Universitat de Girona
Facultat de Medicina

Fundació Salut Empordà

FINAL DEGREE PROJECT

IS BUTYRYLCHOLINESTERASE A POTENTIAL BIOMARKER FOR SUDDEN INFANT DEATH SYNDROME'S PREVENTION?

A pilot, retrospective, case-control study

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January 2023

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Especial agraïment a la doctora Maite Serrando i el doctor Pep Casadesús per tot el temps i bones intencions posades en el treball i per ajudar-me a tirar-lo endavant.

També a en "Chema" del Laboratori del Programa de Cribratge Neonatal de Catalunya per atendre'm de seguida i per resoldre tots els meus dubtes.

Al doctor Marc Saez per dedicar-me el seu temps.

I als meus tutors clínic i metodològic, el doctor Pere Plaja i en Xavi Castells pels consells i les guies durant el treball.

Gràcies als serveis de pediatria de l'Hospital Doctor Josep Trueta i de l'Hospital de Figueres per tractar-me tan bé durant les pràctiques i fer-me sentir una més.

Gràcies sobretot a la Paula, a l'Arnau i a la Clara per acollir-me.

Gràcies a les meves amigues, sobretot la Rocío i la Mar, pels 6 anys que portem creixent juntes.

Al papa, la mama i la Núria, per creure sempre en mi.

I especialment a l'Eloi, per treure el millor de mi i fer-me costat sobretot en els pitjors moments.

ABSTRACT

TITLE: Is butyrylcholinesterase a potential biomarker for sudden infant death syndrome's prevention?

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BACKGROUND: Sudden infant death syndrome is used when a sleeping infant under one year of age, who has presumably been quite well, is found unexpectedly dead and remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history. Safe sleep campaigns and the progress in understanding risk factors have significantly contributed to the decrease in its incidence. Nevertheless, the rate of reduction has now slowed and still remains a leading cause of post neonatal mortality in many developed countries. Strategic action is needed to tackle this problem. Recently, a decreased neonatal cholinesterase activity (BChE) has been suggested to be an important risk to consider.

OBJECTIVES: The main objective is to determine if the absence of BChE's basic monomer in dried heel blood taken after 48-72 hours of birth in the neonatal screening program is associated with sudden infant death syndrome. And the secondary objective is to carry out a descriptive study with the epidemiological information of the cases of the studied period.

STUDY DESIGN: This is a pilot, retrospective, case-control study carried out in Catalunya. Each case will be matched with 10 living controls according to sex and weeks of gestation. A population of 88 cases and 880 controls will be studied. The determination of the monomer will be done with tandem mass spectrometry.

PARTICIPANTS: Cases: Infants who died from sudden infant death syndrome in the last five years. Extracted from forensic records of the "Institut de Patologia Forense de Catalunya". Controls: Alive children born during the month before or after the date of birth of the cases. Obtained from the census of the "Laboratori Central de Cribratge de Catalunya".

KEY WORDS: Sudden infant death syndrome. Prone sleeping. Acetylcholinesterase. Butyrylcholinesterase. Newborn screening. BChE basic monomer.

Abbreviations

- **ACh**: Acetylcholine
- **AChE**: Acetylcholinesterase
- **AEP**: Asociación Española de Pediatría
- **BChE**: Butyrylcholinesterase
- **CEIC**: Comitè Ètic d'Investigació Clínica
- **CO_2**: Carbon dioxide
- **CSF**: Cerebrospinal fluid
- **GEPMSL**: Grupo de trabajo para el Estudio y la Prevención de la Muerte Súbita del Lactante.
- **IMLC**: Institut de Medicina Legal de Catalunya
- **Kg**: Kilograms
- **MS**: Mass spectrometry
- **ND**: Number of dibucaine
- **NF**: Number of fluoride
- **NGS**: Next Generation Sequencing
- **REM**: Rapid eye movement
- **SIDS**: Sudden infant death syndrome
- **SUDI**: Sudden unexpected death in infancy
- **UEC**: Expertise Clinical Unit

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1. INTRODUCTION

1.1. SUDDEN INFANT DEATH SYNDROME

Sudden unexpected death in infancy (SUDI) is a broad term that encompasses all sudden infant deaths including: accidental deaths (such as suffocation and strangulation in a sleeping environment), sudden natural deaths caused from infections, cardiac or metabolic disorders and neurological conditions that were not recognized as life-threatening, acute illnesses of less than 24 hours of duration, homicides and sudden infant death syndrome (SIDS). (1)

SIDS is used when a sleeping infant under one year of age, who has presumably been quite well, is found unexpectedly dead and remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history.

It is recognized that 95% of SIDS deaths occur between one and six months of age, and unexpected deaths after the first year of life are rare. (2)

SIDS is typically associated with the sleeping period, with death presumed to have occurred during sleep itself or in the transition between sleep and wake up.

Finding an apparently healthy baby dead in the place where he slept is a desperate event for parents so it is a universal and serious problem. Despite more and more research on it, it still remains an unpredictable problem. (3)

1.1.1. History

Knowledge and dissemination of the complex subject of SIDS has changed substantially in the last 40 years since SIDS has gone from being anecdotally mentioned in pediatrics textbooks, to being widely treated in Pediatric Congresses and Meetings and appearing as a preferred chapter in books and current Pediatric journals.

In Spain until 1985 this subject was briefly mentioned in the miscellaneous sections of Pediatric textbooks. There were few updated bibliographic reviews on SIDS and care priorities led Spanish pediatricians to knowing the least about this complex entity.

In 1986, the first protocolized clinical study on SIDS cases was carried out in five pediatric Spanish hospitals. They realised that in most countries of the European Union there was a progressive increase in its rate of mortality and bearing in mind the lack of knowledge about the real situation, it aroused the interest of many pediatricians in the subject.

In 1991, the "Asociación Española de Pediatría (AEP)" created a working group for the Study and Prevention of Sudden Infant Death (GEPMSL: "Grupo de trabajo para el Estudio y la Prevención de la Muerte Súbita del Lactante") which included members of different medical specialties (pediatricians, neonatologists, forensics, pathologists, epidemiologists, neuropsychologists, researchers, biologists…). They initiated campaigns to reduce SIDS risk factors such as: promoting infants to sleep on their backs, avoiding overheating the child while sleeping and eliminating smoking in the child's environment. In 1996, GEPMSL launched the first edition of "Libro Blanco de la Muerte Súbita Infantil"(4), carrying out a brief review of the subject, including action protocols for Primary and Hospital Care professionals.

Since then, it has been updated with two other editions, the last one in 2013. (5)

Evolution of SIDS knowledge in Spain	
1985	Spanish pediatricians had little interest in it.
1986	First clinical study on SIDS carried out in Spanish hospitals.
1991	AEP creates the GEPMSL. Beginning of campaigns to reduce SIDS risk factors. Start of a remarkable reduction in incidence.
1996	GEPMSL publishes the "White Book on Sudden Infant Death"
2013	Last update of the "White Book"

Table 1. Evolution of SIDS knowledge in Spain. Adapted from (5)

1.1.2. Epidemiology

The growing interest and recent advances in the understanding of the pathophysiology of SIDS have had an impact on the incidence. The most important advance has been the discovery of the relation between SIDS and the prone sleeping position. The decrease in the prevalence of sleeping in the prone position has been accompanied by a decrease in the incidence of sudden death.

Given this epidemiological evidence, at the beginning of the 1990s, national and international prevention campaigns were launched recommending the supine position for infants during sleep. Since then, it is estimated that the incidence has fallen below 50%, from 1.2 per 1,000 births in 1992 to 0.55 per 1,000 live births in 2006. (1)

Despite the latest advances in research, SIDS still remains one of the main causes of death for children between one month and one year, with 85% occurring in the first 6 months, in developed countries. Current data suggests that approximately between 60-80% of these deaths remain with negative autopsy.

Among developed countries, SIDS rates are widely variable. In the US, SIDS rates are approximately twice high among babies from African American or Indian mothers compared to the Caucasian, and the increase in SIDS risk is also seen in Maori from New Zealand, Australian aborigines and those of mixed ancestry in Cape Town, South Africa.

In Spain according to data from the National Institute of Statistics, SIDS has an incidence of 3-4 cases per 10,000 live births. In the last few years between 45-50 babies under one year died each year due to SIDS and if we compare the incidence with the latest years, the trend is clearly downward: in 1990, sudden death affected 134 babies, 70 in 2000 and 57 in 2010. (5)

1.1.3. Etiology and pathophysiology

In recent decades SIDS has been substantially demystified due to the great advances in understanding its relationship with sleep and homeostasis, overheating, maternal smoking before and after childbirth, genetic risk factors or biochemical and molecular alterations.

The pathophysiology of the SIDS is understood with the **triple risk model**, suggested by Filiano and Kidnney. This theory defends that it is not possible to determine a single SIDS risk factor but that it is the result of the interaction of three overlapping factors: a vulnerable infant, during a critical developmental period in homeostatic control, exposed to an exogenous stressor(6).

Vulnerability*:*

- **Central nervous system and autonomic abnormalities**: Neuropathologic findings from SIDS victims show significant deficits in brainstem and cerebellar structures involved in autonomic

functions such as the regulation of the respiratory drive, cardiovascular control, crying, sleep/wake transition and arousal from sleep.(7)

There are many theories (2,8–10) that suggest alterations in serotonin and acetylcholine:

- The serotonergic system plays a role in the coordination of respiration, sensitivity to carbon dioxide (CO_2), body temperature, blood pressure management and autonomic function. When children sleep on their stomachs or have their faces covered by bedding, they re-breathe the CO_2 they have exhaled and this increase in CO_2 stimulates the breathing and arousal centers in the brain.

