteroid dehydrogenase type 2 (*HSD3B2*). Mutations in the *POR* gene, encoding cytochrome P450 oxidoreductase, disrupt electron transfer to multiple steroidogenic enzymes and result in complex phenotypes that may include virilization of affected females and undervirilization of affected males. Clinically, 21-OHD is traditionally classified into classic and non-classic forms. The classic forms include salt-wasting and simple virilizing variants, whereas non-classic CAH (NCAH) presents later in life with milder manifestations of androgen excess. However, disease severity is better described by the amount of remaining enzyme activity rather than by distinct categories. The incidence of classic CAH ranges from approximately 1:5,000 to 1:15,000 live births and varies across ethnic groups, while NCAH is considerably more prevalent and often underdiagnosed. At the molecular level, the *CYP21A2* gene is located within the highly complex *RCCX* locus on chromosome 6p21.3, adjacent to a homologous pseudogene (*CYP21A1P*). This genomic organization predisposes the locus to gene conversion events and unequal crossover during meiosis, which account for most pathogenic variants. Although over 200 *CYP21A2* mutations have been identified, a limited

increased concentrations of progesterone and 17-OHP are diverted toward androgen biosynthesis, resulting in excess production of adrenal androgens. In severe cases, aldosterone deficiency leads to hyponatremia, hyperkalemia, hypovolemia, and potentially fatal adrenal crises. In addition to the classical steroidogenic pathway, increased flux through an alternative or “backdoor” pathway contributes to androgen excess, particularly during fetal life, and plays a significant role in prenatal virilization of affected females. Clinical manifestations vary across the lifespan. In the neonatal period, classic CAH typically presents with ambiguous genitalia in affected females due to prenatal androgen exposure, whereas affected males often appear phenotypically normal at birth but are at high risk for salt-wasting crises within the first weeks of life. During childhood, patients with simple virilizing CAH or NCAH may present with premature pubarche, accelerated growth velocity, and advanced bone age. In adolescence and adulthood, NCAH frequently manifests with hirsutism, acne, menstrual irregularities, chronic anovulation, and infertility, with substantial overlap with the clinical features of polycystic ovary syndrome ^[4]. A summary of major forms of CAH is elucidated in **Table 5**.

CAH Form	Enzyme Deficiency	Gene	Hormonal Defect	Main Clinical Features	Age of Onset
21-hydroxylase deficiency (classic, salt-wasting)	21-hydroxylase	<i>CYP21A2</i>	↓ Cortisol, ↓ Aldosterone, ↑ 17-OHP, ↑ Androgens	Neonatal adrenal crisis, hyponatremia, hyperkalemia, ambiguous genitalia in 46,XX	Neonatal period
21-hydroxylase deficiency (classic, simple virilizing)	21-hydroxylase	<i>CYP21A2</i>	↓ Cortisol, normal/ ↓ Aldosterone, ↑ Androgens	Prenatal virilization in females, precocious puberty, rapid growth	Infancy / early childhood
21-hydroxylase deficiency (non-classic)	Partial 21-hydroxylase	<i>CYP21A2</i>	Mild ↓ Cortisol, ↑ Androgens	Hirsutism, acne, menstrual irregularities, infertility	Childhood to adulthood
11β-hydroxylase deficiency	11β-hydroxylase	<i>CYP11B1</i>	↓ Cortisol, ↑ DOC, ↑ Androgens	Virilization, hypertension	Infancy / childhood
3β-hydroxysteroid dehydrogenase deficiency	3β-HSD type 2	<i>HSD3B2</i>	↓ Cortisol, ↓ Aldosterone, ↓ Sex steroids	Ambiguous genitalia in both sexes, salt-wasting	Neonatal period
P450 oxidoreductase deficiency (PORD)	Electron transfer to CYP enzymes	<i>POR</i>	Combined steroidogenic defects	Ambiguous genitalia, skeletal anomalies, maternal virilization	Prenatal / neonatal

Table 5. Summary of major forms of CAH.

Early detection of classic CAH has been substantially improved by the implementation NBS programs, which were introduced in the late 1970s and are now widely adopted. Screening is based on measurement of 17-OHP concentrations in DBS collected shortly after birth, allowing early identification of infants at risk for adrenal crises and incorrect sex assignment. NBS has markedly reduced neonatal morbidity and mortality associated with CAH, although false-positive results remain common in premature or stressed neonates, and false-negative results can occur. Adjusted cut-off values, 2-TT steroid profiling, and repeat screening during the second week of life have improved diagnostic accuracy. Notably, NBS is not designed to reliably detect non-classic CAH [4]. Management of CAH focuses on replacing deficient hormones and suppressing excessive ACTH-driven androgen production. Glucocorticoid replacement therapy is the cornerstone of treatment, with mineralocorticoid replacement required in salt-wasting forms. In NCAH, treatment should be individualized and guided by clinical symptoms rather than biochemical abnormalities alone. Therapeutic goals vary by age and include prevention of adrenal crises, normalization of growth and pubertal development, preservation of fertility, and control of hyperandrogenic features. Despite established treatment strategies, optimal management remains challenging due to interindividual variability in glucocorticoid requirements, underscoring the need for continued refinement of therapeutic approaches [4].

2.3 First-tier CAH Screening

Neonatal Screening Center of the Abruzzo Region, located at the Center for Advanced Studies and Technology (CAST), "G. d'Annunzio" University provides the initial screening for CAH measuring 17-OHP levels in DBS using a time-resolved immunofluorescence assay. DBS samples for newborn NBS were obtained 48–72 hours after birth by heel-prick and applied into Ahlstrom 226 filter paper (Revvity, Turku, Finland, OY). First-tier test is performed with the GSP Neonatal 17-OHP kit (Revvity, Turku, Finland, OY) on the GSP Instrument (IVD) (Revvity, Turku, Finland, OY). The method is based on a competitive binding immunoassay: europium-labeled 17-OHP competes with the 17-OHP present in the sample for binding sites on rabbit-derived polyclonal anti-17-OHP antibodies. A secondary antibody specific for rabbit IgG is immobilized on a solid phase to facilitate separation of bound complexes from free antigen. The DELFIA inducer subsequently releases europium ions from the labeled antigen, generating highly fluorescent chelates. The fluorescence

intensity measured in each well is inversely related to the 17-OHP concentration in the sample ^[6] (**Figure 7**).

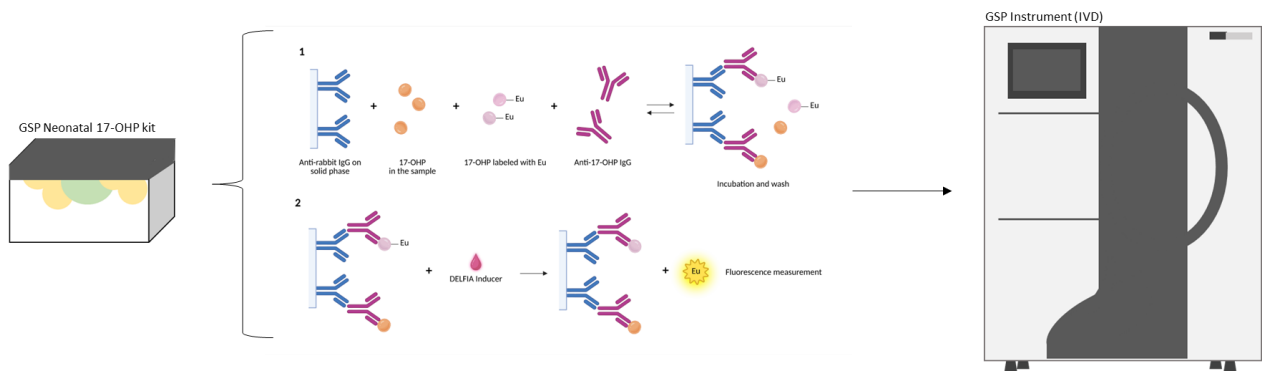


Figure 7. First-tier test for measuring 17-OHP levels on DBS by DELFIA. *Reproduced from Tommolini et al, 2025.*

To improve screening accuracy, we established gestational age-specific cut-off values for 17-OHP: 65 ng/mL for preterm infants (<34 weeks), 35 ng/mL for infants between 34 and 36 weeks, and 11 ng/mL for full-term newborns (>37 weeks). These tailored thresholds help reduce false positives, particularly in preterm or stressed neonates ^[6] (**Table 6**).

Gestational Weeks (GW)	17-OHP Cut-off
< 34 weeks	65 ng/mL
34–36 weeks	35 ng/mL
≥ 37 weeks	11 ng/mL

Table 6. Gestational age-specific 17-OHP cut-off values used for first-tier CAH screening.

2.4 Second-tier CAH Screening: Development and Validation

CAH screening based on first-tier immunoassay measurement of 17-OHP (17-OHP) is characterized by limited specificity, leading to a high rate of false-positive results. This limitation is mainly attributable to antibody cross-reactivity with structurally related steroid precursors, which are frequently present at elevated concentrations in the early neonatal period, particularly in preterm infants. Moreover, physiological conditions such as neonatal stress, illness, and the immaturity of adrenal enzyme activity can further contribute to increased circulating levels of 17-OHP, complicating the interpretation of immunoassay results. To overcome these analytical and biological interferences, a 2-TT performed by an LC-MS/MS approach enables the simultaneous and specific quantification of multiple

adrenal steroids from the same DBS specimen initially identified as screen-positive. Unlike immunoassays, LC-MS/MS is not affected by antibody cross-reactivity, ensuring higher analytical specificity. The implementation of a multi-analyte 2-TT LC-MS/MS, combined with the use of the steroid ratio, has been shown to significantly reduce false positive results compared to measuring 17-OHP alone. This strategy improves the overall accuracy of CAH newborn screening by enhancing both specificity and sensitivity, while simultaneously decreasing the need for repeat sample collection and clinical follow-up, thereby reducing the associated healthcare burden.

To overcome these limitations, a 2-TT based on LC-MS/MS was development and validated in our laboratory. The 2-TT consists of the simultaneous quantification of 4-androstene-3,17-dione (A4), cortisol (CORT), 17-OHP, 11-deoxycortisol (11-DC), 21-deoxycortisol (21-DC) are measured by LC-MS/MS (**Figure 8**), also establishing (17-OHP + 4-A)/cortisol ratio. **Table 7** summarizes the cut-off values applied for 2-TT LC-MS/MS steroid profiling in term newborns within the NBS program.

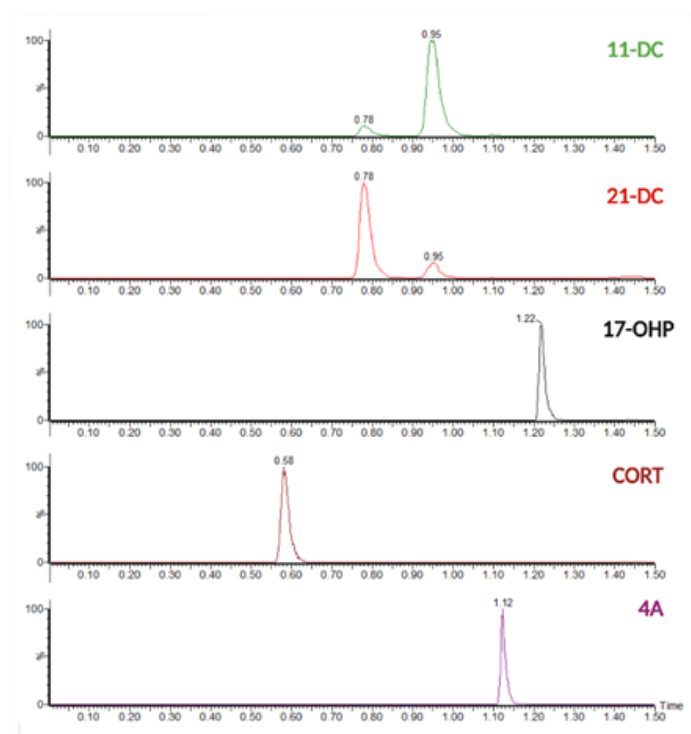


Figure 8. Chromatographic separation of CORT, 21-DC, 11-DC, A4, 17-OHP. *Reproduced from Tommolini et al., 2025.*

Analyte / Ratio	Cut-off
17-OHP	≥ 3.6 ng/mL
(17-OHP + 4A) / CORT	≥ 1
17-OHP	< 3.6 ng/mL
(17-OHP + 4A) / CORT	≥ 4
11-DC	≥ 7.65
21-DC	≥ 1.8 ng/mL
A4	-
CORT	-

Table 7. Cut-off values applied for 2-TT LC-MS/MS steroid profiling in the NBS workflow.

DBS sample preparation for 17-OHP, CORT, 21-DC, 11-DC and 4-A

For 2-TT sample preparation a working solution containing 2.5 ng ISs, with A4 D7, CORT D3, 17-OHP D8, 11-DC D2, and 21-DC D8 purchased from Spectra 2000 S.r.l., is made by diluting the IS stock solutions with 95:5 (v/v) methanol/water. Calibrators and Quality Controls (QCs) are purchased from LabSystem Diagnostics (Helsinki, Finland). 250 μ L of working solution are added to two 3.2 mm diameter punches from DBS samples, calibrators, and QCs. After been incubated at T°C room for 30 minutes at 700 rpm, supernatants are transferred in other tubes and evaporated till dryness. The residues are reconstituted with 100 μ L of 60:40 (v/v) water 0.05 mM ammonium fluoride (NH_4) / methanol and finally transferred into vials for LC-MS/MS analysis. The sample volume injected into the ion source is 20 μ L (**Figure 9**).

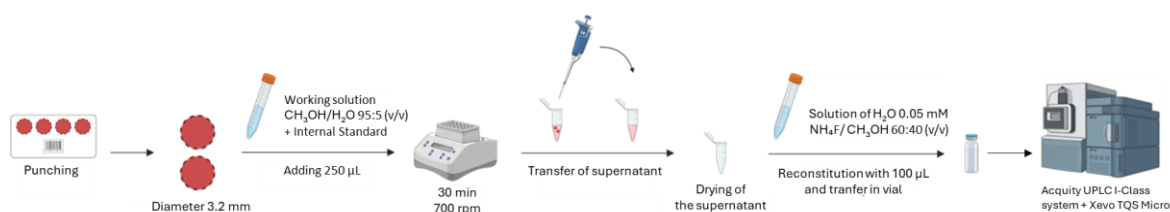


Figure 9. Sample preparation for CAH 2-TT. *Reproduced from Tommolini et al., 2025.*

LC-MS/MS conditions

The LC-MS/MS system consists of an ACQUITY™ UPLC™ I-Class system comprised of a Binary Solvent Manager (BSM) and a Sample Manager with Flow-Through Needle (SM-FTN) coupled to a Xevo® TQ-S micro mass spectrometer (Waters Corporation, Milford, MA, USA). The system operates in positive electrospray ionization (ESI+). The run time is 2.50 minutes, injection-to-injection. For chromatographic separation is employed a CORTECS C18 2.7µM, 50mm x 2.1mm (Waters co.), using a CORTECS C18 2.7µM VanGuard pre-column; column thermostated at 50 °C and the autosampler at 10 °C. Mobile phase (solvents purchased from Carlo Erba Reagents) is composed of water (A) 0.05 mM ammonium fluoride (NH₄) and methanol (B). Flow rate is 1.0 mL/min. LC gradient is elucidated in **Table 8**. Parameters referring to MRM functions for the detection of CORT, 21-DC, 11-DC, A4, 17-OHP are reported in **Table 9**. Data are processed using TargetLynx™ XS software (Waters Corporation, Milford, MA, USA).

N.	Time (min)	Flow Rate (mL/min)	%A	%B	Curve
1	Initial	1.000	60.0	40.0	Initial
2	0.10	1.000	60.0	40.0	6
3	0.25	1.000	55.0	45.0	6
4	0.75	1.000	55.0	45.0	6
5	0.90	1.000	37.5	62.5	6
6	1.15	1.000	5.0	95.0	11
7	1.40	1.000	60	40	11

Table 8. Timetable of gradient elution for detection of CORT, 21-DC, 11-DC, A4, 17-OHP.

MRM Function	Time Window (min)	Analyte	Dwell time (s)	Transitions (m/z)	Cone (V)	Collision Energy (CE)
1	0-2.50	A4 (Quant.)	0.010	287.30 > 97.10	50.0	20.0
2	0-2.50	A4 (Qual.)	0.010	287.30 > 109.10	50.0	20.0
3	0-2.50	A4 D7 IS	0.010	294.30 > 100.00	50.0	20.0
4	0-2.50	17-OHP (Quant.)	0.010	331.30 > 97.10	50.0	20.0
5	0-2.50	17 -OHP (Qual.)	0.010	331.30 > 109.10	50.0	20.0
6	0-2.50	17-OHP D8 IS	0.010	339.30 > 100.10	50.0	20.0
7	0-2.50	11-DC (Quant.)	0.010	347.30 > 97.10	50.0	22.0
8	0-2.50	11-DC (Qual.)	0.010	347.30 > 109.10	50.0	22.0
9	0-2.50	11-DC D2 IS	0.010	349.30 > 97.00	50.0	22.0
10	0-2.50	21-DC (Quant.)	0.010	347.30 > 311.10	50.0	14.0
11	0-2.50	21-DC (Qual.)	0.010	347.30 > 121.20	50.0	22.0
12	0-2.50	21-DC D8 IS	0.010	355.30 > 319.30	50.0	14.0
13	0-2.50	CORT (Quant.)	0.010	363.30 > 121.10	50.0	22.0
14	0-2.50	CORT (Qual.)	0.010	363.30 > 91.10	50.0	50.0
15	0-2.50	CORT D3 IS	0.010	366.30 > 121.00	50.0	22.0

Table 9. Multiple Reaction Monitoring (MRM) functions and settings for detection of CORT, 21-DC, 11-DC, A4, 17-OHP.

Validation of the LC-MS/MS method

The validation of the 2-TT LC-MS/MS – based method was performed by evaluating linearity, precision, and accuracy according to standard analytical criteria. Linearity was assessed by analyzing batches over three non-consecutive days; for all the analytes investigated (4A, 17-OHP, 11-DC, 21-DC, and CORT), the coefficients of determination (R^2) were consistently greater than 0.99, indicating excellent correlation between nominal concentrations and instrumental response. Precision and accuracy were evaluated intra-day and inter-day. The obtained %CV and %RE values were within acceptable limits, demonstrating good method reproducibility and adequate accuracy across the analyzed concentration range (**Tables 10 and 11**).

4A	N.C.	1,32	2,77	5,32	10,7	76,65	154,48	298,58
	Average	1,34	2,72	5,46	10,51	74,92	151,18	308,93
	SD	0,021	0,064	0,26	0,39	3,48	8,96	28,11
	%CV	1,57	2,35	4,76	3,71	4,64	5,93	9,1
	%RE	1,51	-1,81	2,63	-1,78	-2,26	-2,14	3,47
	R2	0.99						
17-OHP	N.C.	1,3	2,62	5,1	10,09	73,02	148,93	283,66
	Average	1,29	2,52	5,2	10,21	72,84	143,68	298,46
	SD	0,075	0,11	0,33	0,76	3,25	8,21	27,34
	%CV	5,81	4,37	6,35	7,44	4,46	5,71	9,16
	%RE	-0,77	-3,82	1,96	1,19	-0,25	-3,53	5,22
	R2	0.99						
11-DC	N.C.	1,46	3,1	6,04	12,22	86,01	172,99	340,23
	Average	1,46	3,05	6,23	12,41	86,14	168,04	340,89
	SD	0,021	0,032	0,39	0,48	3,15	8,54	32,17
	%CV	1,44	1,05	6,26	3,87	3,66	5,08	9,44
	%RE	0	-1,61	3,15	1,55	0,15	-2,86	0,19
	R2	0.99						
21-DC	N.C.	1,27	2,64	5,14	10,27	72,39	148,39	285,93
	Average	1,31	2,47	5,10	10,44	73,17	143,99	298,62
	SD	0,062	0,25	0,27	0,43	2,34	5,74	30,33
	%CV	4,73	10,12	5,29	4,12	3,2	3,99	10,16
	%RE	3,15	-6,44	-0,78	1,66	1,08	-2,97	4,44
	R2	0.99						
CORT	N.C.	1,66	3,65	7	14,1	98,95	201,04	383,84
	Average	1,68	3,46	7,28	14,10	98,52	193,26	399,16
	SD	0,07	0,25	0,55	0,67	3,84	5,15	39,77
	%CV	4,17	7,23	7,55	4,75	3,9	2,66	9,96
	%RE	1,2	-5,21	4	0	-0,43	-3,87	3,99
	R2	0.99						

Table 10. Linearity values of the LC-MS/MS method after analyzing batches over 3 non-consecutive days. R2 values were found to be always greater than 0.99 for 4A, 17-OHP, 11-DC, 21-DC, CORT. N.C.: nominal concentration; SD: standard deviation; %CV: coefficient of variation; %RE: relative error

	N.C.	QC Low 4,15 ng/mL				QC Mid 14,03 ng/mL				QC High 146,11 ng/mL			
4-A		intraday			Inter day	intraday			Inter day	intraday			Inter day
	A	4,17	4,54	3,8	4,17	14,09	15,05	13,44	14,19	138,77	146,64	136,10	140,50
	SD	0,05	0,39	0,30	0,37	1,58	1,79	0,50	0,81	2,54	3,23	3,46	5,48
	%CV	1,2	8,59	7,89	8,87	11,21	11,89	3,72	5,71	1,83	2,2	2,54	3,9
	%RE	0,48	9,4	-8,43	0,48	0,43	7,27	-4,21	1,14	-5,02	0,36	-	-
												6,85	3,84
	N.C.	QC Low 3,98 ng/mL				QC Mid 13,51 ng/mL				QC High 142,52 ng/mL			
17-OHP		intraday			Inter day	intraday			Inter day	intraday			Inter day
	A	4,26	4,67	3,37	4,10	13,78	14,83	13,10	13,90	137,28	145,85	135,39	139,50
	SD	0,062	0,44	0,45	0,66	1,74	2,03	0,41	0,87	0,23	3,94	4,32	5,58
	%CV	1,46	9,42	13,35	16,1	12,63	13,69	3,13	6,26	0,17	2,7	3,19	4
	%RE	7,04	17,34	-15,33	3,03	2	9,77	-3,03	2,89	-3,68	2,34	-5	-
												5,12	2,12
	N.C.	QC Low 4,76 ng/mL				QC Mid 16,17 ng/mL				QC High 162,55 ng/mL			
11-DC		intraday			Inter day	intraday			Inter day	intraday			Inter day
	A	4,74	5,42	4,38	4,85	16,26	17,64	15,98	16,63	158,37	165,93	154,22	159,51
	SD	0,13	0,51	0,29	0,53	2,11	3,08	0,93	0,89	2,49	2,92	5,21	5,94
	%CV	2,74	9,41	6,62	10,93	12,98	17,46	5,82	5,35	1,57	1,76	3,38	3,72
	%RE	-0,42	13,87	-7,98	1,89	0,57	9,09	-1,18	2,84	-2,57	2,08	-	-
												5,12	1,87
	N.C.	QC Low 4,00 ng/mL				QC Mid 13,5 ng/mL				QC High 140,28 ng/mL			
21-DC		intraday			Inter day	intraday			Inter day	intraday			Inter day
	A	4,31	4,22	3,82	4,12	13,93	14,74	12,93	13,87	132,30	147,49	126,5	135,43
	SD	0,08	0,43	0,38	0,26	1,70	2,50	0,86	0,91	1,32	4,27	4,17	10,84
	%CV	1,86	10,19	9,95	6,31	12,2	16,9	6,65	6,56	0,998	2,9	3,3	8
	%RE	7,75	5,5	4,5	3	3,19	9,19	-4,22	2,74	-5,69	5,14	-	3,46
												9,82	
	N.C.	QC Low 4,29 ng/mL				QC Mid 14,42 ng/mL				QC High 147,56 ng/mL			
CORT		intraday			Inter day	intraday			Inter day	intraday			Inter day
	A	4,47	4,94	3,98	4,46	14,65	15,73	14,08	14,82	143,48	148,81	134,70	142,33
	SD	0,07	0,61	0,44	0,48	1,89	2,36	0,65	0,84	1,48	1,27	5,33	7,12
	%CV	1,57	12,35	11,06	10,76	12,9	15	4,62	5,67	1,03	0,85	3,96	5
	%RE	4,2	15,2	-7,23	3,99	1,6	9,08	-2,36	2,77	-2,76	0,85	-	-
												8,72	3,54

Table 11. Intra-day and inter-day precision and accuracy evaluation for 4-A, 17-OHP, 11-DC, 21-DC, and CORT at three quality control levels (QC Low, QC Mid, and QC High). N.C.: nominal concentration; A: Average; SD: standard deviation; %CV: coefficient of variation; %RE: relative error.

2.5 Plasma Steroid Determination by LC-MS/MS

NBS CAH is widely implemented and effective in preventing salt-wasting adrenal crises. First-tier screening in DBS measures 17-OHP by immunoassay test, following the positivity, a 2-TT quantifies additional steroids and steroids ratio to improve specificity. Definitive diagnosis can be established through mutation analysis and also assessing characteristic steroid profiles in plasma, which offer the advantage of providing a rapid, comprehensive and accurate evaluation of adrenal steroidogenesis, with or without an adrenal stimulation test ^[1].

Plasma sample preparation for steroid profile

The plasma steroid profile, in our Laboratory, is determined by UPLC-MS/MS quantifying CORT, 11-DC, 4-A, 17-OHP, testosterone, progesterone, corticosterone and aldosterone using the CHS™ MSMS Steroids Kit, Revvity®, Turku, Finland. 100 µL of the 7 calibrators, 3 quality controls and sample are dispensed into polypropylene tubes, to which 400 µL of acetonitrile 0.1% formic acid solution containing the internal standards mix are added. Samples are vortexed and centrifuged, then supernatants are transferred into other tubes and evaporated until dryness. The residues are reconstituted with 100 µL of 65:35 (v/v) water 0.025% formic acid / methanol 0.025% formic acid and finally transferred into vials for LC-MS/MS analysis. The sample volume injected into the ion source is 20 µL.

LC-MS/MS conditions

The LC-MS/MS system consists of an ACQUITY™ UPLC™ I-Class system comprised of a Binary Solvent Manager (BSM) and a Sample Manager with Flow-Through Needle (SM-FTN) coupled to a Xevo® TQ-S micro mass spectrometer (Waters Corporation, Milford, MA, USA). The system operates in positive electrospray ionization (ESI+). The run time is 12 minutes, injection-to-injection. For chromatographic separation is employed a ACQUITY UPLC HSS T3 1.8 µM, 50mm x 2.1mm (Waters co.) column thermostated at 60 °C. Mobile phase (solvents purchased from Carlo Erba Reagents) is composed of water 0.025% formic acid (A) and methanol 0.025% formic acid (B). Flow rate is 0.25 mL/min. LC gradient is elucidated in **Table 12**. Parameters referring to MRM functions for the determination of plasma steroid profile is reported in **Table 13**. Data are processed using TargetLynx™ XS software (Waters Corporation, Milford, MA, USA) ^[7].

N.	Time (min)	Flow Rate (mL/min)	%A	%B	Curve
1	Initial	0.250	65.0	35.0	Initial
2	1.00	0.250	65.0	35.0	6
3	8.00	0.250	35.0	65.0	6
4	8.10	0.250	00.0	100.0	1
5	9.00	0.250	00.0	100.0	6
6	9.10	0.250	65.0	35.0	1
7	12.00	0.250	65.0	35.0	6

Table 12. Timetable of gradient elution for detection of CORT, 11-DC, 4-A, 17-OHP, testosterone, progesterone, corticosterone and aldosterone. *Reproduced from Ferrante et al., 2024.*

MRM Function	Time Window (min)	Analyte	Dwell time (s)	Transition (m/z)	Cone (V)	Collision Energy (CE)
1	0 – 12	4-A (Quant.)	0.010	287.30 > 97.10	50.0	20.0
2	0 - 12	4-A (Qual.)	0.010	287.30 > 109.10	50.0	20.0
3	0 - 12	4-A-D5 IS	0.010	292.30 > 100.10	50.0	20.0
4	0 – 12	Testosterone (Quant.)	0.010	289.30 > 97.10	50.0	26.0
5	0 - 12	Testosterone (Qual.)	0.010	289.30 > 109.10	50.0	26.0
6	0 - 12	Testosterone-D5 IS	0.010	294.30 > 100.10	50.0	26.0
7	0 – 12	Progesterone (Quant.)	0.010	315.30 > 97.10	50.0	24.0
8	0 - 12	Progesterone (Qual.)	0.010	315.30 > 109.10	50.0	24.0
9	0 - 12	Progesterone -D9 IS	0.010	324.30 > 100.10	50.0	24.0
10	0 – 12	17-OHP (Quant.)	0.010	331.30 > 97.10	50.0	20.0
11	0 - 12	17-OHP (Qual.)	0.010	331.30 > 109.10	50.0	20.0
12	0 - 12	17-OHP-D8 IS	0.010	339.30 > 100.10	50.0	20.0
13	0 – 12	Corticosterone (Quant.)	0.010	347.29 > 97.10	50.0	24.0
14	0 - 12	Corticosterone (Qual.)	0.010	347.29 > 121.10	50.0	24.0
15	0 - 12	Corticosterone-D8 IS	0.010	355.30 > 125.10	50.0	24.0
16	0 – 12	11-DC (Quant.)	0.010	347.30 > 97.10	50.0	22.0
17	0 - 12	11-DC (Qual.)	0.010	347.30 > 109.10	50.0	22.0
18	0 - 12	11-DC-D5 IS	0.010	352.30 > 100.10	50.0	22.0
19	0 – 12	CORT (Quant.)	0.010	363.30 > 91.10	50.0	50.0
20	0 - 12	CORT (Qual.)	0.010	363.30 > 121.10	50.0	22.0
21	0 - 12	CORT-D3 IS	0.010	366.30 > 121.00	50.0	22.0

Table 13. Multiple Reaction Monitoring (MRM) functions and settings for detection of CORT, 11-DC, 4-A, 17-OHP, testosterone, progesterone, corticosterone and aldosterone in plasma. *Reproduced from Ferrante et al., 2024.*

2.6 CAH Screening Outcomes in Abruzzo region: Results from Recent Years

In the Abruzzo region, since 2022, a total of 16,323 newborns were screened for CAH. Among these, 445 samples were positive in the first-tier immunoassay screening, leading to 445 2-TTs (2-TT) performed to enhance diagnostic specificity. Of the 2-TT analyses, 7 samples tested positive, with a recall rate of 0.042%. Following further clinical evaluation, 4 patients underwent confirmatory diagnostic testing and all 4 were diagnosed with classic 21-OHD CAH.

This corresponds to an incidence of approximately 1 case per 4,081 newborns. The screening program demonstrated excellent diagnostic performance with a sensitivity of 100%, specificity of 99.98%, positive predictive value (PPV) of 57.14%, and negative predictive value (NPV) of 100%. These metrics underscore the effectiveness of the two-tier screening approach in accurately identifying affected newborns while minimizing false positives.

This data confirms that the combined first-tier immunoassay and 2-TT LC-MS/MS steroid profiling strategy ensures timely and reliable detection of CAH in the Abruzzo population, facilitating early treatment and improving clinical outcomes (**Table 14**).

Parameter	Value
Newborns screened	16,323
Positive samples at first-tier	445
Samples tested with 2-TT	445
Positive samples at 2-TT	7
Recall rate (%)	0.042
Patients undergoing confirmatory testing	4
Patients with confirmed classic 21-OHD CAH	4
Incidence	1:4,081
Sensitivity (%)	100
Specificity (%)	99.98
PPV (%)	57.14
NPV (%)	100

Table 14. Outcomes of CAH newborn screening in the Abruzzo region (since 2022). The table summarizes first-tier results, 2-TTs, confirmatory diagnostic outcomes and overall diagnostic performance metrics.

2.7 Potential Effects of Neonatal Nutrition on CAH Screening

Since 2022, Abruzzo region has been providing screening NBS for the classic SW form of CAH, quantifying 17-OHP levels, provides differentiated cut-offs based on gestational weeks (GW); in fact, GW is directly associated with the development of the hypothalamic-pituitary-adrenal (HPA) axis ^[8]. In this context, 2-TTs can reduce the recall rate and the false-positive rate. In our laboratory, in these years, we have screened 16,323 newborns for CAH, performing 445 (2.73%) 2-TTs after an initial positive NBS, finding only 4 (0.02%) positive cases for the classic SW form of CAH (**Figure 10**).

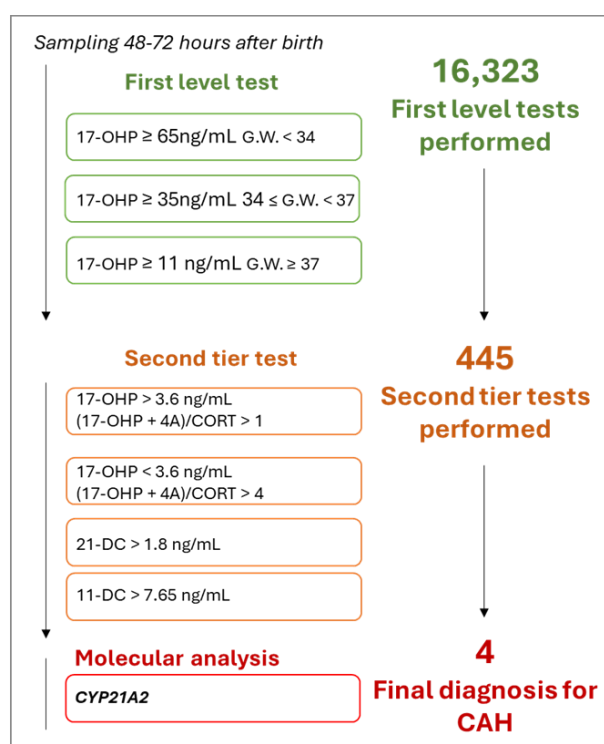


Figure 10. NBS data overview for CAH in the Abruzzo region. *Reproduced from Tommolini et al., 2025.*

In order to reduce 2-TTs, we asked whether external factors could influence 17-OHP ^[6] levels as already observed ^[9].