  Under these conditions, a normal baby would wake up, roll over, and begin to breathe faster when CO_2 levels rise. In contrast, infants who suffer from autonomic abnormalities such as impaired serotonin production and utilisation system, their respiratory reflexes to waking up would be affected resulting in a lack of response to asphyxia with progressive hypoxia, coma and death. (11)

- Acetylcholine (Ach) is the most abundant neurotransmitter of the nervous system. It is a messenger synthesised from a choline and an acetyl CoA that is essential for the communication between neurons.

  Ach regulates attention and memory helping to assimilate new information and to take care of our state of mind. Not only does it act as a messenger, but it also increases the intensity of signals between neurons through theta waves, optimising memory, neuroplasticity, and communication. It is found in both the central and peripheral nervous systems and it has both excitatory and inhibitory functions in order to maintain balance in the organism.

  In the central nervous system, Ach behaves excitatory. Thanks to its interaction with the nerve cells it promotes the processes of motivation, excitement and attention.

  It acts on the cerebral cortex and also in the hippocampus in order to carry out these processes and give structure to higher executive functions like problem-solving and contemplation.

In the basal forebrain Ach promotes rapid eye movement (REM) sleep. During this phase of paradoxical sleep or fast sleep is when memories and information obtained during the day are stored.

Moreover, during this period there is a higher heart rate, a higher blood pressure and also a greater variability, conditioning an increased risk of cardiorespiratory disturbances. It is very important in newborn babies as they spend 70% of the day sleeping and 50% of that time is in REM sleep. (9,12)

It is also a mediator in the perception of the pain, guaranteeing survival. Ach also has an endocrine function on the pituitary gland, a secretory gland located at the base of the skull which is in charge of controlling the activity of other glands such as the thyroid gland or the adrenal glands to regulate certain body functions: being able to control the amount of urine excreted or stimulate the production of thyroid hormones.

In the peripheral nervous system, Ach also has relevant functions for well-being, acting as a bridge between the brain, nerves, muscles and bones, controlling movements. In the cardiovascular system, it mainly acts as a vasodilator reducing and balancing the heart rate. In the gastrointestinal system it favours digestive contractions and in the urinary tract it promotes the voluntary sensation of evacuation.

It will be important to take Ach into account in the pathophysiology of SIDS since a **decrease in the available acetylcholine will result in an impaired arousal response and dysfunction of many systems in the organism**.

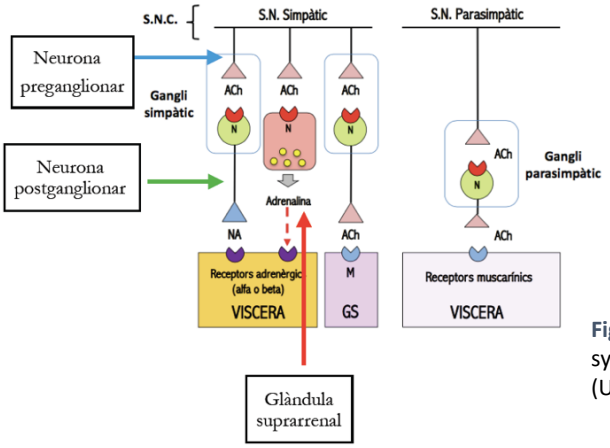

Figure 1. Functioning of the autonomic nervous system. Adapted from Dr. Enric Verdú Navarro (Universitat de Girona)

- **Genetic involvement**: Its implication is justified by the persistence of cases despite the attempt to reduce environmental risk factors. And also because of the higher incidence in families that have already had an affected child, having a recurrence rate of 2%. (11) Nevertheless, there is still much to investigate. Attempts with next generation sequencing (NGS) techniques have shown many variants of uncertain meaning due to the variable penetrance of these genes, entailing a difficult interpretation (2).

- **Maternal and pregnancy related factors**: Intrinsic risk factors.

RISK FACTORS	PROTECTIVE FACTORS
Maternal factors - Smoking - Young mother under 20 years - Drugs consume during pregnancy - Inadequate prenatal control - Complications during pregnancy or delivery: previous placenta, placental abruption, premature rupture of membranes, pre-eclampsia or eclampsia. - Obesity - Anemia	- Breastfeeding - Vaccination - Sleeping in the same room as the parents but without sharing bed - Using a pacifier - Adequate temperature and ventilation.
Baby factors - Gender: Boy - Race: Afro American - Prematurity - Low birth weight - Sibling of an infant who died from SIDS - Multiple gestation - History of apnea	

Table 2. Risk and protective intrinsic factors. Adapted from (3)

A critical developmental period

This point is reinforced by the age distribution of the cases. Between 85-90% of the SIDS occur during the first six months, with a peak in the 2-4 months, a period described as the "developmental window of vulnerability" when dramatic maturational changes in the brain, the autonomic control, ventilation, sleep-waking states, temperature regulation and circadian

rhythms take place in order to complete the transition from fetal life to extrauterine life and maintain the homeostasis. (3,6,11)

External stressor (Extrinsic risk factors)

Physical stressors near death.

Epidemiological studies have identified numerous common factors in SIDS victims such as the prone or side sleeping position, overheating, suffocation from sleeping on soft surfaces, heavily wrapped or with pillows; recent infection or tobacco smoke exposure which may disrupt the homeostasis.

Most of the SIDS occur during the REM sleep, a stage characterised for the dysregulation of various mechanosensory and chemosensory autonomous reflexes that are critical for survival.

These afferent inputs contribute to a phasic activation of the genioglossus (an extrinsic muscle of the tongue) during inspiration, keeping the upper airways open during the inspiratory phase. A dysregulation could be detrimental, triggering a collapse during sleep(2).

Furthermore, anything regurgitated or refluxed from the esophagus must work against gravity to be aspirated into the trachea. When a baby is in the stomach sleeping position, anything regurgitated or refluxed will pool at the opening of the trachea, making it easier for the baby to aspirate or choke(13). That is why it is very important to promote a safe sleeping environment.

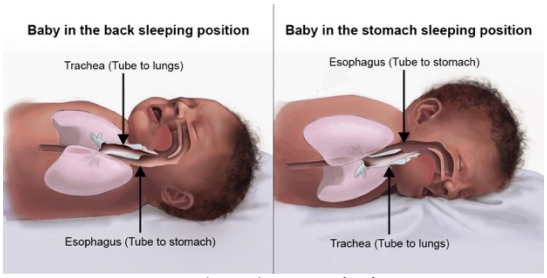

Figure 2. Anatomy when sleeping. (13)

This model suggests that although death is sudden, the putative underlying vulnerability may be present subclinically for days or months before death, even possibly originating during gestation and remaining latent until the vulnerable infant is sufficiently stressed in the critical postnatal period.

According to it, SIDS deaths only occur if all three factors interact. This theory explains the apparently asymptomatic behavior of most SIDS victims prior to death, and helps to justify why eliminating the external stressor may reduce the incidence.

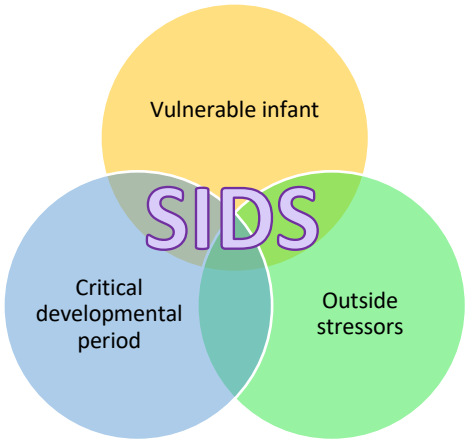

Figure 3. The triple risk model. Adapted from (2)

1.1.4. Prevention

In SIDS the pediatrician is always late so the efforts must be focused at its prevention. As more than 95% of SIDS cases are linked to one or more risk factors, the majority of which are changeable, providing a safe sleeping environment for the newborn, avoiding risk factors and strengthening protective factors is of utmost importance.

However, these factors do not allow the identification in advance of the infants who will suffer from unexpected death. Therefore, its modification should be directed to all infants and should be started as soon as possible.

Recommendations include (14,15): (**Annex 1** – SIDS prevention measures)

1. **Assuring a safe sleeping environment**:
    a. "Back to sleep": Supine sleeping position (on the back)
    b. Use of a hard surface and avoiding soft protectors, pillows or blankets as these objects can invoke the possibility of covering the child's head and cause drowning or suffocation.
    c. Adequate temperature, avoiding excessive heat in the room.
2. **Increase or promote breastfeeding**: Due to the various health advantages for both women and their newborns, breastfeeding is generally accepted as the best approach to feed infants. Breastfeeding encourages shared sleep which enhances breastfeeding frequency and lengthens breastfeeding by months. Together, breastfeeding and sharing a sleeping surface form an integrated care system that is mutually reinforcing. Additionally, there is a significant

correlation between nursing and baby sleep patterns, with breastfed babies displaying night waking behavior that is essential for both nutrition and continued stimulation of the mother's breast milk supply.

3. **Sharing the room with the infant but not the bed**. Co-sleeping is defined as a mother and/or her partner (or any other person) being asleep on the same sleep surface as the infant. It is beneficial to promote breastfeeding.

   However, it is not recommended when there are other risk factors associated since they contribute to worsening the sleeping environment:

   a. Infants under three months of age.
   b. Prematurity and low birth weight.
   c. Parents who consume tobacco, alcohol or other sedative drugs.
   d. Situations of extreme fatigue such as the immediate postpartum period.
   e. Co-sleeping on soft surfaces, water mattresses, sofas or armchairs.
   f. Sharing a bed with other family members, with other children, or with multiple people.

4. Advice **vaccinations**. (16) A 50% decrease in the risk of SIDS is correlated with vaccinations. Biological considerations are the primary justification, yet there may be other essential factors as well.
5. **Consider the use of a pacifier**. Pacifiers are a potential method to reduce the risk of SIDS. It should be offered to the infant up to one year of age when being placed for all sleep episodes including daytime naps and nighttime sleeps.(17)
6. **Toxics**:

   a. Avoid exposure to tobacco in all its forms: active (avoiding or advising the pregnant woman not to smoke) and passive (smoking in her environment).