Consequently, we retrospectively evaluated data from over 6,384 (39.11% of the total population screened for CAH) full-term, normal-weight newborns within the first 120 (five days) hours of life comparing 17-OHP levels between breast- and formula-fed infants. We chose this time frame because breast milk is not static in composition but changes throughout lactation. The first milk is colostrum, which has a primarily trophic function since, unlike formula ^[10], it is rich in bioactive compounds ^[11] that could affect the maturation of adrenal gland in a different way. Subsequently, transitional milk is produced between the

5th and 14th day after birth. After the 14th day, mature milk is produced, which has a composition very similar to formula. Consequently, when we perform NBS for CAH in breastfed infants, we are observing the 17-OHP levels of infants who are consuming colostrum. Colostrum is rich in a wide range of bioactive components, including cytokines and growth factors, which are thought to play a crucial role in the maturation of the neonatal immune system ^[12]. Among these are insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) and epidermal growth factor (EGF), both of which support the development and integrity of the intestinal mucosa. In addition, colostrum provides various hormones and immunoglobulins, such as IgA, IgG, and IgM, as well as pathogen-specific antibodies targeting bacteria, viruses, and fungi, including *Escherichia coli*, *Salmonella*, respiratory syncytial virus, *Candida*, *Streptococcus*, *Staphylococcus*, *Cryptosporidium*, and *Helicobacter pylori*. Compared with mature breast milk, colostrum contains higher concentrations of proteins, minerals, and fat-soluble vitamins, particularly vitamins A, E, and K ^[13].

Univariate comparisons of 17-OHP and other relevant markers (e.g., cortisol) between breastfed and formula-fed newborns were conducted using t-tests in GraphPad Prism (GraphPad Software, Inc., Boston, MA, USA). A threshold of $p < 0.05$ was used to determine statistical significance. Outliers were detected using the ROUT method with a 1% false discovery rate. In addition, personalized cut-off values were defined at the 99.5th percentile for both colostrum-fed and formula-fed groups.

Firstly, we observed results from the first-tier screening level, a statistically significant difference in DBS 17-OHP concentrations was observed between breastfed newborns ($n = 5,945$) and those fed with formula milk ($n = 439$) ($p < 0.0001$; **Figure 11**). Based on the established cut-off values, a higher proportion of formula-fed infants required second-tier testing, with 9.34% ($n = 41$) referred to 2-TT compared with 1.51% ($n = 90$) of breastfed newborns due to elevated 17-OHP levels. This difference persisted within the subgroup undergoing second-tier analysis, where first-tier 17-OHP concentrations remained significantly higher in formula-fed infants than in breastfed newborns ($p = 0.0320$), in agreement with the previously identified pattern (**Figure 12**).

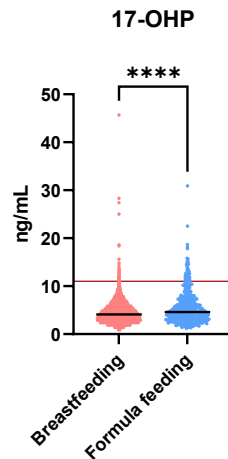


Figure 11. 17-OHP levels (ng/mL) at the first-tier test for formula-fed and breastfed infants. The red line indicates the cut-off (11 ng/mL) for full-term newborns. *Reproduced from Tommolini et al., 2025.*

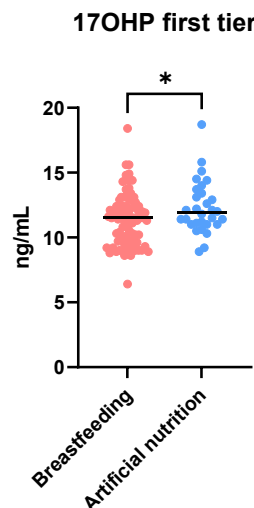


Figure 12. 17-OHP levels (ng/mL) at the first-tier test for formula-fed and breastfed infants who required 2-TT. *Reproduced from Tommolini et al., 2025.*

Subsequently, the effect of feeding type on the steroid markers measured by the second-tier test was assessed in a cohort of 131 full-term, normal-weight newborns who underwent 2-TT analysis. In all samples, 21-deoxycortisol (21-DC) concentrations were below the limit of quantification, while androstenedione (A4) levels did not differ between feeding groups. In contrast, significant differences were detected in the concentrations of 17-OHP ($p = 0.0125$), cortisol (CORT; $p = 0.0048$), 11-deoxycortisol (11-DC; $p < 0.0001$), and in the (17-OHP + A4)/CORT ratio ($p < 0.0001$) (**Figure 13**). Graphical analysis revealed increased 17-OHP and 11-DC levels, together with a higher (17-OHP + A4)/CORT ratio, in formula-fed infants, whereas cortisol concentrations were higher in breastfed newborns.

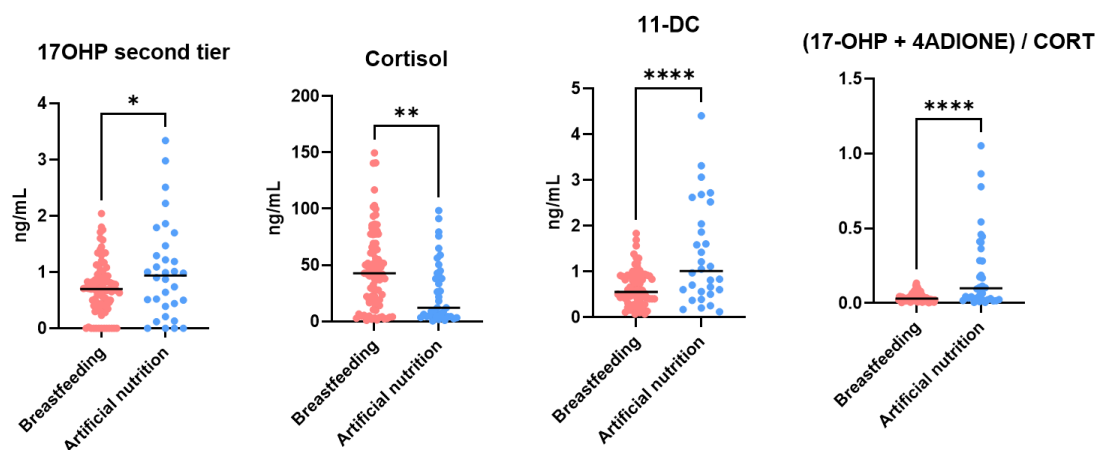


Figure 13. 17-OHP, CORT, 11-DC concentrations and (17-OHP + A4) / CORT ratio for formula fed and breastfed newborns at 2-TT. *Reproduced from Tommolini et al., 2025.*

Given the observed influence of feeding modality on steroid marker concentrations, feeding-customized cut-off values for 17-OHP were established, distinguishing between breastfed and formula-fed newborns (**Table 15**). When applied retrospectively, these tailored thresholds would have reduced the number of 2-TTs to 38, corresponding to 29.8% of all 2-TTs originally performed.

	Breastfeeding	Artificial Nutrition
99.5th percentile	12.33 ng/mL	18.61 ng/mL

Table 15. Feeding-customized cut-off values for 17-OHP calculated at the 99.5th percentile for breastfed and formula-fed infants.

As part of a preliminary investigation into the potential contribution of exogenous steroids, the steroid profile of two commercially available infant formulas and three colostrum samples was characterized. Milk samples were analyzed in analytical triplicate using LC-MS/MS, with quantification of CORT, 11-DC, 4-A, 17-OHP, testosterone, progesterone, corticosterone and aldosterone. Analyses were performed using the CHS™ MSMS Steroids Kit (Revvity®; Turku, Finland, OY) (**Figure 14**).

case report where a 5-year-old boy was referred for endocrine evaluation due to signs of precocious pubertal development, characterized by pubic hair stage P3, genital stage G3, and a testicular volume of 3 mL. Radiographic assessment using the Greulich–Pyle atlas revealed a markedly advanced bone age of 10 years. At presentation, the patient measured 125.2 cm in height and weighed 35 kg, corresponding to a body mass index of 22.3 kg/m².

Genetic and biochemical investigations were carried out on peripheral blood and DBS samples obtained from the patient and his parents at the Pediatric Clinic of SS. Annunziata Hospital (Chieti, Italy). Steroid profiling on DBS was performed by LC-MS/MS to quantify CORT, 21-DC, 11-DC, androstenedione, and 17-OHP. Plasma steroids, including CORT, 11-DC, A4, 17-OHP, testosterone, progesterone, corticosteron, and aldosterone, were measured by UPLC-MS/MS using the CHS™ MSMS Steroids Kit (Revvity®, Turku, Finland). The analytical platform consisted of an ACQUITY™ UPLC™ I-Class system coupled to a Xevo® TQ-S micro mass spectrometer (Waters Corporation, USA), operating in positive electrospray ionization mode. Molecular confirmation of CAH was achieved through genetic testing. Genomic DNA was isolated from peripheral blood and analyzed by Sanger sequencing and multiplex ligation-dependent probe amplification to detect point mutations and large rearrangements affecting the *CYP21A2* locus. Sequence and copy number analyses were performed using validated commercial kits and dedicated software, according to the manufacturers' instructions. Ethics Committee approval was not required for this case report. Written informed consent was obtained from the parents for publication.

Evaluation of the hypothalamic–pituitary–gonadal axis included a gonadotropin-releasing hormone stimulation test, which demonstrated stimulated follicle-stimulating hormone and luteinizing hormone concentrations of 2.4 mUI/mL and 4.3 mUI/mL at 60 minutes, respectively, with a luteinizing hormone–predominant response (**Table 16**).

		Time points		
		0'	+30'	+60'
FSH (mUI/mL)	0,4	1,8	2,4	
LH (mUI/mL)	0,1	3,8	4,3	

Table 16. Results of the LH-RH test at 3 time points. *Reproduced from Ranaudo et al., 2025.*

Circulating 17 β -estradiol levels were below the detection threshold (<10 pg/mL), whereas serum testosterone and prolactin concentrations were 1.19 ng/mL and 21.6 ng/mL, respectively.

Further hormonal investigations revealed elevated adrenocorticotrophic hormone and dehydroepiandrosterone sulfate levels (52.4 pg/mL and 58 pg/mL), exceeding the reference ranges for age and sex. An ACTH stimulation test showed markedly increased baseline 17-OHP concentrations (>20 ng/mL) accompanied by reduced cortisol levels, with no significant CORT response following stimulation (**Table 17**).

	Basal	After stimulation
CORT μg/dL	2,6	2,9
17-OHP ng/mL	>20,00	>20,00

Table 17. Results of the ACTH test at basal and after stimulation. *Reproduced from Ranaudo et al., 2025.*

Plasma renin activity was also found to be increased (168.3 μ U/mL). Routine laboratory analyses, including complete blood count and hepatic, renal, and thyroid function tests, were unremarkable. Taken together, the clinical and biochemical findings were highly suggestive of congenital adrenal hyperplasia, compatible with a non-classic presentation of 21-OHD.

Comprehensive biochemical and molecular investigations were subsequently performed. Steroid profiling demonstrated a pronounced accumulation of 17-OHP confirming a biochemical pattern typical of 21-OHD. Steroid concentrations measured in DBS and plasma are reported in **Tables 18** and **19**, respectively.

Analyte	Value	Cut-off
17-OHP	84.39ng/mL	3.60ng/mL
11-deoxycortisol	0.21ng/mL	7.65 ng/mL
21-deoxycortisol	8.91 ng/mL	1.80ng/mL
4-A	3.52 ng/mL	–
Cortisol	10.51 ng/mL	–
(17OHP + 4-A) / CORT	8.63	1.00

Table 18. Dried blood spotsteroid concentrations of analytes and the related cut-offs to which our laboratory refers, based on a neonatal population. *Reproduced from Ranaudo et al., 2025.*

Analyte	Value	Cut-off
17-OHP	149.96 ng/mL	0.03–2.65 ng/mL
11-deoxycortisol	0.32 ng/mL	0.10–1.56 ng/mL
4-A	4.18 ng/mL	0.06–2.60ng/mL
Cortisol	22.45ng/mL	10–330 ng/mL
Testosterone	1.37 ng/mL	0.03–9.70ng/mL
Progesterone	4.88 ng/mL	0.07–12.94 ng/mL
Corticosterone	1.78 ng/mL	0.80–18.60 ng/mL
Aldosterone	0.30 ng/mL	0.05–0.90ng/mL

Table 19. Plasma steroid concentrations of analytes and the related cut-offs to which our laboratory refers, based on a pediatric population. *Reproduced from Ranaudo et al., 2025.*

Molecular analysis by Sanger sequencing identified the pathogenic c.515T>A variant in exon 4 of the CYP21A2 gene, initially detected in a homozygous configuration (**Figure 15 A**). Multiplex ligation-dependent probe amplification further revealed a heterozygous deletion encompassing exons 1, 3, 4, 6, and 7 of CYP21A2 (**Figure 15 B**).

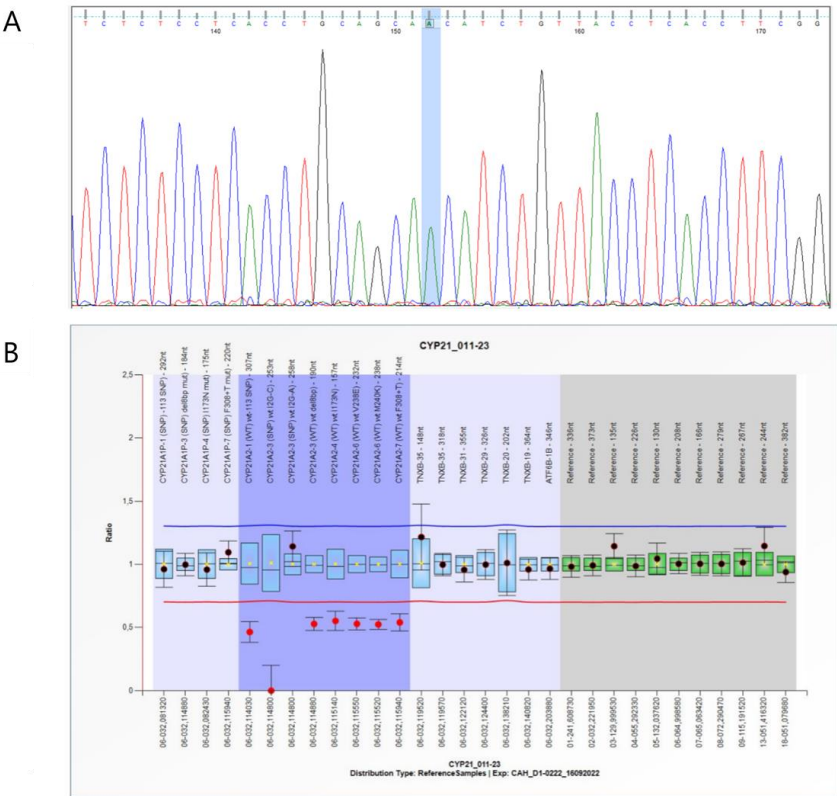


Figure 15. **A**, Sanger Sequencing showed the variant c.515T>A in exon 4 of the CYP21A2 gene in homozygous state in proband; **B**, MLPA analysis detected a heterozygous deletion of exons 1, 3, 4, 6, and 7 in CYP21A2 gene in proband. Source: Original. *Reproduced from Ranaudo et al., 2025.*

To clarify the genetic background, segregation analysis was carried out in both parents. The father was found to carry the c.515T>A variant in a heterozygous state, whereas the mother was heterozygous for the large multiexonic deletion involving CYP21A2 (**Figure 16 A and 16 B**).

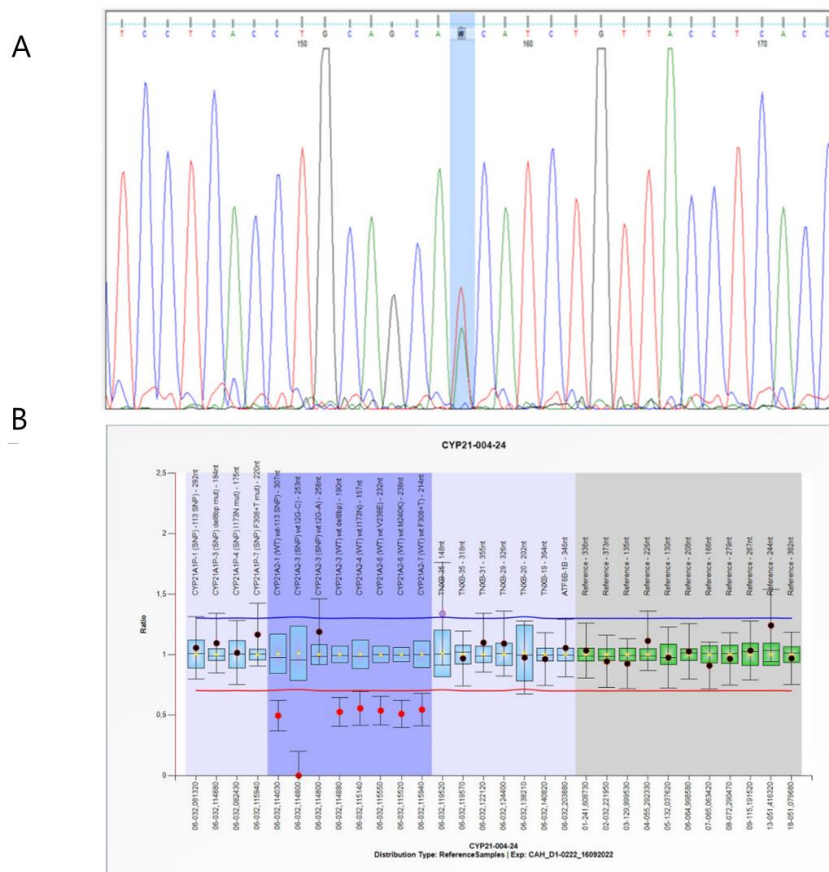


Figure 16. A, Sanger Sequencing identified the variant c.515T>A in exon 4 of the CYP21A2 gene in heterozygous state in proband's father; **B**, MLPA analysis detected a heterozygous deletion of exons 1, 3, 4, 6, and 7 in CYP21A2 gene in proband's mother. Source: Original. Reproduced from Ranaudo et al., 2025.

Based on these findings, the patient was ultimately diagnosed as a compound heterozygote for the c.515T>A mutation and the multiexonic deletion, a genotype consistent with the simple virilizing form of congenital adrenal hyperplasia. According to the EMQN best practice guidelines ^[15], the c.515T>A variant is classified as pathogenic and is associated with the simple virilizing form of congenital adrenal hyperplasia, conferring approximately 1–2% residual 21-hydroxylase activity when present in either homozygous or compound heterozygous states. This molecular characterization was not fully concordant with the initial clinical hypothesis of non-classic CAH, highlighting the limitations of phenotype-based assessment alone.

Following confirmation of the diagnosis, hydrocortisone replacement therapy was initiated at a dose of 10 mg/m²/day, administered in three divided doses, with the aim of suppressing excessive adrenal androgen production. The main therapeutic objectives were to limit further advancement of bone maturation while preserving physiological growth velocity. After one year of treatment, bone age progression was minimal, and no further pubertal progression was observed. This case emphasizes the diagnostic value of early trio-based genetic analysis combined with detailed clinical and biochemical assessment, underscoring the importance of a multidisciplinary approach for the accurate classification of CAH and the prompt initiation of appropriate, targeted therapy.

Consistent with our findings, literature supports the critical role of integrating biochemical profiling with molecular genetic testing to precisely define CAH subtypes ^[16, 17]. For instance, Anastasovska et al. ^[18] described a neonate presenting at 14 days of life with electrolyte disturbances suggestive of salt-wasting CAH. In that case, elevated concentrations of 17-hydroxyprogesterone, ACTH, and testosterone were detected, and subsequent genetic analysis confirmed a genotype consistent with the salt-wasting form. Similarly, Nasir et al. ^[17] reported a 4-year-old boy born to consanguineous parents who initially presented with hyponatremia and hyperkalemia, raising suspicion of salt-wasting CAH. Although biochemical evaluation demonstrated increased 17-OHP and ACTH levels, molecular analysis ultimately revealed compound heterozygosity compatible with a non-classic form, thereby revising the initial clinical diagnosis. Overall, these reports reinforce the necessity of combining biochemical and genetic investigations to achieve accurate CAH subtype classification and to guide optimal clinical management.

2.9 Beyond False Negatives in NBS

The implementation of 2-TTs is considered essential to reduce the high recall rates associated with false-positive findings in NBS. In particular, 2-TT performed by LC-MS/MS assays allow the simultaneous measurement of additional disease-specific biomarkers that are not assessed during first-tier test, thereby improving the interpretation of initial abnormal results and enhancing diagnostic specificity ^[19]. Nevertheless, the presence of a negative NBS result in the setting of a clear or evolving clinical phenotype should not exclude further investigation, as it may mask underlying metabolic or endocrine abnormalities different from those initially suspected.

Below is a case report ^[7] of a newborn with a clinical suspected CAH following no alterations at NBS and given the urgent situation, further genetic investigations and specific biochemical tests were performed.

A female neonate was referred to the Regional Pediatric Diabetology and Endocrinology Center in Chieti for specialist assessment due to atypical development of the external genitalia. The infant was the first child of non-consanguineous parents, with no relevant family history. She was delivered at term following an uncomplicated pregnancy, and the immediate postnatal course was uneventful. At birth, her length was 51 cm and her weight was 3,550 g. On the third day of life, she was admitted to the neonatal unit after the identification of palpable gonadal structures within the labia majora, in the absence of additional clinical signs. Physical and clinical examination showed normally formed labia minora, clitoris, and urethral opening, with a vaginal introitus in the typical anatomical position. Bilateral gonadal structures consistent with testes were palpated within enlarged labia majora. A transabdominal ultrasound performed on day four of life demonstrated the absence of Müllerian structures, including uterus and ovaries, and confirmed the presence of bilateral testes located in the labia majora. Initial laboratory investigations showed normal electrolyte balance and preserved renal, hepatic, glycemic, and thyroid function. In contrast, blood levels of serum anti-Müllerian hormone (AMH), testosterone, and dihydrotestosterone were increased, whereas glucocorticoid and mineralocorticoid levels, as well as 17-OHP, were within age-appropriate reference ranges (**Table 20**).

Laboratory test	Result	Normal Range
AMH (ng/mL)	45.9	/
Testosterone ng/mL	0.73	< 0.025
Dihydrotestosterone pg/mL	438.6	24-368
4-A ng/mL	0.8	0.3-6.5
17-OHP ng/mL	1.7	< 20

Table 20. Serum androgens, AMH and 17-OHP at the time of presentation (compared with the recommended pediatric reference ranges). *Reproduced from Ferrante et al., 2024.*

Besides, cytogenetic analysis performed on metaphase chromosomes obtained from peripheral blood lymphocytes, using G-banding techniques, revealed a 46,XY karyotype.

NBS showed 17-OHP concentrations within the normal range (1.0 ng/mL serum, n.v. < 11 ng/mL serum). Despite the normal screening result, the 2-TT by LC-MS/MS was undertaken. 2-TT demonstrated undetectable 17-OHP levels (0 ng/mL, n.v. < 3.6 ng/mL), along with normal concentrations of 21-DC (0 ng/mL, n.v. < 1.8 ng/mL) and 11-DC (0.1 ng/mL, n.v. < 7.65 ng/mL), and a (17OHP + 4-A) / CORT ratio equal to 1 (n.v. < 1). A subsequent NBS evaluation performed at 339 hours of life confirmed normal 17-OHP levels (2.5 ng/mL serum; reference value <11 ng/mL). 2-TT at this time point again revealed steroid concentrations within physiological limits, including 17-OHP (0.27 ng/mL n.v. < 3.6 ng/mL), 21-DC (0.11 ng/mL n.v. < 1.8 ng/mL), and 11-DC (0.25 ng/mL, n.v. < 7.65 ng/mL). The (17OHP + 4-A) / CORT ratio was 0.04 (n.v. < 1).

Regarding the molecular analysis, genomic DNA was isolated from peripheral blood samples using an automated extraction system (MagPurix 12 EVO, Zinexts Life Science Corp., Taipei, Taiwan) in combination with a commercial whole-blood DNA extraction kit, following the manufacturer's instructions. DNA concentration was subsequently determined using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), according to standard protocols.

Next-generation sequencing was performed using a customized gene panel developed by Thermo Fisher Scientific, targeting 50 genes relevant to the studied condition (**Table 21**). Library preparation included amplicon generation, fragmentation, and adapter ligation, followed by clonal amplification on ion sphere particles. Enriched libraries were loaded onto Ion 510™ chips using the Ion Chef™ system and sequenced on the Ion S5™ platform (Thermo Fisher Scientific, Waltham, MA, USA). Bioinformatic analysis was conducted using Ion Reporter™ Software version 5.12.

Sequencing quality metrics demonstrated a minimum coverage depth exceeding 20X across all target regions, with a mean uniformity of coverage of 98% in all sequencing runs.

GENE	OMIM	REFSEQ	GENE	OMIM	REFSEQ
<i>AKR1C2</i>	600450	NM_001354.5	<i>LEP</i>	164160	NM_000230.2
<i>AKR1C4</i>	600451	NM_001818.3	<i>LHCGR</i>	152790	NM_000233.3
<i>ANOS1</i>	300836	NM_000216.3	<i>MAMLD1</i>	300120	NM_005491.3
<i>AR</i>	313700	NM_000044.3	<i>NR0B1</i>	300473	NM_000475.4
<i>ATRX</i>	300032	NM_000489.3	<i>NR3C1</i>	138040	NM_001018077.1
<i>BMP15</i>	300247	NM_005448.2	<i>NR5A1</i>	184757	NM_004959.4
<i>CHD7</i>	608892	NM_017780.3	<i>POR</i>	124015	NM_000941.2
<i>CYB5A</i>	613218	NM_001914.3	<i>PROK2</i>	607002	NM_00112128.1
<i>CYP17A1</i>	609300	NM_000102.3	<i>PROKR2</i>	607623	NM_144773.3
<i>CYP11B1</i>	610613	NM_000497.3	<i>PROP1</i>	601538	NM_006261.4
<i>CYP19A1</i>	107910	NM_000103.3	<i>RSPO1</i>	609595	NM_001038633.3
<i>DHH</i>	605423	NM_021044	<i>RXFP2</i>	606655	NM_130806.3
<i>DMRT1</i>	601898	NM_004122.2	<i>SOX9</i>	608160	NM_000346.3
<i>DMRT2</i>	602424	NM_021951.2	<i>SRD5A2</i>	607306	NM_000348.3
<i>FGF8</i>	600483	NM_006119.4	<i>STAR</i>	600612	NM_000349.2
<i>FGFR1</i>	136350	NM_023110.2	<i>TAC3</i>	162330	NM_001178054.1
<i>FGFR2</i>	176943	NM_000141.4	<i>WDR11</i>	606417	NM_018117.11
<i>FSHB</i>	136530	NM_000510.2	<i>WT1</i>	607102	NM_024426.4
<i>GATA4</i>	600576	NM_002052.3	<i>ZFPM2</i>	603693	NM_012082.3
<i>GNRH1</i>	152760	NM_001083111.1	<i>HSD17B3</i>	605573	NM_000197.1
<i>GNRHR</i>	138850	NM_000406.2	<i>HSD17B4</i>	601860	NM_000414.3
<i>HESX1</i>	601802	NM_003865.2	<i>HSD3B2</i>	613890	NM_000198.3
<i>AMHR2</i>	600956	NM_020547.3	<i>INSL3</i>	146738	NM_005543.4
<i>CYP11A1</i>	118485	NM_000781.3	<i>MAP3K1</i>	600982	NM_005921.2
<i>FSHR</i>	136435	NM_000145.3	<i>SRY</i>	480000	NM_003140.3

Table 21. Customed NGS panel of 50 genes involved in disorders of sexual development (DSD). *Reproduced from Ferrante et al., 2024.*

The variant identified by NGS was validated through Sanger sequencing using custom-designed primers. Genomic DNA was amplified by polymerase chain reaction (PCR) in a total volume of 30 µL, consisting of 22.25 µL nuclease-free water, 3 µL of 10× PCR buffer, 2.1 µL of 25 mM MgCl₂, 0.5 µL of 10 mM dNTPs, 0.15 µL of AmpliTaq Gold polymerase, 1 µL of DNA template, and 0.5 µL each of forward and reverse primers. PCR amplification was conducted using a SimpliAmp™ thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). PCR products were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics Europe, Düren, Germany) according to the manufacturer's instructions. Purified amplicons were then subjected to direct sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Monza, Italy). Sequencing reactions were purified with the NucleoSEQ Columns Kit (Macherey-Nagel, Düren, Germany) and analyzed in both forward and reverse directions using the SeqStudio™ Genetic Analyzer (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA).

In this study, NGS analysis identified a c.2108C>A (p.Ser703Tyr) variant in the AR gene (**Figure 17**). The AR gene, located on chromosome Xq12, encodes the androgen receptor,

also referred to as the dihydrotestosterone receptor, which plays a central role in the development and maintenance of male sexual characteristics. The AR protein belongs to the class I nuclear receptor family of activated steroid receptors. The detected missense variant, c.2108C>A, involves a single-nucleotide substitution in exon 4, replacing cytosine with adenine at position 2108. This change leads to the amino acid substitution of serine with tyrosine at codon 703 of the encoded protein (p.Ser703Tyr). The AR gene follows an X-linked recessive inheritance pattern, and cytogenetic analysis confirmed a normal 46,XY karyotype in the patient, rendering him hemizygous for this gene. The c.2108C>A variant has not been previously reported in the literature or in the ClinVar database. In silico analyses using predictors available on the Varsome platform suggest that this variant is likely pathogenic.

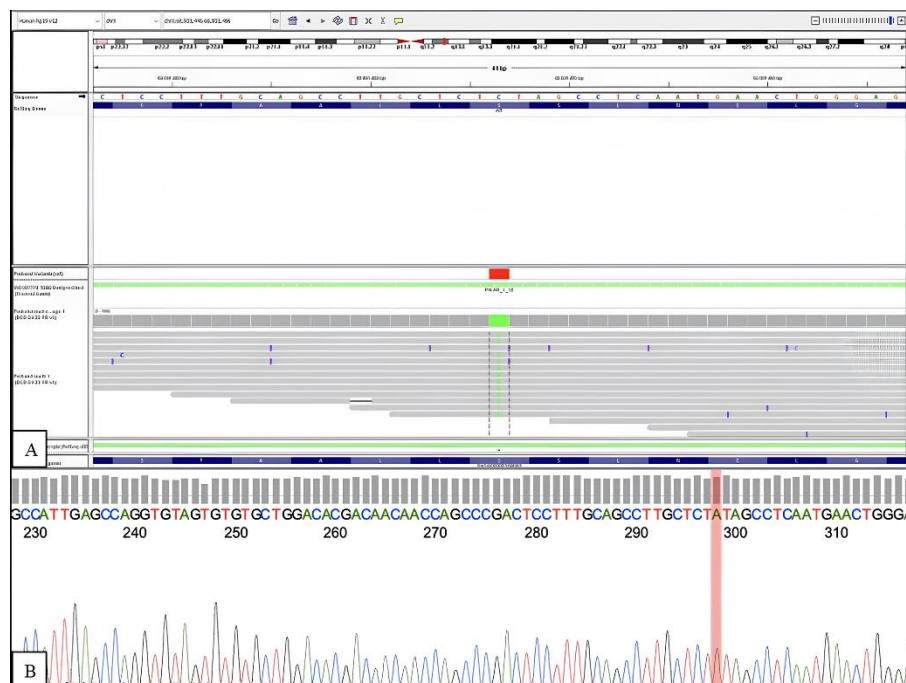


Figure 17. A, Visualization of identified variant, c.2108C>A in AR gene, on IGV. **B**, in the sequence obtained by Sanger Sequencing. *Reproduced from Ferrante et al., 2024.*

Based on the integration of the clinical and molecular findings, a preliminary diagnosis of complete androgen insensitivity syndrome (CAIS) was established. The patient underwent regular endocrinological follow-up, which documented normal growth parameters. During surgical assessment, transperineal ultrasonography revealed a left gonad with typical testicular echotexture located in the inguinal canal/left labia majora, while the right gonad was positioned intra-abdominally, approximately 15 mm from the internal inguinal ring, also showing characteristic testicular structure. A short, blind-ending vagina was identified,

whereas no uterus or ovaries were visualized. No abnormalities were detected in other organs.

Androgen insensitivity syndrome (AIS) arises from mutations in the AR gene, leading to variable degrees of atypical sexual differentiation in 46,XY individuals. The clinical spectrum ranges from a normal female phenotype in complete AIS (CAIS) to partial under-virilization or infertility in phenotypic males with partial AIS (PAIS). Newborns may present with inguinal hernias, ambiguous genitalia, or isolated clitoral hypertrophy, whereas pubertal girls typically present with primary amenorrhea, normal breast development, and sparse pubic or axillary hair ^[20]. Differential diagnoses include gonadal dysgenesis, Mayer-Rokitansky-Kuster-Hauser syndrome, and defects in androgen biosynthesis, such as 5 α -reductase or 17 β -hydroxysteroid dehydrogenase deficiencies. Hormonal evaluation in AIS reflects androgen resistance, with testosterone often within or above male reference ranges, elevated LH, and normal FSH and inhibin. Estrogen levels may be mildly increased due to peripheral aromatization. In infancy, dynamic tests such as hCG stimulation help differentiate AIS from other conditions, and LC-MS/MS steroid profiling has proven useful for distinguishing AIS from 5 α -reductase deficiency ^[21,22]. This case underscores not only the discovery of a previously unreported AR mutation but also highlights the necessity of a multidisciplinary approach, integrating clinical, biochemical and genetic data, to ensure timely and accurate diagnosis of AIS.