      Tobacco smoke increases the risk of SIDS by 400%. Nicotine acts by decreasing the autonomic arousal border in the cerebellum and the brainstem. (8)

      Several studies have revealed abnormal cardiovascular responses to various stimuli such as hypoxemia or CO_2 exposure and difficulty awakening after stimulation in infants born from mothers who smoked during pregnancy.(14)

b. Discourage the consumption of alcohol or any other type of drug by both parents since they can trigger a state of sedation in parents, reducing the children's awareness and responsibility.

Many times the risk is not determined by a single factor but rather by a combination of several. For example, there is a higher incidence of prematurity with low birth weight and/or other postnatal conditions coexisting such as: an unfavourable socioeconomic environment, risk behaviours such as co-sleeping, tobacco consume or other drugs, psychiatric problems... All these risk factors added together condition a vulnerable child.

Therefore, the demonstration that the supine position during sleep is the safest for infants has been an important milestone in pediatrics. A simple and efficient parenting recommendation to avoid deaths from SIDS has derived from it and has had an impact on infant's mortality (18).
In 1994, with the implementation of preventive educational campaigns focused on promoting the supine position during sleep, directed to parents and to health care professionals, achieved a decrease in the prevalence of SIDS in the United States of America by 1.3 per 1,000 live births in 1990 to 0.38 per 1,000 live births in 2016, which meant avoiding approximately 1,500 deaths from SIDS in 2016 (1).

U.S. SIDS Rate and Sleep Position, 1988—2010 (Deaths per 1,000 Live Births)

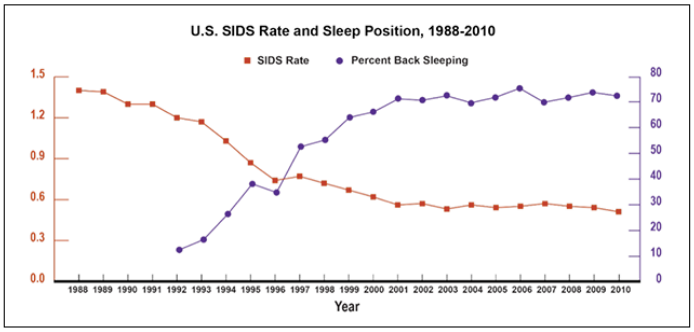

Figure 4 Relation between sleeping position and SIDS (1)

1.1.5. Postmortem investigation

According to the "Ley de Enjuiciamiento Criminal" Articles 340 and 343, if the investigation takes place due to a violent or suspicious death, before proceeding with the burial of the corpse or

immediately after its exhumation, it will be identified by means of witnesses who give a satisfactory reason for their knowledge. Even when the cause of death can be presumed by external inspection, the autopsy of the corpse will be carried out by the Forensic Doctors or, where appropriate, by those designated by the Judge, who, after exactly describing said operation, will report on the the origin of the death and its circumstances.(19)

Bearing the legislation in mind, a forensic autopsy with judicial investigation is required in all violent and suspicious deaths, including sudden and unexpected natural deaths in non-hospitalized children whereas in natural deaths it is only needed a medical death certificate signed by the doctor who was treating the patient.

In Catalunya, the "Institut de Medicina Legal de Catalunya (IMLC)" is the technical body attached to the Department of Justice whose mission is to assist the courts, tribunals and prosecutors' offices and Civil Registry offices through the practice of medical expert tests, both thanatological and clinics and laboratories, provided for in the current regulations on forensic medicine, that are related to forensic medicine.

The IMLC has a centralised forensic pathology service with five autopsy rooms distributed in Girona, Barcelona, Tarragona, Lleida and Terres de l'Ebre, that follow a common protocol to perform autopsies. They have an established guide of specific recommendations for the unification of the judicial autopsy. It is a specific protocol that must be followed in the 5 autopsy rooms in order to rule out all the possible causes of the SUDI. (20)

The protocol designed by the IMLC includes: (21,22) : (**Annex 2** – Epidemiological survey).

1. **Clinical History**: family history, social aspects, obstetric history, pathological history.
2. **Removal of the body**:
    a. Time when last seen alive and time when found dead.
    b. Analysis of the death scene: room temperature, layers of clothing, overheating...
    c. Check whether the place where the child was found was where they regularly slept, position of the body in the bed, sharing bed or not, rule out suffocation by bedding or clothing, hazardous objects near the child that could obstruct airways.
    d. Findings of alcohol, drugs, medication or toxic substances near the child.
    e. Check whether resuscitation has been attempted (start time and duration).

f. Food: breastfeeding, artificial milk, mixed… and last shot (time, time, quantity).

g. Photography during the removal.

3. **Preliminary diagnostic tests**: Radiological studies of the whole body (skull, spine, chest, abdomen, limbs), photographic studies and taking fingerprints or footprints for genetic identification.

4. **Autopsy**:

   a. External examination:

      i. Description of race, apparent age, gender, hair colour, iris colour, birthmarks

      ii. Nutritional (normal, deficient, obesity) and hydration status (skinfold, fontanelle, eyes, tongue).

      iii. Weight and measures (bodyweight, total length, head circumference, biparietal diameter, chest and abdominal circumference, limb length…), sexual characteristics, natural orifices.

      iv. Rule out airway obstruction due to foreign bodies, food or vomiting. Assess the presence or absence of secretions or blood in the mouth.

      v. Rule out signs of violence or suspected child abuse (old scars, ecchymosis, hematoma, abrasions…)

      vi. Presence of petechiae on skin and mucous membranes, conjunctiva…

      vii. Evidence of cardiopulmonary resuscitation: marks on face, chest ecchymosis, ECG suckers, defibrillator marks, venipuncture…

   b. Internal examination: Complete autopsy preferably using bloc evisceration.

   c. Sampling:

      i. Histopathological study: respiratory, cardiovascular, digestive, mononuclear phagocyte system, endocrine, urinary, brain, musculoskeletal and skin.

      ii. Microbiological and virological studies: nasopharyngeal aspirate, throat exudate, feces, blood, serum, cerebrospinal fluid (CSF), body fluids and swabs of any lesions.

      iii. Study to detect botulinum toxin

iv. Chemical-toxicological study: peripheral blood, urine, gastric contents and vitreous humor.
v. Genetic studies: peripheral blood.
vi. Study of metabolic diseases: serum, plasma, CSF and urine.

Diagnosis is essentially by exclusion after performing the autopsy, studying the medical history and investigating the circumstances of death.

Differential diagnosis (**Table 3**) of situations that must be excluded:

General	- Sepsis - Asphyxia - Anaphylaxis - Metabolic decompensation - Sickle cell anemia
Cardiac	- Subendocardial fibroelastosis - Congenital heart disease - Myocarditis - Channelopathies
Pulmonary	- Pneumonia - Bronchiolitis, Severe tracheobronchitis - Idiopathic pulmonary hypertension - Impaction of a foreign body
Renal	- Intoxication - Pyelonephritis
Digestive	- Bacterial enterocolitis - Intestinal obstruction - Intestinal perforation with peritonitis
Liver	- Intoxication - Hepatitis - Fatty deposits (metabolopathies)
Pancreas	- Pancreatitis - Boric acid poisoning - Cystic fibrosis
Adrenal	- Congenital adrenal hyperplasia
Cerebral	- Encephalitis, meningitis - Trauma (fractures, hemorrhages)

	- Arteriovenous malformations, intracerebral hemorrhage.
Musculoskeletal	- Fractures, dislocations - Soft tissue and bone: infections, inflammation

Table 3. Situations that can act like a SIDS. Adapted from (11)

1.1.7. Classification

In January 2004, a meeting was held in San Diego, California, with the participation of pediatric pathologists, forensic pathologists and pediatricians with extensive experience in sudden infant death from Europe, North America, and Australia.

In this meeting they established a new definition of SIDS where they did not limit saying the diagnosis was one of exclusion, but they defined it as a syndrome itself with a common cause or mechanism of death since researchers saw that there were a series of characteristics that were repeated in most of the cases such as the association with sleep or the higher incidence in the period of 2-4 months.

As a result, they created the San Diego classification to try to stratify and separate typical cases from atypical ones taking into account the presence or absence of a series of histopathological findings as inclusion criteria in different categories: IA, IB, II or indeterminate. (22,23)

	Clinical criteria	**Circumstances of death**	**Autopsy**
Definition	Sudden and unexpected death. Less than 1 year. During sleep.	Unexplained by circumstances	Unexplained by a full autopsy
Category IA	>21 days - <9 months Normal medical history Full-term pregnancy (>37SG) Normal development and growth Absence of similar deaths in siblings/relatives	Investigation of the scene and circumstances Safe environment	No fatal findings No trauma Not thymic stress Additional studies negative
Category IB	>21 days - <9 months Normal medical history Full-term pregnancy (>37SG) Normal development and growth Absence of similar deaths in siblings/relatives	No investigation	One or more complementary studies are missing

Category II	<21 days, >9 months Preterm Resolved neonatal pathology Similar deaths in siblings/close relatives	Suspected mechanical asphyxia or suffocation (not proven)	Abnormal development and growth. Marked inflammatory changes.
Indeterminate	Criteria for categories I or II are not met	Doubtful differential diagnosis between natural or violent death	No autopsy is performed

Table 4. San Diego classification (22,23)

1.2. NEONATAL SCREENING PROGRAM FOR CONGENITAL METABOLIC DISORDERS IN CATALUNYA (HEEL PRICK TEST)

Neonatal screening tests are widely and internationally recognized secondary prevention interventions in the field of public health. It is evolving very rapidly in the Western world due to the expansion of knowledge of diseases, improvements in treatment and the emergence of new technologies that make it possible to detect them during the first days of life.