1. Cicalini I, Tumini S, Guidone PI, Pieragostino D, Zucchelli M, Franchi S, Lisi G, Lelli Chiesa P, Stuppia L, Laurenzi V, Rossi C. Serum Steroid Profiling by Liquid Chromatography-Tandem Mass Spectrometry for the Rapid Confirmation and Early Treatment of Congenital Adrenal Hyperplasia: A Neonatal Case Report. *Metabolites*. 2019 Nov 21;9(12):284. doi: 10.3390/metabo9120284. PMID: 31766536; PMCID: PMC6950672.
2. Kater CE, Giorgi RB, Costa-Barbosa FA. Classic and current concepts in adrenal steroidogenesis: a reappraisal. *Arch Endocrinol Metab*. 2022 Mar 8;66(1):77-87. doi: 10.20945/2359-3997000000438. PMID: 35263051; PMCID: PMC9991025.
3. Bruce M. Carlson, Chapter 9 - The Endocrine System, Editor(s): Bruce M. Carlson, The Human Body, Academic Press, 2019, Pages 241-269, ISBN 9780128042540, <https://doi.org/10.1016/B978-0-12-804254-0.00009-0>. (<https://www.sciencedirect.com/science/article/pii/B9780128042540000090>)
4. Witchel SF. Congenital Adrenal Hyperplasia. *J Pediatr Adolesc Gynecol*. 2017 Oct;30(5):520-534. doi: 10.1016/j.jpap.2017.04.001. Epub 2017 Apr 24. PMID: 28450075; PMCID: PMC5624825.
5. de Hora M, Heather N, Webster D, Albert B, Hofman P. The use of liquid chromatography-tandem mass spectrometry in newborn screening for congenital adrenal hyperplasia: improvements and future perspectives. *Front Endocrinol (Lausanne)*. 2023 Oct 2;14:1226284. doi: 10.3389/fendo.2023.1226284. PMID: 37850096; PMCID: PMC10578435.
6. Tommolini ML, Frisco A, Zucchelli M, Di Carlo G, Natale L, Petruccioli R, Semeraro D, Verrocchio S, Foley D, Chiavaroli V, Di Valerio S, Cicalini I, Cufaro MC, Valentinuzzi S. The effect of neonatal nutrition on 17- α -hydroxyprogesterone levels in Classic Congenital Adrenal Hyperplasia Screening: a retrospective study. *JIM* 2025; 2 (3): e1005 DOI: 10.61012_20258_1005.
7. Ferrante R, Tumini S, Saltarelli MA, Di Rado S, Scorrano V, Tommolini ML, Zucchelli M, Lauriola F, Lisi G, Lauriti G, Marino N, Stuppia L, Rossi C, Bucci I. A Very Early Diagnosis of Complete Androgen Insensitivity Syndrome Due to a Novel Variant in the AR Gene: A Neonatal Case Study. *Biomedicines*. 2024 Aug 2;12(8):1742. doi: 10.3390/biomedicines12081742. PMID: 39200206; PMCID: PMC11351560.
8. Matharu PK, Held PK, Allen DB. Multiple 17-OHP Cutoff Co-Variates Fail to Improve 21-Hydroxylase Deficiency Screening Accuracy. *Int J Neonatal Screen*. 2022 Oct 25;8(4):57. doi: 10.3390/ijns8040057. PMID: 36412583; PMCID: PMC9680344.
9. Tommolini ML, Cufaro MC, Valentinuzzi S, Cicalini I, Zucchelli M, Frisco A, Simonetti S, Perrone Donnorso M, Moccia S, Bucci I, Aricò M, De Laurenzi V, Federici L, Pieragostino D, Rossi C. Propionyl Carnitine Metabolic Profile: Optimizing the Newborn Screening Strategy Through Customized Cut-Offs. *Metabolites*. 2025 May 6;15(5):308. doi: 10.3390/metabo15050308. PMID: 40422885; PMCID: PMC12112895.
10. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am*. 2013 Feb;60(1):49-74. doi: 10.1016/j.pcl.2012.10.002. PMID: 23178060; PMCID: PMC3586783.
11. Purkiewicz A, Stasiewicz M, Nowakowski JJ, Pietrzak-Fiećko R. The Influence of the Lactation Period and the Type of Milk on the Content of Amino Acids and Minerals in Human Milk and Infant Formulas. *Foods*. 2023 Oct 6;12(19):3674. doi: 10.3390/foods12193674. PMID: 37835327; PMCID: PMC10572789.
12. Munblit D, Treneva M, Peroni DG, Colicino S, Chow L, Dissanayeke S, Abrol P, Sheth S, Pampura A, Boner AL, Geddes DT, Boyle RJ, Warner JO. Colostrum and Mature Human Milk of Women from London, Moscow, and Verona: Determinants of Immune Composition. *Nutrients*. 2016 Nov 3;8(11):695. doi: 10.3390/nu8110695. PMID: 27827874; PMCID: PMC5133082.
13. Shah R, Sabir S, Alhawaj AF. Physiology, Breast Milk. 2022 Sep 19. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan-. PMID: 30969612.
14. Ranaudo M, Dell'Elce A, Navicella A, Minelli M, Tommolini ML, Zucchelli M, Ferrante R, Mohn A, Gatta V. A multidisciplinary biochemical and genetic approach for the diagnosis of congenital adrenal hyperplasia: a pediatric case. *JIM* 2025; 2 (1): e705 DOI: 10.61012_20252_705.

15. Baumgartner-Parzer S, Witsch-Baumgartner M, Hoepfner W. EMQN best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency. *Eur J HumGenet* 2020;28:1341-1367.
16. Baumgartner-Parzer S, Witsch-Baumgartner M, Hoepfner W. EMQN best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency. *Eur J HumGenet* 2020;28:1341-1367.
17. Nasir H, Ali SI, Haque N, Grebe SK, Kirmani S. Compound heterozygosity for a whole gene deletion and p.R124C mutation in CYP21A2 causing nonclassic congenital adrenal hyperplasia. *AnnPediatrEndocrinolMetab* 2018;23:158-161.
18. Anastasovska V, Kocova M, Zdravetska N, Stojiljkovic M, Skakic A, Klaassen K, Pavlovic S. A novel 9 bp deletion (c.1271_1279delGTGCCGCG) in exon 10 of CYP21A2 gene causing severe congenital adrenal hyperplasia. *Endocrine* 2021; 73: 196-202.
19. Rossi C, Cicalini I, Rizzo C, Zucchelli M, Consalvo A, Valentinuzzi S, Semeraro D, Gasparroni G, Brindisino P, Gazzolo D, Dionisi-Vici C, De Laurenzi V, Pieragostino D. A False-Positive Case of Methylmalonic Aciduria by Tandem Mass Spectrometry Newborn Screening Dependent on Maternal Malnutrition in Pregnancy. *Int J Environ Res Public Health*. 2020 May 20;17(10):3601. doi: 10.3390/ijerph17103601. PMID: 32443888; PMCID: PMC7277087.
20. Delli Paoli, E.; Di Chiano, S.; Paoli, D.; Lenzi, A.; Lombardo, F.; Pallotti, F. Androgen Insensitivity Syndrome: A Review. *J Endocrinol Invest* 2023, 46, 2237–2245, doi:10.1007/s40618-023-02127-y.
21. Hughes, I.A.; Davies, J.D.; Bunch, T.I.; Pasterski, V.; Mastroyannopoulou, K.; MacDougall, J. Androgen Insensitivity Syndrome. *The Lancet* 2012, 380, 1419–1428, doi:10.1016/S0140-6736(12)60071-3.
22. Han, B.; Zhu, H.; Yao, H.; Ren, J.; O'Day, P.; Wang, H.; Zhu, W.; Cheng, T.; Auchus, R.J.; Qiao, J. Differences of Adrenal-Derived Androgens in 5 α -Reductase Deficiency versus Androgen Insensitivity Syndrome. *Clin Transl Sci* 2022, 15, 658–666, doi:10.1111/cts.13184.

Chapter 3

Salivary Cortisol: Biological Relevance and Methodological Development for Its Quantification

Stress is implicated in the genesis and progression of numerous chronic diseases. Under physiological conditions, acute stress represents an essential adaptive response that allows the body to cope with environmental stimuli. Conversely, prolonged or excessive exposure to stressors can lead to persistent alterations in biological regulatory systems, contributing to the development of multisystem dysfunction through so-called allostatic load. From this perspective, reliable monitoring of biological stress mediators plays a central role in the early identification of risk conditions and the implementation of preventive strategies. Among the main mediators of the stress response, cortisol plays a key role. This glucocorticoid, secreted by the adrenal cortex following activation of the hypothalamic-pituitary-adrenal (HPA) axis, is involved in the regulation of numerous physiological processes, including energy metabolism, immune response, and cardiovascular function. Variations in cortisol levels reflect the activity of the HPA axis and make this hormone one of the most widely used biomarkers in the study of stress ^[1].

Cortisol can be measured in various biological matrices, serum and salivary cortisol allow for the assessment of acute and diurnal variations in the hormone. Salivary cortisol is a noninvasive measurement, making it particularly suitable for studies in vulnerable populations, such as newborns, and in clinical and experimental settings where repeatable sampling and minimal invasiveness are essential. In recent years, hair cortisol analysis has also emerged as an innovative approach for the retrospective assessment of chronic stress ^[1]. In this context, the development of highly sensitive, specific and reproducible analytical methodologies for the quantification of salivary cortisol is extremely important.

As part of this doctoral work, an LC-MS/MS method was developed for the measurement of salivary cortisol in preterm and full-term infants exposed to 432 Hz music (Translational Music). Therefore, we attempted to understand the effects of music on neonatal stress, using salivary cortisol as a biomarker in clinical and translational settings.

3.1 Cortisol as a Biomarker in Stress Assessment

Stress is a complex physiological phenomenon that can profoundly impact an individual's physical and psychological health. In transient conditions, it can have an adaptive effect, providing the energy and motivation needed to face challenging situations. Conversely, intense or prolonged exposure to stress is associated with negative effects on numerous biological systems, including the immune, cardiovascular, neuroendocrine, and central nervous systems. In particular, chronic stress is considered a major risk factor for alterations in endocrine homeostasis, especially through dysregulation of the HPA axis. ^[1].

The HPA axis is a paradigmatic example of hierarchical control in the endocrine system and is the main mediator of the hormonal response to stress. As illustrated in Figure 18, stressful stimuli, initially processed at the level of the cerebral cortex, activate neurons in the paraventricular nucleus of the hypothalamus, inducing the secretion of corticotropin-releasing hormone (CRH). CRH stimulates the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH), which, in turn, acts on the adrenal cortex, promoting the synthesis and secretion of glucocorticoids, particularly cortisol. ^[2]. Cortisol plays a central role in regulating energy metabolism, promoting hepatic gluconeogenesis and the degradation of lipids and proteins, ensuring adequate availability of energy substrates during stressful conditions. At the same time, cortisol exerts a powerful immunosuppressive and anti-inflammatory effect, modulating cytokine production and reducing immune system activation ^[2]. These effects, while beneficial in the short term, can be harmful in cases of chronic exposure to high levels of glucocorticoids.

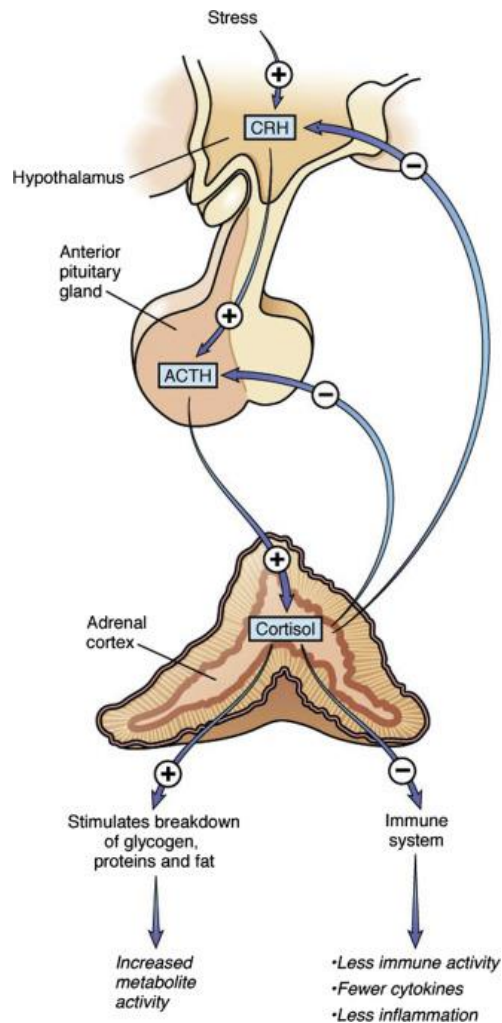


Figure 18. The HPA axis and regulation of cortisol secretion. The sequential activation of CRH, ACTH, and cortisol regulates energy metabolism and immune activity through negative feedback mechanisms. *Reproduced from Bruce et al., 2019.*

A crucial aspect of cortisol physiology is its circadian rhythm. Under physiological conditions, serum cortisol levels increase in the early morning hours, peaking around 7:00 - 8:00 a.m., and then progressively decline throughout the day, reaching approximately 10% of morning levels in the evening and nighttime hours. This pattern makes sampling timing a crucial factor in correctly interpreting cortisol levels and highlights the importance of standardized measurement protocols [3].

From a biological perspective, only a small fraction of circulating cortisol is present in a free and biologically active form, while the majority is bound to plasma proteins. Due to its low molecular weight and lipophilic nature, free cortisol passively diffuses across cell membranes, making it possible to measure it in various biological fluids. In this context,

salivary cortisol has gained increasing relevance as a noninvasive tool for assessing HPA axis activity, demonstrating several advantages over serum and urinary cortisol. Numerous studies have demonstrated that salivary cortisol reliably reflects the free fraction of the hormone and is particularly sensitive to changes associated with acute stress ^[1]. Hence, the term stress refers to stimuli that alter homeostasis, causing adaptive responses. These adaptive responses are called allostasis, which can be beneficial or harmful depending on their intensity and duration. Allostatic load represents the accumulation of chronic stress responses that alter the physiological "set point," favoring the onset of pathologies such as diabetes. Among the mediators of allostasis, cortisol is crucial: in acute conditions, it supports energy adaptation, but chronically elevated levels damage brain structures involved in negative feedback, maintaining elevated cortisol levels even in the absence of stress. For this reason, cortisol is a key biomarker of allostatic load, although its measurement is complex due to circadian and environmental variations. Recently, hair cortisol has emerged as a reliable indicator of cumulative stress, with clinical applications ranging from early childhood to metabolic and neuropsychiatric disorders (**Figure 19**) ^[1, 4].

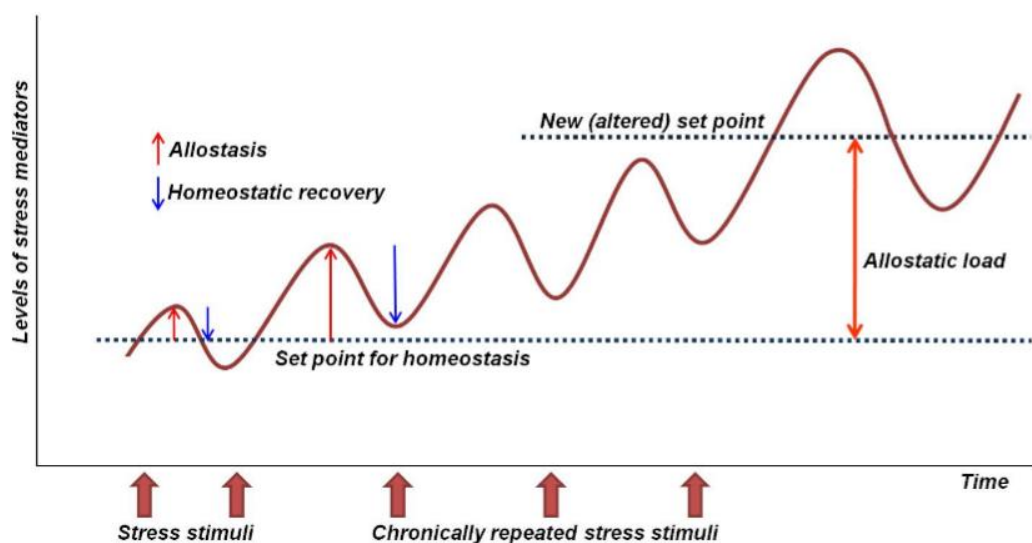


Figure 19. Stress, allostasis, and allostatic load. Stress induces adaptive or maladaptive changes (allostasis) in stress mediators. Repeated stressful stimuli can prevent a return to the original homeostasis, establishing a new altered "set point," called allostatic load. *Reproduced from Lee et al., 2015.*

Hair cortisol has recently been classified as a reliable indicator of cumulative stress, useful for monitoring long-term exposure from childhood to metabolic and neuropsychiatric disorders. With growth of approximately 1 cm/month, hair allows for a noninvasive retrospective measurement of chronic stress, overcoming the limitations of short-term

biological samples. Because over 70% of diseases are stress-related, hair cortisol represents an important biomarker for predicting the risk of metabolic syndrome, obesity, and cardiovascular disease. Studies show an association between elevated hair cortisol levels and childhood obesity, cardiovascular risk, and conditions such as adrenocortical carcinoma. Furthermore, in patients with adrenal insufficiency and undergoing replacement therapy, hair cortisol correlates with hydrocortisone dosage and levels of perceived stress, suggesting potential clinical use in therapeutic management (**Figure 20**) ^[1, 5].

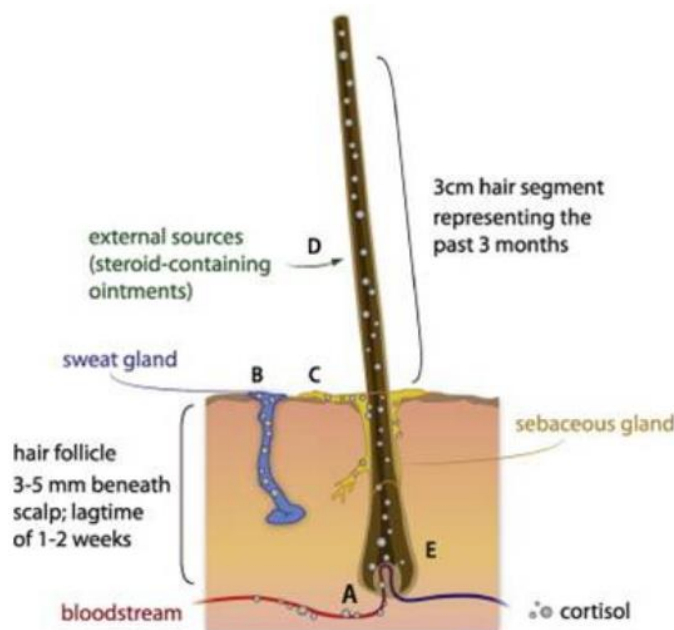


Figure 20. Cortisol incorporation into hair and retrospective reflection of its chronic secretion. Cortisol may be incorporated into hair via passive diffusion from (A) blood capillary, (B) sweat, and (C) sebum, as well as from (D) external sources. *Reproduced from Lee et al., 2015.*

In addition to cortisol, stressful conditions can produce other organic chemicals that can subsequently be found in the blood, serum and saliva, such as immunoglobulin A, lysozyme, melatonin, α -amylase (sAA), chromogranin A (CgA), and/or fibroblast growth factor 2 (FGF-2). Stress can weaken the immune system and inhibit the production of immunoglobulins, salivary IgA levels can vary depending on whether your mood is positive (increase) or negative (decrease). Lysozyme, a protein with antibacterial and antiviral properties present in saliva, significantly decreases under stress conditions resulting in a potential biomarker of chronic stress. Melatonin, produced by the pineal gland, regulates the sleep-wake cycle and has antioxidant properties, salivary melatonin levels can be used as biomarkers for disorders such as depression, anxiety and post-traumatic stress. sAA, a digestive enzyme with immune function, has been shown to increase in patients subjected to chronic stress.

CgA, an acidic glycoprotein released by the adrenal medulla and sympathetic nerve endings, can be measured in saliva, an increase in salivary CgA has been shown to occur in response to various stressors (within 1 hour) and remain constant throughout the day. FGF-2, present in salivary glands and saliva, is involved in neuroregeneration and stress response, salivary glands that contain FGF-2, can secrete it in response to stress ^[3].

3.2 Methodological Development for Salivary Cortisol Quantification

Given the central role of cortisol as a biomarker of HPA axis activity and allostatic load, accurate and reliable measurement of its concentration in biological fluids is essential for both research and clinical applications. Saliva represents a particularly attractive matrix due to its noninvasive collection, reflection of the biologically active free cortisol fraction, and suitability for repeated sampling. Thus, the low concentrations of cortisol in saliva, coupled with potential interference from other salivary components, require the development of highly sensitive and specific analytical methods.

In this context, LC-MS/MS has emerged as the gold standard for steroid quantification, offering superior specificity, sensitivity, and reproducibility compared with immunoassays. The following section describes the methodological development of a LC-MS/MS assay for salivary cortisol.

Salivary sample preparation for cortisol quantification

Salivary samples were prepared for cortisol quantification by LC–MS/MS. A working internal standard (IS) solution containing 10 ng of deuterated cortisol (cortisol-d₃; Spectra 2000) was prepared in methanol. Calibrators and quality control (QC) were prepared in water over a concentration range of 0.25–100 ng/mL. 80 µL of the working IS solution were added to 20 µL of salivary samples, calibrators, and QC samples. The mixtures were vortex-mixed and subsequently centrifuged. The resulting supernatants were transferred to clean tubes and evaporated to dryness. The dried residues were reconstituted with 100 µL of a 60:40 (v/v) mixture of water 0.05 mM ammonium fluoride (NH₄F) and methanol. The reconstituted samples were then transferred into vials for LC–MS/MS analysis. A sample volume of 5 µL was injected into the ion source.

LC-MS/MS conditions

The LC–MS/MS analyses were performed using an ACQUITY™ UPLC™ I-Class system equipped with a Binary Solvent Manager (BSM) and a Sample Manager with Flow-Through Needle (SM-FTN), coupled to a Xevo® TQ-S micro triple quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The mass spectrometer was operated in positive electrospray ionization mode (ESI+). The total run time was 4 minutes per sample, from injection to injection. Chromatographic separation was achieved using a CORTECS C18 column (2.7 µm, 50 × 2.1 mm; Waters Corporation) with a CORTECS C18 VanGuard pre-column (2.7 µm). The column temperature was maintained at 50 °C. The mobile phase consisted of water containing 0.05 mM ammonium fluoride (NH₄F) (solvent A) and methanol (solvent B), with solvents supplied by Carlo Erba Reagents. The flow rate was set at 0.5 mL/min. The gradient elution program is detailed in **Table 22**. Cortisol detection was performed using multiple reaction monitoring (MRM), the optimized MRM transitions and related parameters are reported in **Table 23**. Data acquisition and processing were carried out using TargetLynx™ XS software (Waters Corporation, Milford, MA, USA).

N.	Time (min)	Flow Rate (mL/min)	%A	%B	Curve
1	Initial	0.500	60.0	40.0	Initial
2	0.10	0.500	60.0	40.0	6
3	0.25	0.500	55.0	45.0	6
4	0.75	0.500	55.0	45.0	6
5	0.90	0.500	37.5	62.5	6
6	1.15	0.500	5.0	95.0	11
7	1.40	0.500	60	40	11

Table 22. Timetable of gradient elution for detection of CORT.

MRM Function	Time Window (min)	Analyte	Dwell time (s)	Transitions (m/z)	Cone (V)	Collision Energy (CE)
1	0 – 4	CORT (Quant.)	0.050	363.30 > 121.10	50.0	22.0
2	0 – 4	CORT (Qual.)	0.050	363.30 > 91.10	50.0	50.0
3	0 – 4	CORT D3 IS	0.050	366.30 > 121.00	50.0	22.0

Table 23. Multiple Reaction Monitoring (MRM) functions and settings for detection of cortisol.

3.3 Salivary Cortisol Applications

Salivary cortisol is a well-established, non-invasive biomarker widely used to assess HPA axis activity and physiological stress responses in both adult and pediatric populations. In neonatal settings, and particularly in preterm infants, salivary cortisol measurement offers a valuable tool for monitoring stress exposure while minimizing additional discomfort, an essential consideration in this vulnerable population. The use of salivary cortisol has therefore gained increasing attention in neonatal research as an objective indicator of stress and adaptation to environmental and clinical stimuli ^[1]. Preterm and hospitalized term neonates are exposed to multiple stressors during the early postnatal period, including medical procedures, environmental stimuli, and maternal separation, all of which may influence short- and long-term neurodevelopmental outcomes ^[6 - 13]. In this context, salivary cortisol has been extensively applied to evaluate the impact of both pharmacological and non-pharmacological interventions aimed at reducing neonatal stress ^[14]. Among non-pharmacological approaches, sensory-based interventions such as exposure to maternal voice and music have shown promise in modulating stress responses, as reflected by changes in behavioral state, physiological parameters, and salivary cortisol levels ^[15 - 20]. In this scenario, the assessment of salivary cortisol represents a key outcome measure for investigating the potential stress-modulating effects of auditory interventions, including music therapy, in neonatal populations. In particular, salivary cortisol analysis provides an integrative measure that complements clinical and behavioral assessments, enabling a more comprehensive evaluation of neonatal stress regulation and adaptation to environmental stimuli.

Overall, the findings of our study indicate that exposure to 432 Hz music was associated with a measurable reduction in salivary cortisol levels, particularly in preterm infants, supporting its potential as a non-pharmacological intervention for stress modulation in neonatal populations. Although baseline cortisol levels did not differ significantly between preterm and term infants, a statistically significant decrease was observed in the preterm group after the 5-day intervention, while a similar trend was seen in term infants, though not reaching statistical significance. The reduction in cortisol was most pronounced between Day 1 and Day 3, suggesting that repeated exposure to music may have an early and cumulative effect on HPA axis activity. In addition to hormonal responses, physiological parameters also demonstrated positive changes. Heart rate and respiratory rate were reduced during individual music sessions, with significant within-day improvements in both preterm and term infants. Oxygen saturation increased progressively, with the most marked

improvements observed over the five days of exposure. Blood pressure also decreased, particularly in term infants, both within single sessions and over the total intervention period. Behavioral responses, as assessed by BPSN-R scores, showed a consistent decrease during and after music exposure, highlighting a concomitant improvement in observable stress-related behaviors.

Taken together, these results suggest that 432 Hz music may exert multi-level modulatory effects on neonatal stress, encompassing hormonal, physiological, and behavioral domains. The integration of salivary cortisol measurements with continuous monitoring of vital signs and behavioral scores provides a comprehensive evaluation of stress regulation in neonates and underscores the utility of salivary cortisol as a sensitive biomarker for assessing the effectiveness of environmental and therapeutic interventions.

1. Lee DY, Kim E, Choi MH. Technical and clinical aspects of cortisol as a biochemical marker of chronic stress. *BMB Rep.* 2015 Apr;48(4):209-16. doi: 10.5483/bmbrep.2015.48.4.275. PMID: 25560699; PMCID: PMC4436856.
2. Bruce M. Carlson, Chapter 9 - The Endocrine System, Editor(s): Bruce M. Carlson, *The Human Body*, Academic Press, 2019, Pages 241-269, ISBN 9780128042540, <https://doi.org/10.1016/B978-0-12-804254-0.00009-0>. (<https://www.sciencedirect.com/science/article/pii/B9780128042540000090>)
3. Chojnowska S, Ptaszyńska-Sarosiek I, Kępka A, Knaś M, Waszkiewicz N. Salivary Biomarkers of Stress, Anxiety and Depression. *J Clin Med.* 2021 Feb 1;10(3):517. doi: 10.3390/jcm10030517. PMID: 33535653; PMCID: PMC7867141.
4. Juster RP, McEwen BS, Lupien SJ. Allostatic load biomarkers of chronic stress and impact on health and cognition. *Neurosci Biobehav Rev.* 2010 Sep;35(1):2-16. doi: 10.1016/j.neubiorev.2009.10.002. Epub 2009 Oct 12. PMID: 19822172.
5. Stalder T, Kirschbaum C. Analysis of cortisol in hair--state of the art and future directions. *Brain Behav Immun.* 2012 Oct;26(7):1019-29. doi: 10.1016/j.bbi.2012.02.002. Epub 2012 Feb 15. PMID: 22366690.
6. Anderson DE, Patel AD. Infants born preterm, stress, and neurodevelopment in the neonatal intensive care unit: might music have an impact? *Dev Med Child Neurol.* 2018;60(3):256-66.
7. Perlman JM. Neurobehavioral deficits in premature graduates of intensive care - Potential medical and neonatal environmental risk factors. *Pediatrics.* 2001;108(6):1339-48.
8. ruz MD, Fernandes AM, Oliveira CR. Epidemiology of painful procedures performed in neonates: Asystematic review of observational studies. *Eur J Pain (United Kingdom).* 2016;20(4):489-98.
9. Mörelius E, Theodorsson E, Nelson N. Salivary cortisol and mood and pain profiles during skin-to-skin care for an unselected group of mothers and infants in neonatal intensive care. *Pediatrics.* 2005;116(5):1105-13.
10. Grunau RE, Haley DW, Whitfield MF, Weinberg J, Yu W, Thiessen P. Infants Born At Extremely Low Gestational Age. *Methods.* 2007;150(2):151-6.
11. Buss C, Entringer S, Swanson JM, Wadhwa PD. The Role of Stress in Brain Development: The Gestational Environment's Long-Term Effects on the Brain. *Cerebrum [Internet].* 2012;2012(April):4. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23447790>0Ahttp://www.pubmedcentral.nih.gov/articlerend er.fcgi?artid=PMC3574809
12. Schwilling D, Vogeser M, Kirchhoff F, Schwaiblmair F, Boulesteix AL, Schulze A, et al. Live music reduces stress levels in very low-birthweight infants. *Acta Paediatr Int J Paediatr.* 2015;104(4):360-7.
13. Standley JM. A meta-analysis of the efficacy of music therapy for premature infants. *J Pediatr Nurs.* 2002;17(2):107-13.
14. Aucott S, Donohue PK, Atkins E, Allen MC. Neurodevelopmental care in the NICU. *Ment Retard Dev Disabil Res Rev.* 2002;8(4):298-308.
15. Lejeune F, Lordier L, Pittet MP, Schoenhals L, Grandjean D, Hüppi PS, et al. Effects of an early postnatal music intervention on cognitive and emotional development in preterm children at 12 and 24 months: Preliminary findings. *Front Psychol.* 2019;10(MAR):1-12.
16. Chirico G, Cabano R, Villa G, Bigogno A, Ardesi M, Dioni E. Randomised study showed that recorded maternal voices reduced pain in preterm infants undergoing heel lance procedures in a neonatal intensive care unit. *Acta Paediatr Int J Paediatr.* 2017;106(10):1564-8.

17. Sajjadian N, Mohammadzadeh M, Alizadeh Taheri P, Shariat M. Positive effects of low intensity recorded maternal voice on physiologic reactions in premature infants. *Infant Behav Dev* [Internet]. 2017;46:59–66. Available from: <http://dx.doi.org/10.1016/j.infbeh.2016.11.009>
18. Loewy J, Stewart K, Dassler AM, Telsey A, Homel P. The effects of music therapy on vital signs, feeding, and sleep in premature infants. *Pediatrics*. 2013;131(5):902–18.
19. Arnon S, Shapsa A, Forman L, Regev R, Bauer S, Litmanovitz I. Live music is beneficial to preterm infants in the neonatal intensive care unit environment. *Birth*. 2006;33:131–6.
20. Hartling L, Shaik MS, Tjosvold L, Leicht R, Liang Y, Kumar M. Music for medical indications in the neonatal period: A systematic review of randomised controlled trials. *Arch Dis Child Fetal Neonatal Ed*. 2009;94(5).

Discussion

NBS represents one of the most effective prevention strategies in public health, as it allows for the early identification of IEMs in the very first days of life. Diagnosis in the presymptomatic phase allows for timely therapeutic interventions that can prevent irreversible damage, significantly reducing morbidity and mortality. Over the decades, NBS has benefited from significant technological progress, including various analytical methods.

In Italy, NBS has gradually developed into a structured and regulated national program. NBS became mandatory in 1992 for a limited number of conditions, including congenital hypothyroidism, phenylketonuria, and cystic fibrosis. A key turning point came with the introduction of tandem mass spectrometry, which facilitated the simultaneous identification of numerous IEMs from a single DBS. Law No. 167 of 2016 made NBS mandatory and free for all newborns in Italy, while subsequent legislative measures provided for the periodic updating of the panel of conditions and the extension to new categories of rare diseases. In this context, the Abruzzo Region has played an active role, activating extended neonatal screening in 2017 at the Neonatal Screening Center of the Abruzzo Region, located at the CAST of the "G. d'Annunzio" University, and launching a pilot project in 2022 to include additional rare diseases. This initiative led, in 2025, to the definitive integration of new diseases into the regional program, with a total panel of 56 diseases guaranteed to all newborns.

Although individually rare, IEMs as a whole represent a significant public health problem, with an estimated prevalence of approximately 1 in 2,500 in Italy. They include aminoacidopathies, urea cycle disorders, organic acidurias, and fatty acid β -oxidation disorders, generally characterized by acute onset in the neonatal period, as well as later-onset disorders such as lysosomal storage diseases. Clinical manifestations frequently involve the central nervous system and gastrointestinal tract and, if untreated, can rapidly lead to severe disability or death. The inclusion of these disorders in screening programs is based on criteria such as clinical severity, the availability of reliable tests, and the demonstrated benefit of early diagnosis.

Nevertheless, metabolic complexity and the potential sharing of biomarkers across different pathologies necessitate accurate cut-off definition and the integration of 2-TTs. 2-TTs, often

based on LC-MS/MS, significantly reduce false positives and unnecessary recalls, increasing the PPV of screening. A particularly relevant area is steroid profiling, which is essential in CAH. Although immunoassays are still widely used due to their simplicity, they have specificity limitations due to cross-reactivity and interference with similar steroids. Mass spectrometry-based methods, particularly LC-MS/MS, allow for highly specific and accurate multiplex analysis, overcoming many of the limitations of immunoassays and now representing the reference method for assessing complex steroid profiles.