Figure 5 Heel Prick test(24)

Its aim is to detect, diagnose and treat, early from birth, certain serious diseases which are minority and of genetic origin in order to apply before the appearance of the symptoms, an appropriate treatment to avoid sequelae and future complications and guarantee a better life quality (24). The Program is a very effective tool offered to all children born in Catalunya. However, it is not mandatory **Annex 3** – "Heel prick test".

1.2.1. Phases

The program consists of several phases: (**Annex 4** - Algorithm of the Neonatal Screening Program of Catalunya)

1. **Universal screening** of all boys and girls born in maternity centres in Catalunya from both public and private centres by obtaining a blood sample from the baby's heel and carrying out screening analytical tests.

   Procedure:

   A superficial puncture is taken on the posterolateral region of the heel. Current guidelines recommend that samples are taken from a limited area on the lateral borders of the plantar surface of the heel to avoid puncture (and thus infection) of the calcaneum. Tissue injury caused by the lance results in inflammatory change which makes the area tender and therefore hypersensitive to further pricks (25).

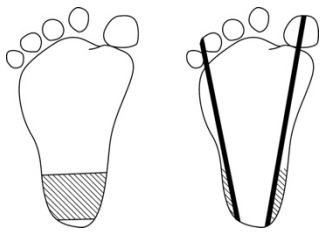

Figure 6. Heel prick punction (25)

   The resultant drops of blood are extracted and soaked in an approved absorbent paper.

   This paper is sent to the laboratory to be analyzed with a baby and family data sheet.

The sample can be taken between 48 and 72 hours after birth. It is usually carried out at the maternity hospital where the child was born.

The puncture causes minor discomfort to the baby which can be alleviated by putting him to the mother's breast.

The analysis of the sample is performed in the "Laboratori Central de Cribratge Neonatal de Catalunya" in the "Centre de Diagnòstic Biomèdic de l'Hospital Clínic de Barcelona".

Screening can also detect healthy disease carrier children who have a related genetic alteration but will never develop it.

The parents have the right to be informed, but they can also express their wish not to know.

Performing the test only takes a few minutes, and the results will be received approximately three weeks later. In most cases the test results are normal and in the event that an abnormality is detected, medical professionals will contact the family.

2. **Definitive diagnosis** of babies with a positive screening result.

   Sometimes a second sample may be requested. This does not mean that the newborn has the disease but that new analyses are needed to complete the process as early detection is just a prior step to diagnosis which makes it possible to identify among all newborns in a population those who are most likely to suffer from a certain disease.

   In the event that a second examination came out positive again, more specific clinical, biochemical and in some cases genetic diagnostic tests would be carried out by Clinical Expertise Units (UEC).

3. **Early treatment** to avoid complications and sequelae and guarantee a better quality of life.
4. **Follow-up** of babies from UEC. Controls are coordinated between the regional reference hospital, the primary care center and the UEC.

1.2.2. Screened diseases

Newborn screening seeks to rule out any inborn errors that could have lasting effects or elevated mortality rates. Many of these disorders result from enzyme defects within metabolism, leading to unnatural accumulation of intermediate metabolites that can be toxic. (26)

Inclusion criteria for the Neonatal Screening Program:

In 1970, the National Academy of Sciences wrote the Wilson and Jungner Criteria, a series of characteristics that a disease must meet so that it can be included in a screening program.
Later, the "Hospital Sant Joan de Deu" revised and updated them. Currently, in order to include a disease in the neonatal screening program it must accomplish: (27)

- A disease that progresses with serious mental and physical damage or vital risk during the neonatal period.
- No possibility of effective clinical diagnosis in the neonatal period.
- Available effective or palliative treatment that is affordable and that improves the quality and/or life expectancy of the patient.
- Early initiation of treatment improves clinical prognosis.
- Fast, sensitive, reliable and reasonable cost analytical methodology.
- There must be diagnostic, treatment and clinical follow-up units for the pediatric patient and also when the patient reaches adulthood.
- There must be coordination between the maternity centers where the sample is taken, the screening laboratory, the confirmation laboratory (when different from the screening laboratory) and the diagnostic, treatment and clinical follow-up units.
- Relatively high incidence (1/10,000-15,000) by itself or in combination with those that are detected in the same analytical process.
- The beneficiaries of the inclusion of the disease in the screening programs must be: the child in the first place, but also the family and society.

The early neonatal detection program in Catalunya has been expanding over the years and it currently includes up to 24 minority diseases integrated into more than 7,000 clinical conditions that have a low incidence in the population.
Screening began, in its early years, by being able to detect phenylketonuria, an hereditary genetic disease. It expanded in the 80s with congenital hypothyroidism, the early treatment of which allows the prevention of serious disabilities, and by the year 2000, with cystic fibrosis, a disease that causes important digestive and respiratory disturbances.
The impact on the detection of these serious diseases with such a simple test was so great that its use has expanded over time.

	2003	2004	2005	2006	2007	2008	2009	2010
Number of births	73.295	77.219	80.448	82.329	89.448	89.327	85.347	84.071
Phenylketonuria	7	8	5	14	7	15	5	16
Congenital Hypothyroidism	25	41	48	33	32	43	30	35
Cystic Fibrosis	12	17	16	11	12	11	9	5

Figure 7. Newborn babies diagnosed with the screening program in Catalunya (24)

In 2013, the detection of a group of 19 metabolic diseases was included, and in 2015, sickle cell disease. Finally, in 2017, the Catalan public health system became the first in Europe to be able to detect severe combined immunodeficiency, a genetic disease of the immune system that every year could affect between 1 and 4 babies in Catalunya. (Annex 5 – Metabolopathies detected in the neonatal screening program)(24).

Summing up, there are twenty-four diseases that can be assessed: congenital hypothyroidism, cystic fibrosis, phenylketonuria and five other disorders of amino acid metabolism, eight disorders of organic acid metabolism, six disorders of the fatty acid metabolism, sickle cell disease and severe combined immunodeficiency.

After the analysis, the samples are **stored for at least five years** in the laboratory in case they could be useful for carrying out new analyses, for the benefit of the child himself (28).

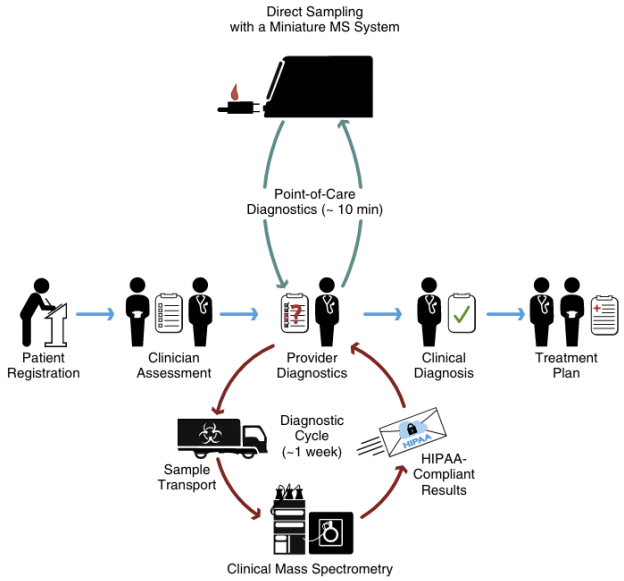

Figure 8 Screening process. (26)

1.2.3. Analytical techniques

Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) is a technique that identifies compounds from a biological sample based on the mass-to-charge ratio. An important benefit of MS in the field of metabolomics and lipidomics is its high sensitivity and structural characterization of metabolites and lipids (29). Mass analysis is performed by a dissociation process or a chemical reaction that allows the change of the mass or the charge of an ion. The most common dissociation method is collision-induced dissociation (CID) in which the target molecule exposed in a first mass analyzer (MS1) undergoes a fragmentation process so that a second analyzer (MS2) can analyse the product ions and forms.

There are several scan modes (26):

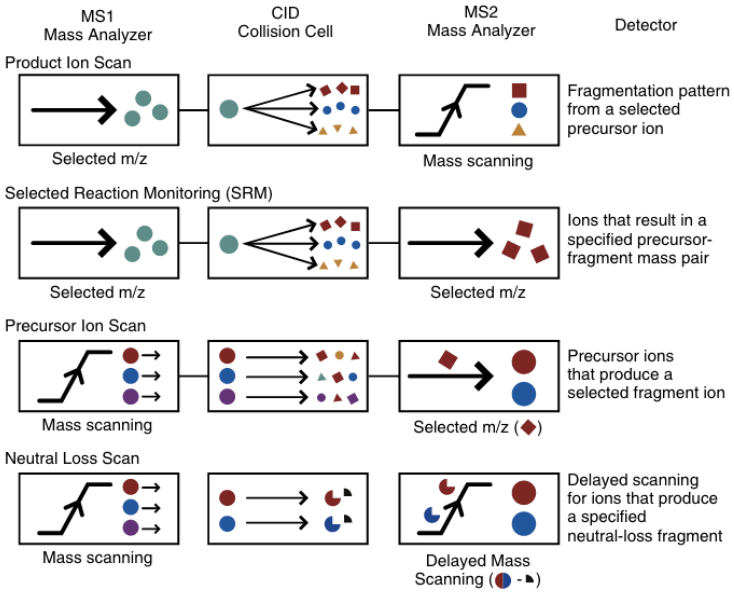

Figure 9 Scan modes for MS/MS spectrometry. Extracted from (26)

This technique can assess the presence of a metabolite based on its concentration. It is a very useful technique as it allows the evaluation of several metabolites simultaneously. **Targeted analytic mass spectrometry** focuses on identifying known diagnostic molecules within patient samples, being an optimal resource for metabolite screening.

Drs Millington and Chace's groups (Millington et al., 1990; Chace et al. 1999) were the first to implement MS/MS in neonatal screening by analyzing amino acids and acylcarnitines, which

allowed the identification of a good number of aminoacidopathies (including phenylketonuria), organic acidurias and deficiencies of mitochondrial β-oxidation of fatty acids.

It is remarkable that this multiple measurement allows one analyte to be assessed against another. Thus, in the case of phenylketonuria, the concentration of phenylalanine and the phenylalanine/tyrosine ratio can be measured, improving the specificity of the screening and avoiding false positives and above all false negatives. (30)

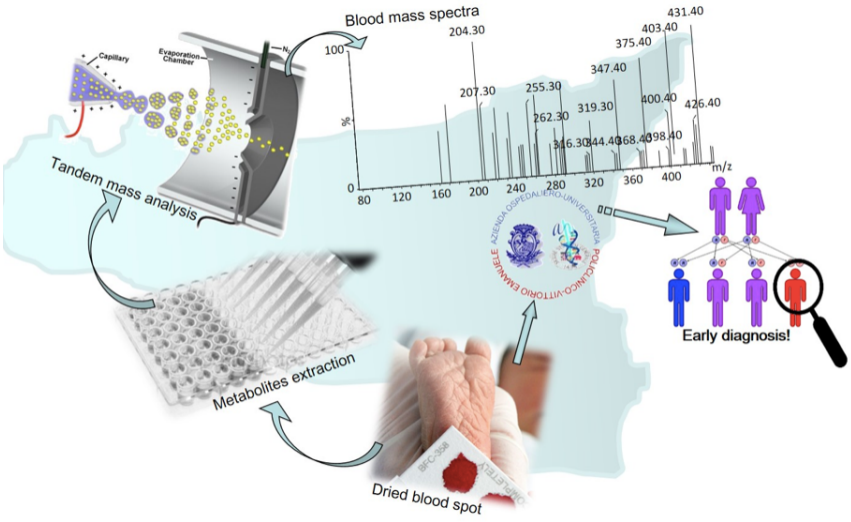

Figure 10. Newborn screening. Tandem mass spectrometry (30)

MS/MS is necessary to detect a significant number of the diseases proposed in the screening program. Nevertheless, other analytical techniques should be used for other diseases.

Other techniques (24)

- **Fluoro Immuno Analysis**: Fluoroimmunoanalysis is an immunometric analytical method in solid phase in which monoclonal antibodies recognize two antigenic determinants of the molecule we want to measure. With this technique, congenital hypothyroidism, cystic fibrosis and congenital adrenal hyperplasia are detected.
- **Spectrophotometry**: Spectrophotometry is the most common method of optical analysis in clinical laboratories. The spectrophotometer is an instrument that allows the measurement of light radiation, in the ultraviolet and visible spectrum, absorbed by a solution containing

an unknown amount of substance, by comparing it with another solution containing a known amount of the same substance.

This technique is the most used to detect biotinidase deficiency and galactosemia.

- **Fluorimetry**: Analytical method based on the absorption of light radiation by the molecule we want to measure. We currently use this method to detect phenylalanine.
- **Capillary electrophoresis**: Separation technique based on the mobility of the analytes in an electric field. Sickle cell anemia and other hemoglobinopathies can be detected with this technique.
- **High performance liquid chromatography**: Chromatographic technique that uses a column to separate the components of a mixture based on different types of chemical interactions between the substances to be analyzed and the chromatographic column. This technique detects sickle cell anemia and other hemoglobinopathies.

1.3. BUTYRYLCHOLINESTERASE

Esterases are enzymes that catalyze the hydrolysis of ester bonds, they belong to the group of hydrolases.

The term cholinesterase was proposed in 1932 in order to describe an enzyme capable of hydrolyzing acetylcholine and other choline esters. Cholinesterases have an extraordinary ability to hydrolyze choline esters faster than other esters under optimal conditions of substrate concentration, pH and ionic strength.

In 1940, Alies and Hawes (1940) found out that there were two different enzymes hydrolyzing choline esters in human tissues with different origins, structures, specificities of action and physiological actions. Later in 1943, Mendel and Rudney (31) classified them into Acetylcholinesterase or true cholinesterase (AChE, EC 3.1.1.7) and Butyrylcholinesterase or pseudocholinesterase (BChE, EC 3.1.1.8).

AChE and BChE have numerous physiological functions depending on their localization and time of expression (32).

1.3.1. Acetylcholinesterase

Acetylcholinesterase or intracellular cholinesterase is found in the white matter of the central nervous system, cholinergic ganglia, cholinergic neuromotor plaques, membrane-bound neurons, ganglion synapses in the neuromuscular structure and erythrocytes (33).

It is an asymmetric protein composed of simple and soluble oligomers (monomers, dimers or tetramers) or complex molecular forms of three tetramers linked by a filament. Its fundamental structure is a catalytic subunit of 70,000 daltons.

It optimally hydrolyzes acetylcholine catalyzing the following reaction: ACh + H2O = Choline + Acetate (34) in a very fast reaction of only 150 microseconds approximately. (33)

Therefore, it plays a fundamental role in neuromuscular transmission. Its inhibition in cholinergic synapses and neuromuscular junctions leads to a potentially fatal major cholinergic syndrome.

Its regulation is through negative feedback. Under conditions of high brain activity, raised concentrations of local synaptic Ach can reach micromolar levels that approach inhibitory levels for AChE activity. (32)

	AChE	BChE
Name	Acetylcholinesterase	Butyrylcholinesterase
Enzyme number	3.1.1.7	3.1.1.8
Response to substrate excess	Inhibition	No inhibition
Optimum pH	7'5-8	8'5
Tissues with high activity	Erythrocytes Nervous tissue Thymus	Blood Pancreas Heart Liver

Figure 11. Properties of cholinesterases. Adapted from (31)

1.3.2. Butyrylcholinesterase

Butyrylcholinesterase is also called serum, plasma or pseudocholinesterase as it is extracellular. Synthesised by hepatocytes, it is present in most tissues and organs such as pancreas, liver or the central nervous system (mainly in the astrocytes and microglia (34)), with the exception of erythrocytes (32).

The gene encoding BChE is located on the long arm of chromosome 3 (3q26.1- q26.2). It has four exons, three of which are translated (exons 2 to 4).

The major and functional circulating molecular form of BChE is a **tetramer** of identical **monomeric subunits** which have a **molecular weight of 84,000 daltons** each (33).

Every associated protein sequence of polypeptides comprises 602 amino acids of which 28 correspond to a signal peptide and 574 to the mature protein (35), and 9 carbohydrate chains attached to 9 asparagine molecules.

The four subunits interact via their C-terminal domain, a polyproline-rich peptide located in the center of the bundle composed by the four subunits. The oligomeric structure of BChE and its strong glycosylation (25% of its mass) determine its pharmacokinetic properties.

The catalytic triad (Ser198/ His438/ Glu325) is located at the heart of the protein, the catalytic serine being accessible by a deep groove. Different motifs play a fundamental role in enzymatic activity. The binding of a substrate on the peripheral anionic site (Asp70/Tyr332) leads to a conformational modification of the enzyme with a bringing together of the two ends of the loop. The substrate then slides to the cationic site (Trp82). The oxyanion hole (Gly116/Gly197/Ala199) and the acyl pocket (Trp231/Leu286/Val288) ensure the presentation of the substrate to the catalytic triad allowing its hydrolysis. (33,35,36)

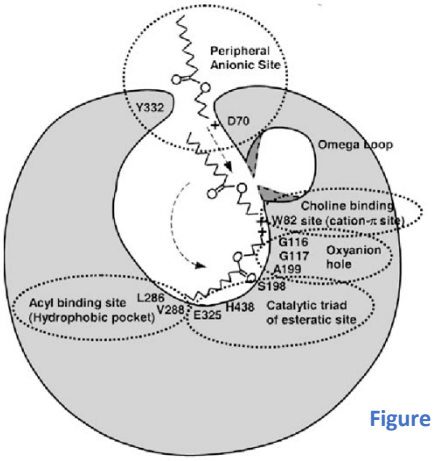

Figure 12. Schematic structure of the active site of BChE (36)

The biosynthesis of the enzyme is controlled by the locus E located on chromosome 3. The type of cholinesterase synthesized by the individual depends on the E1 and E2 loci, both polymorphs. Each loci can have several allelic and codominant genes; depending on which gene is expressed, a different type of cholinesterase is translated. These variations can determine an increase or a decrease in enzymatic activity, with some mutations being capable of altering the ability to hydrolyze ester bridges or even inhibiting it. (33)

Different varieties of BChE enzymes:

Usual BChE (Eu)	Normalcy. Present in 96% of the population. Capable of hydrolyzing ester bridges very quickly.
Atypical BChE (Ea)	Active enzyme with little inhibition by dibucaine[1]
Fluoride resistant BChE (Ef)	Active enzyme with little inhibition by fluoride[2]

[1] Dibucaine is a local anesthetic that under standard conditions inhibits BChE. The dibucaine number (ND) measures the percentage of the enzyme activity that is inhibited by dibucaine. The normal value oscillates around 80% while the atypical variants have it decreased. (33)

[2] Similar to the ND, sodium fluoride is an inhibitor of cholinesterase activity. We define the fluoride number (NF) as the percentage of enzyme inhibition resulting from putting the two substances in contact. Under normal conditions the NF is 60% and when there is resistance to fluoride the value decreases. (33)

Silent BChE (Es)	Very low or no enzyme activity
K Variant (EK)	Most frequent mutation. It associates a decrease in ACh hydrolyzing activity of approximately 30-60%.
Johannesburg Variant (EJ)	Responsible for a higher activity of the BChE.
Hammersmith variant (EH)	It concerns very few individuals. Its significance is not exactly known.

Table 5. Genetic variations. Adapted from (33)

Depending on genetic variability and inherited mutations transmitted in an autosomal recessive pattern, a different type of BChE with more or less action will be expressed, being the homozygous combination EuEu the normal condition with efficient and rapid enzymatic activity with a ND of 80 and a NF of 60. On the contrary, the homozygous combination EsEs entails the silent pattern associated with a drastic reduction in its expression (low or even zero BChE activity)(37).