CAH is a group of autosomal recessive disorders of adrenal steroidogenesis, most commonly caused by 21-hydroxylase deficiency due to mutations in the *CYP21A2* gene. The defect impairs the synthesis of cortisol and, in the classic salt-wasting forms, also aldosterone, leading to chronic ACTH hypersecretion, adrenal hyperplasia, and accumulation of steroid precursors, particularly 17-OHP, resulting in hyperandrogenism. This imbalance results in a clinical spectrum ranging from classic salt-wasting forms with the risk of potentially fatal adrenal crises to simple virilizing and non-classical variants. The global incidence of classic CAH is estimated to be between 1:5,000 and 1:15,000 live births, justifying its inclusion in NBS programs.

The Abruzzo Region NBS Laboratory screens for CAH by measuring 17-OHP on DBS collected in the first 48–72 hours of life, allowing for the early identification of newborns at risk of adrenal crisis and misassignment of sex. The first level of screening uses a fluorimetric immunoassay with differentiated cut-offs based on gestational age. Anyway, the limited specificity of immunoassays requires the use of 2-TT based on LC-MS/MS, which allows for the simultaneous quantification of multiple steroids, including 17-OHP, 4-A, CORT, 11-DC, and 21-DC, (17-OHP + 4-A) / CORT ratio is also determines, improving diagnostic specificity and reducing unnecessary recalls.

Since 2022, 16,323 newborns have been screened for CAH; 445 first-level positive samples were analyzed with 2-TT, 7 tested positive, and after clinical and diagnostic confirmation, 4 newborns were definitively diagnosed with classic CAH, corresponding to approximately 1 in 4,081 births. The program showed 100% sensitivity, 99.98% specificity, 57.14% PPV, and 100% NPV, confirming the effectiveness of the two-tiered approach. A retrospective study conducted in 2025 at our laboratory highlighted that non-pathological factors, such as neonatal feeding (breast milk vs. formula), can influence 17-OHP and other steroid levels, suggesting the possibility of personalized cut-offs to further reduce false positives.

The integration of first-tier testing, 2-TT, plasma steroid profiling, and genetic analysis currently represents the most effective model for the early and accurate diagnosis of CAH and the main conditions in the differential diagnosis. This multidisciplinary approach allows for an accurate diagnosis of CAH and the main conditions in the differential diagnosis.

We have therefore demonstrated that the presence of an apparently positive clinical picture for CAH in the presence of a negative newborn screening requires further investigation, as well as the use of next-generation sequencing techniques to provide a correct and timely diagnosis.

In addition to diagnosing congenital disorders such as CAH, precise quantification of steroids by LC-MS/MS also opens the possibility of studying the physiology of the HPA axis and the stress response. In particular, cortisol, one of the key steroids assessed in endocrine panels, can be measured not only in DBS or plasma, but also in noninvasive matrices such as saliva, offering valuable tools for research on neonatal stress and adaptive response modulation strategies.

Stress is a major determinant of health, contributing to the development and progression of numerous chronic diseases. Under physiological conditions, the stress response is adaptive; anyhow, its persistence causes allostatic overload with long-lasting alterations in biological systems and an increased risk of multisystem dysfunction. Monitoring biological mediators of stress is essential for the early identification of risk factors and the implementation of preventive strategies. Among the main mediators, cortisol plays a central role: secreted by the adrenal cortex in response to HPA axis activation, it regulates energy metabolism, immune response, and cardiovascular function. Variations in cortisol levels reflect HPA axis activity, making it one of the most widely used biomarkers in stress studies. Cortisol is closely linked to the circadian rhythm, making the choice of biological matrix and timing of sampling crucial.

Salivary cortisol is a noninvasive measure of the free fraction of the hormone, highly sensitive to acute stress-related changes and particularly suited to the study of vulnerable populations such as newborns. Salivary cortisol was measured as a biomarker to assess neonatal stress in preterm and full-term infants exposed to 432 Hz music (Translational Music), using an LC-MS/MS method developed in our laboratory. In addition to cortisol, other salivary biomarkers such as IgA, α -amylase, chromogranin A, melatonin, and FGF-2 contribute to the characterization of the stress response, reflecting the interaction between the endocrine, immune and autonomic nervous systems.

Overall, measuring salivary cortisol represents a key tool for assessing the impact of early life stressors and for studying the effectiveness of environmental and sensory interventions, such as music therapy, aimed at promoting adaptation and well-being in early life.

Therefore, steroid characterization using advanced methods such as LC-MS/MS is now an essential tool both in the clinical setting, for the early diagnosis of diseases such as CAH, and in translational research, for the study of stress biomarkers such as salivary cortisol. The ability to accurately measure steroids in various biological matrices allows for the integration of diagnostic and physiological assessments, providing a comprehensive picture of the HPA axis in newborns and supporting timely and targeted interventions from both a clinical and neurobehavioral perspective.

Conclusions

This PhD work highlights NBS as a fundamental tool for the prevention and early diagnosis of metabolic and endocrine disorders, with a direct impact on the reduction of neonatal morbidity and mortality. In particular, the integration of 2-TTs based on LC-MS/MS methods significantly improves diagnostic specificity, reducing false-positive results and unnecessary recalls, as demonstrated by the CAH screening program implemented in the Abruzzo Region. Moreover, the adoption of personalized screening cut-offs, accounting for clinical and environmental factors may further optimize NBS performance reducing time, costs and number of 2-TTs.

The multidisciplinary approach combining first-tier immunoassays, 2-TT, plasma steroid profiling and genetic analysis currently represents the most effective model for the early and accurate diagnosis of both classical and non-classical forms of CAH, as well as of the main differential diagnoses.

Beyond its clinical relevance, accurate steroid quantification, especially cortisol measurement, opens new perspectives in the study of neonatal stress responses. The use of salivary cortisol as a non-invasive biomarker allows detailed characterization of HPA axis activity in the neonatal period.

In conclusion, steroid profiling represents as a key tool to understand neuroendocrine regulatory mechanisms, supporting both clinical diagnostics and the investigation of physiological processes.

PART TWO

Academic and Scientific Activities

List of publications

- Cicalini I, Moffa S, **Tommolini ML**, Valentinuzzi S, Zucchelli M, Bucci I, Chiacchiaretta P, Fontana A, Federici L, De Laurenzi V, Del Boccio P, Rossi C, Pieragostino D. Impact of Maternal Lifestyle and Dietary Habits during Pregnancy on Newborn Metabolic Profile. *Nutrients*. 2023 May 13;15(10):2297. doi: 10.3390/nu15102297. PMID: 37242180; PMCID: PMC10221063.

ABSTRACT– Expanded newborn screening (NBS) is a preventive program that allows for the early identification of over 40 congenital endocrine-metabolic diseases by analyzing dried blood spot samples collected from the newborn’s heel within 48–72 h of birth. The determination of amino acids and acyl-carnitines by Flow Injection Analysis Tandem Mass Spectrometry (FIA-MS/MS) may also highlight metabolic alterations resulting from external factors, such as maternal nutrition. In the present study, we developed a questionnaire to investigate the eating habits of 109 women during pregnancy and statistically correlated the results from the investigation on dietary habits with the data obtained by the NBS laboratory of Abruzzo region (Italy). Parameters such as smoking, physical activity, and the intake of iodized salt, drugs, and supplements were analyzed. This study aimed to highlight how maternal lifestyle, diet, and drug intake during pregnancy may affect the neonatal metabolic profile, possibly generating false positive or false negative results in the NBS test. The results pointed out how the knowledge of maternal nutrition and lifestyle may also be precious in preventing misinterpretations of the neonatal metabolic profile, thereby reducing unnecessary stress for newborns and their parents and limiting costs for the health system.

- Valentinuzzi S, **Tommolini ML**, Frisco A, Zucchelli M, Cicalini I, Cufaro MC. Second Tier - Tests do not always require chromatographic separation: the case of hydroxylated and dicarboxylic acyl – carnitine couples. *JIM* 2024; 1(1): e456 DOI: 10.61012_20242_456.

ABSTRACT– Newborn screening is used to diagnose inherited metabolic diseases (IEMs), many of which are diagnosed via flow injection analysis coupled with tandem mass spectrometry (FIA-MS/MS). Some metabolites are isobaric and thus are not distinguished. Second-tier tests have been developed to facilitate this separation, often involving liquid chromatography. Some isobaric acyl-carnitines are associated with IEMs characterized by the suppression of ketogenesis. These are acquired as a sum of signals at the first screening, as in the case of dicarboxylic and hydroxylated acyl-carnitines (C3DC\C4OH, C4DC\C5OH, C5DC\C6OH). In this regard, a second-tier test based on FIA-MS/MS has been developed. Internal standards (ISs) of the NeoBase™ 2 non-derivatized MSMS kit (Revvity, ex PerkinElmer) were employed; for the isobaric couples, the corresponding ISs were 2 H3 -C4,

2 H9-C5, and 2 H6 -C5DC. A derivatization step using 3N HCl in n-butanol was added to the normal sample preparation of the kit. Samples were analyzed by FIA-MS/MS through the RenataDX™ screening system coupled to a Xevo™ TQD IVD mass spectrometer. This approach allowed rapid differentiation between isobaric couples without a chromatographic separation. The method is accurate and precise for routine diagnostics. In this context, a clinical suspect was identified between 3-methylcrotonylglycinuria, multiple carboxylase deficiencies, 3-methylglutaconic aciduria type I, and 3-hydroxy-3-methylglutaric aciduria in a newborn showing elevated methylmalonyl-carnitine (C4DC)/3-hydroxyisovaleryl-carnitine (C5OH) levels at the first screening. The second-tier test highlighted alterations in the levels of C5OH rather than C4DC. For the clinical approach, the availability of this simple second-tier test supports a rapid differential diagnosis among IEMs that differ in the degree of metabolic decompensation through suppression of ketogenesis.

- Ferrante R, Tumini S, Saltarelli MA, Di Rado S, Scorrano V, **Tommolini ML**, Zucchelli M, Lauriola F, Lisi G, Lauriti G, Marino N, Stuppia L, Rossi C, Bucci I. A Very Early Diagnosis of Complete Androgen Insensitivity Syndrome Due to a Novel Variant in the AR Gene: A Neonatal Case Study. *Biomedicines*. 2024 Aug 2;12(8):1742. doi: 10.3390/biomedicines12081742. PMID: 39200206; PMCID: PMC11351560.

ABSTRACT – Androgen insensitivity syndrome (AIS) is one of the most common Disorders of Sexual Differentiation (DSDs). AIS is characterized by an X-linked recessive inheritance pattern associated with variants in the androgen receptor (AR) gene that affects the masculinization process in individuals with XY karyotype. Here, we report a neonatal case of a very early diagnosis of complete AIS due to a novel variant in the AR gene. In the present case, after the clinical evaluation, the infant has undergone the following tests: biochemical analyses, including newborn screening workflow, karyotype analysis, and Next-Generation Sequencing (NGS) panel of 50 genes involved in DSDs. The NGS analysis identified a missense variant, c.2108C>A, in the AR gene. According to a cytogenetic analysis, the patient presented a 46, XY karyotype, thus the resulting hemizygote for the AR gene variant. The variant is not currently described in the literature nor in the ClinVar database. However, according to computational models, the variant could have a pathogenetic effect. This clinical case reveals a novel variant of the AR gene with a possible pathogenetic effect associated with AIS and highlights the importance of a multidisciplinary approach for the timely diagnosis and appropriate follow-up of the patient.

- Verrocchio S, Semeraro D, Cicalini I, Valentinuzzi S, **Tommolini ML**, Cufaro MC, Ferrante R, Bucci I. Galactosemia: insight into newborn screening strategies. *JIM* 2024; 1 (4): e630 DOI: 10.61012_202411_630.

ABSTRACT – Galactosemia is a group of hereditary disorders that affect galactose metabolism, potentially causing fatal multi-organ toxicity. However, the morbidity and mortality associated with galactosemia can be prevented through a galactose-restricted diet. The earlier this diet is implemented, the better the prognosis, although some long-term complications may persist despite early dietary intervention. Early diagnosis and treatment, achieved through newborn screening (NBS), are crucial. NBS for galactosemia enables timely diagnosis and intervention, significantly reducing early neonatal complications and mortality. This study aims to highlight the importance of NBS for galactosemia and review the various screening strategies implemented worldwide. We conducted a literature review to summarize the inclusion of galactosemia in NBS programs,

the methodologies used, and the screening modalities applied. Alongside phenylketonuria, galactosemia was among the first conditions for which neonatal screening tests became available. Despite this long history, controversy persists, and the inclusion of galactosemia in NBS panels varies across countries. The two primary types of assays used in galactosemia screening are total galactose tests and enzyme activity tests, applied either as single-tier or two-tier approaches. This review outlines the key characteristics of galactosemia, the principles and methodologies underlying screening tests, the strategies for their implementation, and the performance of various screening programs worldwide.

- Ranaudo M, Dell'Elce A, Navicella A, Minelli M, **Tommolini ML**, Zucchelli M, Ferrante R, Mohn A, Gatta V. A multidisciplinary biochemical and genetic approach for the diagnosis of congenital adrenal hyperplasia: a pediatric case. *JIM* 2025; 2 (1): e705 DOI: 10.61012_20252_705.

ABSTRACT – This study aimed to demonstrate the potential of integrating genetic and biochemical approaches for the rapid and definitive diagnosis of congenital adrenal hyperplasia. We report the case of a 5-year-old boy presenting with early puberty and significantly advanced bone age. Due to a strong suspicion of congenital adrenal hyperplasia, the patient underwent diagnostic evaluation incorporating both biochemical and genetic analyses. Biochemical analysis using ultra-performance liquid chromatography–tandem mass spectrometry revealed a marked accumulation of 17-hydroxyprogesterone, with the steroid profile confirming a pattern characteristic of 21-hydroxylase deficiency. Sanger sequencing identified the pathogenic variant c.515T>A (exon 4) in a heterozygous state, while multiplex ligation-dependent probe amplification detected a heterozygous deletion of exons 1, 3, 4, 6 and 7 in the CYP21A2 gene. Parental segregation analysis showed that the c.515T>A variant was inherited from the father, whereas the large heterozygous deletion originated from the mother. A multidisciplinary approach integrating biochemical and genetic analyses enables a definitive and early diagnosis of congenital adrenal hyperplasia, facilitating appropriate treatment and preventing serious or fatal complications associated with the classic form of the disease.

- **Tommolini ML**, Cufaro MC, Valentinuzzi S, Cicalini I, Zucchelli M, Frisco A, Simonetti S, Perrone Donnorso M, Moccia S, Bucci I, Aricò M, De Laurenzi V, Federici L, Pieragostino D, Rossi C. Propionyl Carnitine Metabolic Profile: Optimizing the Newborn Screening Strategy Through Customized Cut-Offs. *Metabolites*. 2025 May 6;15(5):308. doi: 10.3390/metabo15050308. PMID: 40422885; PMCID: PMC12112895.

ABSTRACT– The advent of tandem mass spectrometry (MS/MS) had an essential role in the expansion of newborn screening (NBS) for different inborn errors of metabolism (IEMs). Nowadays, almost 50 IEMs are screened in Italy. The use of second-tier tests (2-TTs) in NBS minimizes the false positive rate; nevertheless, the metabolic profile is influenced not only by the genome but also by environmental factors and clinical variables. We reviewed the MS/MS NBS data from over 37,000 newborns (of which 8% required 2-TTs) screened in the Italian Abruzzo region to evaluate the impact of neonatal and maternal variables on propionate-related primary biomarker levels. Expanded NBS and 2-TT analyses were performed using MS/MS and liquid chromatography–MS/MS methods. We set up layered cut-offs dividing all 37,000 newborns into categories. Statistical analysis was used to create alarm thresholds for NBS-positive samples. Statistically significant differences were found in both neonatal and maternal conditions based on the 2-TTs carried out. According to the stratified cut-offs, only 1.47% of the newborns would have required a 2-TT while still

retaining the ability to recognize the true-positive case of methylmalonic acidemia with homocystinuria, which has been identified by NBS. To further support the clinical applicability, we performed an external evaluation considering nine positive cases from an extra-regional neonatal population, confirming the potential of our model. Interestingly, the setting of alarm thresholds and their application would allow for establishing the degree of priority/urgency for 2-TTs. Tailoring NBS by customized cut-offs may enhance the application of precision medicine, focusing on true-positive cases and also reducing analysis costs and times.

- **Tommolini ML**, Frisco A, Zucchelli M, Di Carlo G, Natale L, Petruccioli R, Semeraro D, Verrocchio S, Foley D, Chiavaroli V, Di Valerio S, Cicalini I, Cufaro MC, Valentinuzzi S. The effect of neonatal nutrition on 17- α -hydroxyprogesterone levels in Classic Congenital Adrenal Hyperplasia Screening: a retrospective study. *JIM* 2025; 2 (3): e1005 DOI: 10.61012_20258_1005.