Tabla 4
Variantes genóticas de las colinesterasas plasmáticas

Genotipo	Incidencia	Respuesta a succinilcolina	Actividad enzimatica	Concentración de la enzima (u/l)	Número de dibucaina	Numero de fluoruro
E_1^u E_1^u	96%	Normal	Normal	690-1.560	80	60
E_1^a E_1^a	1:2.800	Muy prolongada	Muy baja	14-27	20	20
E_1^u E_1^a	1:25	Ligeramente prolongada	Moderadamente baja	433-1.197	40-60	45
E_1^f E_1^f	1:150.000	Moderadamente prolongada	Moderadamente baja	500-1.230	70	30
E_1^s E_1^s	1:100.000	Muy prolongada	Ninguna	–	–	–
E_1^u E_1^f	1:200	Ligeramente prolongada	Ligeramente baja	514-1.150	75	50
E_1^u E_1^s	1:190	Ligeramente prolongada	Moderadamente baja	329-870	80	60
E_1^a E_1^f	1:20.000	Moderadamente prolongada	Moderadamente baja	25-33	45	35
E_1^s E_1^a	1:29.000	Muy prolongada	Muy baja	19-30	20	19
E_1^f E_1^s	1:150.000	Moderadamente prolongada	Muy baja	26-38	60	35

u: gen de la enzima normal. a: gen de la enzima resistente a la dibucaína. f: gen de la enzima resistente al fluoruro. s: gen silente.

Figure 13. Genetic variants of BChE and its activity. (33)

BChE belongs to a group of enzymes (examples: catalase, fumarase, acetylcholinesterase) which have almost reached catalytic perfection. Its catalytic efficiency is so great with respect to its preferred substrate butyrylthiocholine, that it is only limited by the diffusion coefficient of the molecules in a liquid.

No physiological function has yet been clearly attributed to it. Individuals with deficient BChE do not develop any particular clinical disease. So far it has only been demonstrated a greater sensitivity to certain toxins.

BChE plays an important role in the metabolism of many pharmacologically active substances comprising an ester function. A deficiency in the enzyme activity would lead to a prolongation of the neuromuscular block for up to several hours (37).

In the healthy human brain, AChE predominates over BChE.

It is important to take into account the difference in their kinetic response to concentrations of Ach. BChE is less efficient in Ach hydrolysis at low concentrations but highly efficient at high ones, at which AChE becomes substrate inhibited. So it is important to have a synergistic BChE-mediated hydrolysis assistance in the regulation of local Ach levels to maintain a normal cholinergic function. An alteration in the levels of BChE in situations where there is a deficiency or an absence of AChE could lead to significant clinical repercussions such as: (8)

- Alteration of Ach regulation in the neuromuscular junction.
- Alteration of Ach modulation at the presynaptic level predisposing to neurodegenerative diseases. Reduced Ach hydrolyzing capacity has been related to Alzheimer's disease in the adult's brain(38).
- Severe systemic inflammation.
- Alteration in arousal responses carrying instability of the cardiovascular and respiratory responses.
- Sleep-wake cycle alteration.
- Cholinergic-serotonergic interaction: Decreased serotonergic activity which can lead to an alteration of the protective autonomic respiratory and cardiac reactions through preganglionic broncho vagal response.

2. JUSTIFICATION

Finding an apparently healthy baby dead in the place where he slept is a desperate event for parents, causing a great emotional impact. It is a universal serious problem and despite the success of sleep campaigns and the progress in understanding its risk factors, the rate of reduction in the cases of SIDS has slowed, and still remains a leading cause of post neonatal mortality in many developed countries. (3,39)

This is why it is important to go a little further and try to rule out all possible etiologies and tackle this problem.

Bearing in mind the triple risk model, we can predict that Ach plays a relevant role in the pathophysiology. An alteration in the presence or functionality of BChE causes a decrease in the degradation of the ACh altering the regulation in the neuromuscular junction, empowering severe systemic inflammation, dysregulating the sleep-wake cycle or remodeling the arousal autonomic responses carrying instability in the cardiovascular and respiratory reactions, which can lead to self-resuscitation failure under unfavorable circumstances such as hypoxia or suffocation. (8)

The newborn screening program is a very well implemented test that is already done routinely in Catalunya in order to rule out some inborn metabolic errors that could have lasting effects or elevated mortality rates(26). It could be a great tool to make a first screening to start the diagnosis of the BChE deficiency.

The newborn screening samples are stored for five years, allowing its recovery for the future study of some diseases. For example, the detection of cytomegalovirus (CMV) DNA by real time polymerase chain reaction (rt-PCR) in dried blood spots recovered from routinely metabolic screening has been assessed for the retrospective diagnosis of congenital CMV infection (40).

Considering this fact, these screening samples could also be recovered in order to evaluate the presence or absence of the basic subunit of BChE in infants who died from SIDS, since we assume that if we have the correct monomer, a normo-functioning enzyme has to be translated.

It could be very useful for the medical community, especially the pediatric one, to know the results of the post-mortem investigation of sudden infant death in our country in order to promote prevention measures(14).

To try to relate alterations in BChE, specifically an alteration in its basic monomer, with an increased risk of sudden infant death syndrome, this study would be a good test to do an initial screening. In the event that the value of this test is altered, confirmation examinations will be carried out to determine not only the presence or absence of the functional enzyme but also its activity and epidemiological data. For this second step, other more specific tests should be used, such as the homogeneous ELISA.

The performance of this first screening test would be based on the fact that we are screening for a disease with a low prevalence but which can pose a vital risk for the baby. This is why it is necessary to detect it before it is expressed. Therefore, a test with a very high negative predictive value is needed so that most of the results come out healthy.

The disease is not being diagnosed, but an attempt is being made to detect all or most of the affected children. To do it, a very large population sample is needed and for this reason it must be taken into account that the test must be very sensitive but also easily accessible, non-invasive, fast and with low risk of direct side effects in the early hours, so the use of the "heel prick test" would be justified.

This test does not need to be specific since we will already perform more precise tests in those children in which the first screening comes out altered.

It is also justified to value the presence or absence of the enzyme's basic monomer and not its concentration because it is a pathway that matures over the years and therefore in a newborn infant it would be of little relevance.

In addition, if it was detected that this alteration really existed, as it is a metabolic disease of genetic origin with autosomal recessive inheritance, a family study could be carried out and genetic counseling would be offered.

3. HYPOTHESIS OF THE STUDY

The absence of the basic monomer of butyrylcholinesterase in dried heel blood taken after 48-72 hours of birth in the neonatal screening program is a risk factor for sudden infant death syndrome.

3.1. Justification of the hypothesis

Determining whether the basic BChE monomer is present or absent will be useful as a first step in justifying the relationship between the absence of functional BChE enzyme and SIDS.

We know that the enzyme is fundamentally inhibited in pathological situations, for example, in poisoning by organophosphates, these bind to the catalytic subunit causing its inhibition and reducing its activity, causing neurotoxicity. Therefore, we assume that under normal conditions, with an EuEu genotype, its physiological activity must be between 90-100%. If other mutations are inherited, they will lead to an alteration of its basic monomer making the tetrametric component non-normofunctional and as a result, decreasing the physiological activity of the enzyme.

The purpose of the screening will therefore be to assess in the first place the presence of the basic subunit of the enzyme in dried blood samples from the heel of the newborn babies.

If we take into account the techniques used to analyze these samples, the most optimal procedure to make this determination is tandem mass spectrometry (MS/MS).

We must bear in mind that this technique does not allow us to assess whole proteins since MS/MS relies on volatile and thermally stable samples to be analyzed by using a gas-phase ionization method. For samples that do not have these characteristics, extensive derivation is needed for the analytes to be sufficiently volatile, so MS/MS is not suitable for the analysis of most biological molecules, especially proteins (26). That is why we will have to look for the basic monomer (a subunit of approximately 84,000 daltons).

Nevertheless, it does not pose a problem for us since in the event of a genetic mutation, the monomer would also be affected and would already be reflected in the study.

We assume that if we have a genetic mutation, the basic structure of the amino acid chain will change, and therefore MS/MS will not detect the presence of this 84,000 dalton monomer.

In case we detect this alteration, we will have to continue the study:

1. Repeat the test to rule out that the result was a false positive.
2. Confirmatory diagnosis: We will have to do more specific tests to characterise the alteration:
    a. Activity percentage study
    b. Enzyme concentration study
    c. Molecular study: see which mutation is inherited, what is the genotype...
3. Epidemiological study: study the parents and offer genetic counseling.
4. Clinical translation.

4. OBJECTIVES

4.1. Main objective

The aim of this study is to determine if the absence of BChE's basic monomer in dried heel blood taken after 48-72 hours of birth in the neonatal screening program is associated with enzyme dysfunction and consequently with sudden infant death syndrome.

4.2. Secondary objective

To carry out a descriptive study with the epidemiological information of the cases of the studied period.

5. MATERIAL AND METHODS

5.1. STUDY DESIGN

It is a pilot, retrospective, case-control study.

The study will be carried out in Catalunya.

Cases will be extracted from forensic databases and matched in a 10:1 ratio with living controls selected anonymously.

5.2. SITUATION TO FINISH THE STUDY

If none of the samples from the SIDS children showed a reduced BChE, the study will be forced to cease.

5.3. STUDY POPULATION

The study population is composed of pediatric patients from all Catalunya from whom we have the results of the neonatal screening test and the access to their computerized birth records.

5.3.1. Inclusion criteria

- **Cases**:
    - Infants under one year of age who died due to sudden death of unknown cause.
    - Forensics must have performed a deep case investigation including performance of a complete autopsy, examination of the death scene and extensive review of the clinical history.
    - Children whose parents have signed the informed consent.
- **Controls**:
    - Children included in the neonatal screening program of Catalunya.
    - Children born during the month before or after the date of birth of the cases.