ABSTRACT – Newborn screening (NBS) on dried blood spots (DBS) is mandatory under Italian law (No. 167/2016), despite a fair heterogeneity in the panel of diseases being monitored. Classical Congenital Adrenal Hyperplasia (CAH) has been recently included in the NBS program of the Abruzzo region, providing screening tests to over 16,000 newborns to date. According to our workflow, 445 (2.73%) second-tier tests (2-TT) were performed, 7 (0.04%) newborns required further attention, and 4 (0.02%) positive cases were found. Our NBS lab has already set customized cut-offs based on gestational weeks; however, other factors, such as different types of nutrition, could influence 17- α -hydroxyprogesterone levels (17-OH). We retrospectively evaluated the CAH screening performed on DBS specimens collected within the first 120 hours (colostrum production time) after birth. We compared 17-OHP and associated markers levels between newborns who consumed formula milk and those who received colostrum. The analysis focused on 17-OHP levels in 6,384 infants, of which 439 were formula-fed while 5,945 breast-fed. 17-OHP levels showed statistically significant differences ($p < 0.0001$) depending on the different types of nutrition, as well as some markers quantified by the second-tier test (2-TT). Then, we set customized cut-off values for 17-OHP according to the types of nutrition. As a preliminary approach, we analyzed steroids in two infant formulas and three colostrum samples, observing the absence of cortisol in formula milk. These results lay the foundation to remodulate the current cut-offs in order to reduce 2-TTs, thus optimizing our analytical performance.

- **Tommolini ML**, Zucchelli M, Frisco A, Ferrante R, Dufrusine B, Palmarini C, Natale L, Ballerini P, Stuppia L, Federici L, Pieragostino D, Cicalini I. Phenotyping and genotyping for the dihydropyrimidine dehydrogenase test in Italy: a precise diagnostic strategy to detect rare variants and improve drug administration. *Clin Biochem.* 2025 Dec;140:111010. doi: 10.1016/j.clinbiochem.2025.111010. Epub 2025 Sep 10. PMID: 40939752.

ABSTRACT – Dihydropyrimidine dehydrogenase (DPD) is the enzyme implicated in the catabolism of the fluoropyrimidines (FP), a class of chemotherapeutics used to treat many cancers. The DPYD gene is known to have a huge number of variants potentially associated with DPD deficiency. Therefore, phenotypic and genotypic characterization of DPD is fundamental for cancer patients before undergoing treatment with FP. The AIOM (Italian Association of Medical Oncology) requires genetic analysis, with the purpose to identify a panel of 5 single nucleotide polymorphisms associated with 5-fluorouracil (5-FU)-induced toxicity, while the biochemical test, which measures uracil levels to predict the residual

activity of DPD, is only recommended and not mandatory. Single nucleotide polymorphisms were analyzed by real-time polymerase chain reaction (PCR) from peripheral blood. Uracil and dihydrouracil were quantified using an ultra-performance liquid chromatography / tandem mass spectrometry system in plasma obtained from patients before 5-FU treatment. Sanger sequencing was performed for the DPYD gene. Here, we describe the case of a 70-year-old Caucasian male patient with pancreatic cancer for whom real-time PCR highlighted a heterozygous pathogenic variant c.1905 + 1G > A associated with a 50 % reduction of DPD enzymatic activity. This finding was not confirmed by the biochemical test which revealed a complete absence of DPD activity. By performing Sanger sequencing, we highlighted the concomitant presence of a likely pathogenic variant c.2622 + 1G > A, not compatible with the administration of 5-FU. Thus far, guidelines provide a limited panel for the identification of pathogenic or likely pathogenic variants of the DPYD gene, therefore we encourage the mandatory use of biochemical tests as a precise diagnostic strategy to detect rare variants and improve drug administration.

Research and Clinical Activities

Laboratory of Analytical Biochemistry, Proteomics and Endocrinology – C.A.S.T. (Center for Advanced Studies and Technology), University "G. d'Annunzio" of Chieti–Pescara

Method Development and Validation:

- Development and validation of a second-tier test (2-TT) for steroid profiling of 4-androstene-3,17-dione (4-A), cortisol (CORT), 17- α -hydroxyprogesterone (17-OHP), 11-deoxycortisol (11-DC), and 21-deoxycortisol for Congenital Adrenal Hyperplasia from dried blood spots (DBS) by LC-MS/MS
- Development and validation of a DBS-based LC-MS/MS 2-TT for two lysosomal storage diseases: simultaneous quantification of LysoGB3 (Fabry disease) and LysoGB1 (Gaucher disease).
- Development of an LC-MS/MS method for salivary cortisol measurement.

Main Laboratory Activities:

- Newborn Screening (NBS) by FIA-MS/MS from DBS using the NeoBase™ 2 Non-derivatized MSMS Kit (Revvity).
- First-tier enzymatic assays for three lysosomal storage diseases (Fabry Disease, Gaucher Disease, Mucopolysaccharidosis Type I) using NeoLSD™ MSMS Kit (Revvity) for quantitative assessment of ABG, GLA and IDUA enzymatic activities.
- Plasma steroid panel by LC-MS/MS (17-OHP, CORT, 4-A, 11-DC, testosterone, progesterone, corticosterone, aldosterone) using the CHS™ MSMS Steroids Kit (Revvity).
- Second-tier tests (2-TT):
Propionate-related metabolic profile: simultaneous quantification of methylmalonic acid, methylcitric acid and homocysteine from DBS by LC-MS/MS. Steroid panel profiling 4-A, CORT, 17-OHP, 11-DC, 21-DC from DBS by LC-MS/MS. Analysis of C5 isomeric acylcarnitines (isovaleryl-carnitine, valeryl-carnitine, pivaloyl-carnitine and methylbutyryl-carnitine) from DBS by LC-MS/MS. Carboxylated-hydroxylated acylcarnitines analysis (C3DC/C4OH, C4DC/C5OH, C5DC/C6OH) acylcarnitines from

DBS by FIA-MS/MS. Lysosomal Storage Diseases: simultaneous quantification of LysoGB3 (Fabry disease) and LysoGB1 (Gaucher disease) from DBS by LC-MS/MS. Dihydropyrimidine dehydrogenase (DPD) testing: quantification of uracil and dihydrouracil from plasma in cancer patients before fluoropyrimidine therapy (LC-MS/MS).

Participation in seminars, lectures, courses, journal clubs, conferences, and research training abroad.

Research Activities Abroad

Visiting PhD Student, Research & Development Department, REVVITY (PerkinElmer), Turku, Finland

- Sep – Nov 2023 (3 months)
- Feb – Apr 2024 (3 months)

NeoBase2 and NeoLSD on QSight – Product Specialist Training, Research & Development Department, REVVITY (PerkinElmer), Turku, Finland — April 2023

Teaching Activities

Subject Expert (Cultore della materia) - A.Y. 2024–2025:
Chemistry, biochemistry, and physics applied to motor sciences
Chemistry and Biochemistry Module – CFU (Crediti Formativi Universitari) = 7
Bachelor Degree Programme in Motor and Sports Sciences (DM 270),
University "G. d'Annunzio" of Chieti–Pescara
Module coordinator: Prof. Claudia Rossi

Third Mission Activities

- Expanded Newborn Screening and Pilot Project for the Expansion of Diseases Subject to Neonatal Screening for Congenital Metabolic Diseases. Newborn Screening Laboratory - CAST (Center for Advanced Studies and Technology) - University "G. d'Annunzio" of Chieti and Pescara.

- Participation in the European Researchers' Night 2025 with a dedicated stand on Newborn Screening, aimed at science communication and public awareness on genetic and metabolic disorders. – University "G. d'Annunzio" of Chieti and Pescara, Italy.

- STEM Orientation Project (PNRR) – I.C. Pescara, “Tinozzi” Middle School (6 hours). National initiative to promote Science, Technology, Engineering and Mathematics (STEM) disciplines.
- University “G. d’Annunzio” of Chieti and Pescara, Italy.
- Green Lab Ambassador and Accredited Professional for the Newborn Screening Laboratory, Medical Genetics Laboratory, and Proteomics and Metabolomics Core Facility, as part of the My Green Lab Certification Program – University “G. d’Annunzio” of Chieti-Pescara, Italy. – University “G. d’Annunzio” of Chieti and Pescara, Italy.
- Involved in the “Nonno Ascoltami” public health initiative, with a dedicated stand on Newborn Screening, aimed at prevention and health education for the general public – Pescara, Italy
- Involved in the “La Salute scende in Piazza” public health initiative, with a dedicated stand on Newborn Screening, aimed at prevention and health education for the general public – Chieti, Italy.
- Participation in the European Researchers’ Night 2024 with a dedicated stand on Newborn Screening, aimed at science communication and public awareness on genetic and metabolic disorders. – University “G. d’Annunzio” of Chieti and Pescara, Italy.

Conferences and Scientific Dissemination

- 1st Interregional Congress of Central Italy in Biochemistry and Molecular Biology – SIB section, Italy, July 21–22, 2025.
- 13th ISNS European regional meeting, Luxembourg, 23-26 March 2025
- XIV SIMMESN National Congress (Società Italiana per lo Studio delle Malattie Metaboliche Ereditarie e lo Screening Neonatale) – Montesilvano, Italy 2024
- ItPA Italian Proteomics Association XVII International Congress, “Proteomics and Metabolomics towards Global Health” - Roma, Italy – 2023.

Oral Presentation

- The potential effects of neonatal nutrition on 17- α -hydroxyprogesterone levels in Classic Congenital Adrenal Hyperplasia screening. Oral presentation at the 1st Interregional Congress of Central Italy in Biochemistry and Molecular Biology – SIB section, Italy, July 21–22, 2025.

Conference Posters

International Congress of Inborn Errors of Metabolism ICIEM, Kyoto, Japan, 2 – 6 September 2025:

- Enhancing Neonatal Screening Strategies: A Machine Learning Approach for Improved Diagnostic Process. **M. L. Tommolini***
- The effects of neonatal nutrition on 17-hydroxyprogesterone levels in Classic Congenital Adrenal Hyperplasia screening: a retrospective study. **M. L. Tommolini***

- An innovative analytical platform to decode novel IDUA variants of uncertain significance identified by newborn screening for mucopolysaccharidosis type 1.
- Untargeted metabolomic and proteomic profiling on dried blood spot to shed light on acid sphingomyelinase deficiency and the effects of enzyme replacement therapy.

12th MS J-day 1st International Edition (Società Chimica Italiana – SCI), Pisa, Italy 22-23 May 2025:

- Metabolomic approach for unravelling novel Gaucher disease biomarkers on dried blood spot

13th ISNS European regional meeting, Luxembourg, 23-26 March 2025:

- The effects of neonatal nutrition on 17- α -hydroxyprogesterone levels in Classic Congenital Adrenal Hyperplasia screening: a retrospective study. **M. L. Tommolini*** (<https://doi.org/10.3390/ijns11010021>).

62nd Annual ESPE (European Society for Pediatric Endocrinology), Liverpool, UK, 16 - 18 November 2024:

- Effects of Music at 432 Hz on Biological Stress Responses in Infants born Preterm and at Term

XIV SIMMESN National Congress (Società Italiana per lo Studio delle Malattie Metaboliche Ereditarie e lo Screening Neonatale) – Montesilvano, Italy 2024:

- Comprendere l'influenza delle variabili cliniche sul metabolismo della propionil-carnitina per una migliore interpretazione dei risultati nello screening neonatale. **M. L. Tommolini***
- Analisi ICP-MS di elementi in traccia ed ultratraccia da campioni DBS: una piccola goccia di sangue nell'oceano dell'"esposomica"
- Dalla Biochimica alla Genetica: identificazione di una nuova variante del gene AR associata alla Sindrome da insensibilità agli androgeni
- Progetto Pilota in Abruzzo: una panoramica sull'attività di screening neonatale per le malattie di accumulo lisosomiale
- L'importanza della fase preanalitica negli screening neonatali: Survey sugli errori comuni e le strategie di miglioramento nella regione Abruzzo.

XXX Congresso Nazionale Società Italiana di Neonatologia – Padova, Italy – 2024:

- Effetti della musica a 432 Hz su parametri fisiologico-comportamentali e cortisolo salivare in neonati pretermine e a termine

SSIEM - Society for the Study of Inborn Errors of Metabolism - Porto, Portugal - 2024 Annual Symposium:

- Understanding the influence of clinical variables on propionyl carnitine metabolism for a better comprehension of newborn screening results. (<https://doi.org/10.1002/jimd.12788>)

ItPA Italian Proteomics Association XVII International Congress, "Proteomics and Metabolomics towards Global Health" - Roma, Italy – 2023:

- Proteomics assessment of early treated adults with Phenylketonuria: a perspective study to assess the disease status. **M. L. Tommolini***

The 2023 APHL/ISNS Newborn Screening Symposium - Sacramento, California:

- The first year of NBS pilot study for seven further disorders including neuromuscular and metabolic diseases, immunodeficiencies, and lysosomal storage diseases in Abruzzo: An Italian experience.

XIII SIMMESN National Congress (Società Italiana per lo Studio delle Malattie Metaboliche Ereditarie e lo Screening Neonatale) – Verona, Italy 2023:

- Oltre lo SNE: il primo anno di screening neonatale in Abruzzo per immunodeficienze congenite severe, malattie neuromuscolari genetiche, metaboliche e da accumulo lisosomiale.

62° SIB Congress – Società Italiana di Biochimica e Biologia Molecolare – Firenze, Italy:

- Understanding the influence of clinical variables on neonatal metabolic profiling for an improved interpretation of newborn screening results. **M. L. Tommolini***
- Characterization of lymphocytes protein cargo in Covid-19: unveiling the impaired coagulation.

26° Convegno Multidisciplinare Abruzzo e Molise: Biomarcatori Emergenti nella Medicina di Laboratorio – SIBioC Società Italiana di Biochimica Clinica e Biologia Molecolare Clinica e Medicina di Laboratorio – Chieti 2023:

- Understanding the influence of clinical variables on neonatal metabolic profiling for an improved interpretation of newborn screening results. **M. L. Tommolini***

HUPO (Human Proteome Organization) World Congress 2023 – Busan, South Korea:

- Proteomics assessment of early treated adults with Phenylketonuria: a perspective sight to personalized medicine. **M. L. Tommolini***
- Characterization of lymphocytes protein cargo in Covid-19: unveiling the impaired Coagulation.
- Proteomics profile of BAG3 knockout in pancreatic β -cells and hepatocyte-specific BAG3 knockout mouse model.

ABCD 2023 National Congress – Associazione di Biologia Cellulare e del Differenziamento - Paestum, Italy:

- Microgravity effects on testicular physiology: study of enzymatic and hormonal parameters in mouse embryonic testis.

XXVI Congresso Nazionale SIGU (Società Italiana di Genetica Umana) - Rimini, Italy:

- A case of congenital adrenal hyperplasia diagnosis through careful biochemical and molecular characterization.
- A family case of Gaucher disease by a multidisciplinary approach for lysosomal storage Diseases.

XII SIMMESN National Congress (Società Italiana per lo Studio delle Malattie Metaboliche Ereditarie e lo Screening Neonatale) – Bari, Italy 2022:

- Impatto dello stile di vita e delle abitudini alimentari materne durante la gravidanza sul profilo metabolico del neonato.

Professional Awards and Merits

- Poster Selected for the Guided Poster Walk of Highest Ranked Posters at the SSIEM 2024 Symposium in Porto, Portugal - Understanding the influence of clinical variables on propionyl carnitine metabolism for a better comprehension of newborn screening results. (<https://doi.org/10.1002/jimd.12788>)
- Paper selected by Metabolites (international, peer-reviewed, open access journal of metabolism and metabolomics) Editors to be featured on the cover of the issue 5 (2025). Tommolini, M.L.*; Cufaro, M.C.*; Valentinuzzi, S.*; Cicalini, I.; Zucchelli, M.; Frisco, A.; Simonetti, S.; Perrone Donnorso, M.; Moccia, S.; Bucci, I.; et al. Propionyl Carnitine Metabolic Profile: Optimizing the Newborn Screening Strategy Through Customized Cut-Offs. Metabolites 2025, 15, 308. <https://doi.org/10.3390/metabo15050308>.
- Paper selected as Title Story for Metabolites (international, peer-reviewed, open access journal of metabolism and metabolomics). Tommolini, M.L.*; Cufaro, M.C.*; Valentinuzzi, S.*; Cicalini, I.; Zucchelli, M.; Frisco, A.; Simonetti, S.; Perrone Donnorso, M.; Moccia, S.; Bucci, I.; et al. Propionyl Carnitine Metabolic Profile: Optimizing the Newborn Screening Strategy Through Customized Cut-Offs. Metabolites 2025, 15, 308. <https://doi.org/10.3390/metabo15050308>.

Professional Memberships - Scientific Societies

- SIB member - Italian Society of Biochemistry and Molecular Biology
- SIMMESN member - Italian Society for the Study of Hereditary Metabolic Diseases and Newborn Screening
- SIBioC member - Laboratory Medicine Association
- ItPA member - Italian Proteomics Association
- SIPMeL member - Italian Society of Clinical Pathology and Laboratory Medicine