5.3.2. Exclusion criteria

For cases and controls:

- Patients for whom we do not have access to the sample of the "heel prick test".

5.3.3. Withdrawal criteria for cases

- Patients unwilling to attend the program or who revoke the informed consent.
- Delayed identification of a violation of the inclusion and/or exclusion criteria.

5.4. SAMPLING AND SAMPLE

5.4.1. Sample size determination

Bearing in mind that the incidence of SIDS cases is 3-4/10,000 live newborn babies per year and that the birth rate in recent years in Catalunya is approximately: 57.700 (2022), 57.600 (2021), 58.400 (2020)(41), we assume an average of about 20 cases a year. We also know that records of neonatal screening tests are kept minimum for 5 years.

Therefore, if we take all the cases, we start with an initial sample of approximately 100 cases.

Taking this information into consideration we have calculated the size of the sample with the GRANMO program:

- Proportion of controls exposed to the factor: 4% (33)
- Minimum odds ratio to detect: 300%. Regarding to the clinical impact, we assume that having the mutation triples the risk of having SIDS.
- Ratio between the number of controls and cases: 10. Every case will be paired with ten controls.
- Expected loss-to-follow-up ratio: 10%. We consider a risk of loss of 10%.

Accepting an alpha risk of 0.05 and a beta risk of less than 0.2 in a bilateral contrast, **88 cases and 880 controls** are needed to detect a minimum odds ratio of 3. The rate of exposures in the control group is assumed to be 0.04. A rate of loss to follow-up was estimated at 10%. The POISSON approximation was used.

5.4.2. Sample selection

Cases

The "Institut de Patologia Forense de Catalunya" will be consulted for information on all SIDS cases over the previous five years. If parents of the patients agree, the principal investigator will give them an information sheet and the consent form to be signed.

Controls

Dried blood spot samples from anonymous children from the records of the "Laboratori Central de Cribratge Neonatal de Catalunya" of the last 5 years. Sampling will be random.

5.4.3. Matching

Matching of cases and controls is often used to increase the statistical efficiency of the analysis and to attempt to control for unmeasured confounders.(42)

In this study cases and controls will be paired up according to **sex** and **weeks of gestation** in order to try to create similar situations and reduce variability.

5.5. VARIABLES

5.5.1. Independent variable

The independent variable will be the **presence of the basic monomer from BChE** in dried blood samples taken between 48-72 hours of life from a newborn baby and analyzed by tandem-mass spectrometry.

It is a qualitative nominal dichotomous categorical variable since it has only two categories: to be present on the blood's samples or not.

5.5.2. Outcome variable

The dependent outcome variable will be **death from SIDS**.

It is also a qualitative nominal dichotomous categorical variable since it has only two categories which are death from SIDS or not.

5.5.3. Covariates

Name of the variable	Definition	Level of measurement	Operating level
Covariates for CASES and CONTROLS:			
Sex	Sex as reported in the clinical history	Qualitative nominal	0: Boy 1: Girl
Gestational age	Weeks at birth	Quantitative continuous but expressed as discrete	Numeric
Birth weight	Weight registered in kg	Quantitative continuous	Numeric with decimal
Multiple gestation	Single or multiple gestation	Qualitative nominal	0: Single 1: Multiple
Breastfeeding	Feeding the infant by breastfeeding	Qualitative nominal	0: No 1: Yes
Smoking environment	Maternal smoking during pregnancy o smoking in her environment	Qualitative nominal	0: No 1: Yes
Mother's age	Mother's age under 20 years	Qualitative nominal	0: No 1: Yes
Complications during pregnancy	Presence or absence of : previous placenta, placental abruption, premature rupture of membranes, pre-eclampsia or eclampsia	Qualitative nominal	0: No 1: Yes
Other exclusive CASE variables collected for the secondary objective:			
Sleeping position	Sleeping in prone position	Qualitative nominal	0: No 1: Yes
Sleeping surface	Presence of blankets or pillows in the infant's bed	Qualitative nominal	0: No 1: Yes
Co-sleeping	Parents sleeping in the same room as the infant but not in the same bed	Qualitative nominal	0: No 1: Room sharing 2: Bed sharing
History of SIDS	Antecedent of a brother or sister dead from SIDS	Qualitative nominal	0: No 1: Yes
Origin	Human community with similar physical characteristics, genetic	Qualitative nominal	1: Caucasian 2: Asian

	features, common history, language and cultural traits.		3: Black African 4: Maghreb 5: Others
Forensic registry	Forensic databases from the 5 autopsy rooms of the centralised forensic pathology service of the "Institut de Patologia Forense de Catalunya.	Qualitative nominal	1: Girona 2: Barcelona 3: Tarragona 4: Lleida 5: Terres de l'Ebre

5.6. DATA COLLECTION

All data will be collected retrospectively.

Once the cases are identified from forensic records, the research team will be responsible for contacting the families in order to explain the project and provide an information sheet (**Annex 6** – Protocol Information Sheet). If they agree to participate, they must sign the consent form (**Annex 7** – Consent form) so that the research team can access to the samples from the "heel prick test" and reanalyze them looking for this new analyte.

Information from the anonymous controls will be extracted from the census of the "Laboratori Central del Cribratge Neonatal de Catalunya" or through the "Programa d'analítica de dades per a la recerca i la innovació en salut (PADRIS)" a program with the mission of making related health data available to the scientific community in order to promote research, innovation and evaluation in health through access to the reuse and cross-referencing of health data generated by the integral health system of public use of Catalunya (SISCAT), in accordance with the legal and regulatory framework, ethical principles and transparency towards the program's citizens (43).

In order to gain statistical power, information from both cases and controls will come from the same census.

We will ask for the samples of the neonatal screening test and also for the information regarding the birth which is acquired in the same leaflet where the drops of blood from the heel are impregnated.

5.7. STATISTICAL ANALYSIS

The statistical analysis will be carried out by the statistical analyst. The Statistical Package for Social Sciences (SPSS) software, version 28.1, will be used to complete it.

We will define a 95% confidence interval for all analyses and define a p value of 0.05 as statistically significant.

5.7.1. Descriptive analysis

Tandem-mass spectrometry will determine whether the basic monomer of the enzyme is present or absent. This information will be summarised using proportions and the 95% confidence interval (CI) and expressed as a categorical dichotomous variable (presence or absence of the basic monomer of 84,000 daltons).

These ratios will be divided into case and control groups (live or death).

SIDS will be also summarized using proportions and the 95%.

In this case, these proportions will be stratified by presence or absence of the BChE's basic monomer.

Additional stratification will be done by the covariates.

Gestational age, although continuous, it is asymmetrically distributed and behaves like a quantitative discrete variable. This is the reason why this variable will not be categorised.

Birth weight will be categorised in low birth weight (below percentile 10) or normal birth weight.

5.7.2. Bivariate analysis

The **chi-square test** or, if the expected number of cases/controls was less than 5 in any cell, the Fisher's exact test, will be used to determine the difference between the proportions of monomer's presence or absence between the cases and controls as well as the proportions of SIDS between the presence or absence of BChE's monomer.

The relationship between the presence/absence of BChE's monomer on SIDS will be determined by calculating the odds ratio.

5.7.3. Multivariate analysis

To assess our objectives, we will use logistic regressions with dependent variables presence or absence of the monomer and SIDS or other death causes, explanatory variables: cases/controls and presence or absence of the monomer, respectively, controlling for all the covariates.

In this case, we will also be interested in the risk associated to each covariate in addition to being case and the presence of BChE's basic monomer.

6. ETHICAL CONSIDERATIONS

The study will be performed respecting the human rights and the four bioethics principles defined by Beauchamp and Childress in 1979, as well as the medical ethics considerations gathered in the World Medical Association Declaration of Helsinki of "Ethical Principles for Medical research Involving Human Subjects" (1964 last reviewed in October 2013). (44)

The principle of **justice** will be taken into consideration since all patients, both cases and controls, will receive the same access to all medical resources, providing the same opportunity for diagnosis. Patient's **autonomy** will be respected as the parents of the cases will receive a thorough explanation of the study's methodology and its rationale. They will be the ones who choose whether or not they wish to take part and will sign an informed consent. It is expected that the **non-maleficence** principle will be respected since no malicious intent is being done to the patients participating in the study. Finally, it is also expected the respect of the principle of **beneficence** as we are trying to implement a screening strategy that we hope will help to detect early the risk in order to prevent and reduce SIDS mortality. Fulfilling the moral obligation to act for the benefit of others.

All the cases who are interested in taking part in the study will be requested to sign a voluntary informed consent. They will be given the necessary information about the study prior to being included in it via a personal discussion with the principal investigator and an information sheet. On the other hand, data from controls will be recorded as a "number of controls" instead of the patient's name, so the anonymity will be guaranteed according to "Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales" (45) and "Reglamento (UE) 2016/679 del Parlamento Europeo y del Consejo, de 27 de abril de 2016, relativo a la protección de las personas físicas en lo que respecta al tratamiento de datos personales y a la libre circulación de estos datos y por el que se deroga la directiva 95/46/CE (Reglamento general de protección de datos)"(46).

Before the study starts, the protocol will be presented to the "Comissió de Docència i Investigació de l'Institut de Medicina Legal i Ciències Forenses de Catalunya". If they approve the study, and with it the data transfer from judicial records, in a second phase the protocol will be presented to the Ethics Committee of the "Hospital Doctor Josep Trueta" (CEIC), claiming that an information sheet and an informed consent will be given to the families of the cases.
Their recommendations will be considered and added to the protocol.
It will not be until we have both approvals that we will request the data from the "Oficina de Cribratge Neonatal de Catalunya".

Transparency will be maintained throughout data publication, and undesirable events that may occur during the study will not be excluded.
We hereby declare there is no bias, no economic interest neither any other conflict of interest.

7. LIMITATIONS

This study has some limitations that have been found throughout the design process and that must be acknowledged:

PREVALENCE

SIDS is an uncommon condition with low incidence thanks to the strong influence of the preventative methods. This implies that the control group will require a sizable patient population. Since records of neonatal screening tests are retained for five years and we have access to them, we will attempt to address this limitation by expanding the sample by five years.

HETEROGENEITY OF THE CASES

It is possible that there is heterogeneity between registries which will imply information bias. Nevertheless, the "Institut de Patologia Forense de Catalunya" has a centralized forensic pathology service with a standardized protocol for recording cases.

On the other hand, I will control it by including the record as a covariate in the multivariate analysis.

EPIDEMIOLOGICAL APPROACH

We must study the same data from the same source when comparing cases and controls in order to increase statistical power. Because of this, the attached data in the "heel prick test" is the information that will be contrasted. Nevertheless, the epidemiological study will be constrained because we can only evaluate a limited common number of covariates.

Since controls are anonymous, we will not have access to their information and hence we will not be able to rule out confounding comorbidities that could interfere with the protocol, restricting the epidemiological study. For the cases, however, we will be able to do a more thorough epidemiological investigation in the future as we will have their informed consent.

In order to resolve this, we presume that the SIDS registry maintained by the Catalan forensic team is accurate and that all cases have been well recorded in it.

CLINICAL CORRELATION

Given that there is no previous data of a direct relation, there is no guaranteed clinical correlation. We start with the evidence of a single study so the result can be difficult to predict, we lack a certainty value to attribute the risk. This is why, in the event that the study came out with conclusive results, subsequent protocols should be drawn up to continue with the diagnostic process aimed at determining in the following step, concentration and enzyme activity.

DE-ANONYMIZATION

We acknowledge that our ability to de-anonymize will be limited because we lack the information from the controls. However, it would not be necessary to alert them just yet because the study will not yet be conclusive and further testing will be required to get the final diagnosis. The fact that 80% of the children at the time of the study will already be older than one year old and will therefore be at low risk of SIDS also lends credibility to our argument.

OVERMATCHING

Overmatching occurs when the matching variable is strongly associated with the risk factor but not (or only weakly) with the illness, resulting in a loss of statistical power. In the study there will be no risk of overmatching since the covariates will be very well controlled and there will be no excessive matching, we will simply control for sex and weeks of gestation.

PREANALYTIC VARIABILITY

Pre-analytical variability is a factor that cannot be controlled since it is a retrospective study and the samples to be analysed have already been extracted. However, it is a point that we must also take into consideration.

It must be ensured that a proper circuit must have been followed: obtaining the sample (pricking the heel in the proper location, within the allotted timeframe and ensuring that the blood matches the patient's file), filling out all the paper's circles, transporting in the proper circumstances and opportune storage.

8. WORK PLAN AND CHRONOGRAM

Personnel involved in the study include:

- **Research team (RT):** Composed by medical physician Bet Solé (principal investigator) and Doctor Pere Plaja (Co-investigator). In charge of: general coordination, economic management, analysis of the results, publication and dissemination.
- **Data manager (DM):** Database creation and control carried out by the "Programa d'analítica de dades per a la recerca i la innovació en salut (PADRIS)".
- **Medical analysts** from the "Laboratori Central de Cribratge de Catalunya". They will be responsible for the analysis of the "heel prick test" samples.
- **Collaborators**:
    - PhD Medicine and Clinical Physician Doctor Maite Serrando. Head of the ICS-IAS Girona Clinical Laboratory and Assistant Professor of Laboratory Medicine in Girona University School of Medicine.
    - PhD Medicine and Forensic pathologist Doctor Josep Maria Casadesús. President of the "Junta Directiva de l'Associació Catalana de Metges Forenses" and Assistant Professor of Legal and Forensic Medicine in Girona University School of Medicine.
    - Doctor José Manuel González de Aledo. Specialist in the "Programa de Cribratge Neonatal de Catalunya" in "Servei de Bioquímica y Genètica Molecular Centre de Diagnòstic Biomèdic (CDB)".
- **Independent statistician (IS):** in charge of the statistical analysis of collected data, interpretation of results and publication.

With anterior sample calculation, the study is expected to be carried out over a period of 21 months (start November 2022, end July 2024). Edition and publication will take approximately 5 months.

The study will be divided in the following 5 stages:

STAGE 0: PREPARATION OF THE STUDY (*November 2022 - April 2023*)

- **Bibliographic research**: Based on the "Libro Blanco de la SMSL" and scientific evidence published during the latest years.
- **Elaboration of the protocol**: Carried out by the RT.
- **Ethic committee evaluation and approval**: protocol will be presented to the "*Comissió de Docència i Investigació de l'Institut de Medicina Legal i Ciències Forenses de Catalunya"* and to the Ethics Committee (CEIC) of the *"Hospital Doctor Josep Trueta"* for its revision and approval.
- **Permission request**: The RT will present the protocol to the "Oficina de Cribratge de Catalunya" and ask for permission to make this new determination.

STAGE 1: PREPARATION, COORDINATION AND TRAINING (*March 2023*)

- **Informative sessions and study members**: The RT will firstly meet with the other members included in the program with the aim of explaining the protocol and its goals.
- **Database creation**: Carried out by the RT.

STAGE 2: DATA COLLECTION AND PRESERVATION (*May 2023 – August 2023*)

- **Cases recruitment**: Carried out by the RT.
- **Controls recruitment**: Carried out by the DM
- **Sample collection**: Carried out by the DM.
- **Sample processing**: Samples will be analysed in the "Laboratori Central de Cribratge Neonatal de Catalunya" in Barcelona.

STAGE 3: STATISTICAL ANALYSIS (*September 2023 – December 2023*)

- **Data quality control and Statistical Analysis**: The Independent Statistician will take time to perform the statistical analyses, which is expected to approximately last 4 months as he/she will have to take into account many confounders.

STAGE 4: PUBLICATION AND DISSEMINATION (*January 2023 – July 2024*)

- **Interpretation and discussion of the results**: there will be a last meeting where the investigators will discuss the data collected and analyzed by the statistician.
- **Final report**: Final discussion by all the group, generating a final report showing the study's results and conclusions.
- **Publication and dissemination of the results**: The results will be presented in two congresses, one national and one international, and the article will be published in an open access journal. We will also suggest a collaboration to the AEP SIDS working group.

CHRONOGRAM

TASKS	TIME																					RESPONSIBLE PERSONNEL
	2022		2023												2024							
	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	
0. PREPARATION OF THE STUDY																						
Bibliographic research																						RT
Elaboration of the protocol																						RT
Ethic committee evaluation and approval																						CEIC
Permission request																						Oficina de cribratge de Catalunya
1. PREPARATION, COORDINATION AND TRAINING																						
Informative sessions and study members																						RT
Database creation																						RT
2. DATA COLLECTION																						
Cases recruitment																						RT
Controls recruitment																						Data manager
Sample collection																						Data manager
Sample processing																						Physicians of the laboratory
3. STATISTICAL ANALYSIS																						
Data quality control																						Statistical
Statistical analysis																						Statistical
4. PUBLICATION AND DISSEMINATION																						
Interpretation and discussion of the results																						RT
Final report																						RT
Publication and Dissemination of the results																						RT

9. BUDGET

To calculate the needed budget for this protocol we have divided the costs in human needs, execution and publication and dissemination costs.

EXPENSES	UNIT COST	UNITS	TOTAL
PERSONNEL			
Data manager	35€/h	10 h	350 €
Laboratory staff	35€/h	15 h	525 €
Statistician	35€/h	30 h	1.050€
EXECUTION			
Tandem mass spectrometry	35€/sample	968 samples	33.880€
PUBLICATION			
Article publication (Open access)			1.500€
DISSEMINATION			
Inscription to national congresses	600€/congress	2 congresses	1.200€
TOTAL			**38.505€**

Since the heel prick test is an already realized procedure, it will not be necessary to take out insurance to cover any possible adverse effects that patients may suffer attributable to their participation in the study.

The highest cost of the study will be the analysis of the samples since the assessment of the monomer is not part of the automated study and the samples will have to be reanalyzed looking for this new analyte.

10. FEASIBILITY AND CLINICAL AND HEALTH IMPACT

10.1. FEASIBILITY

This study will collect data from Catalunya.

Regarding the reason why we chose it, we consider that there is a sufficient sample, according to the incidence of SIDS per year, to do a pilot study.

Being a retrospective study where we analyse dried heel blood samples that have already been taken, we are not causing any harm to the patients and greatly reduce the risk of losses.

We know that the "Laboratori Central de Cribratge Neonatal de Catalunya" keeps the "heel prick test" samples for 5 years in case future analysis are needed for the benefit of the patient.

This gives us a sufficient sample of 80 cases and 880 controls to carry out our study.

Accordingly, we believe that it is possible to develop this case-control pilot study and that it might benefit many patients in the long term.

10.2. CLINICAL AND HEALTH IMPACT

SIDS is the leading cause of mortality in the first year of life despite the emphasis on its prevention. This supports the necessity to keep pushing for the identification of its pathophysiological responsible mechanisms.

This investigation will be a very good starting point for the early detection of the disease if it turns out that changes in the degradation of acetylcholine are significantly implicated.

If statistically significant results were obtained, this would not necessarily infer a cause-and-effect link but would rather support the need for more protocols to accurately identify which are the specific alterations.

In the event that all the studies were conclusive in the end, this determination could be introduced in routine neonatal screening studies and would help to detect early, a few hours after birth, the risk of sudden death in infants, allowing to carry out intensive monitoring and anticipating the desperate event.

This simple, non-invasive and painless test, which is already routinely performed for the detection of other metabolic diseases, would greatly assist many families given that discovering an apparently healthy baby dead in his place of sleep is a desperate event for parents that causes a significant emotional impact.

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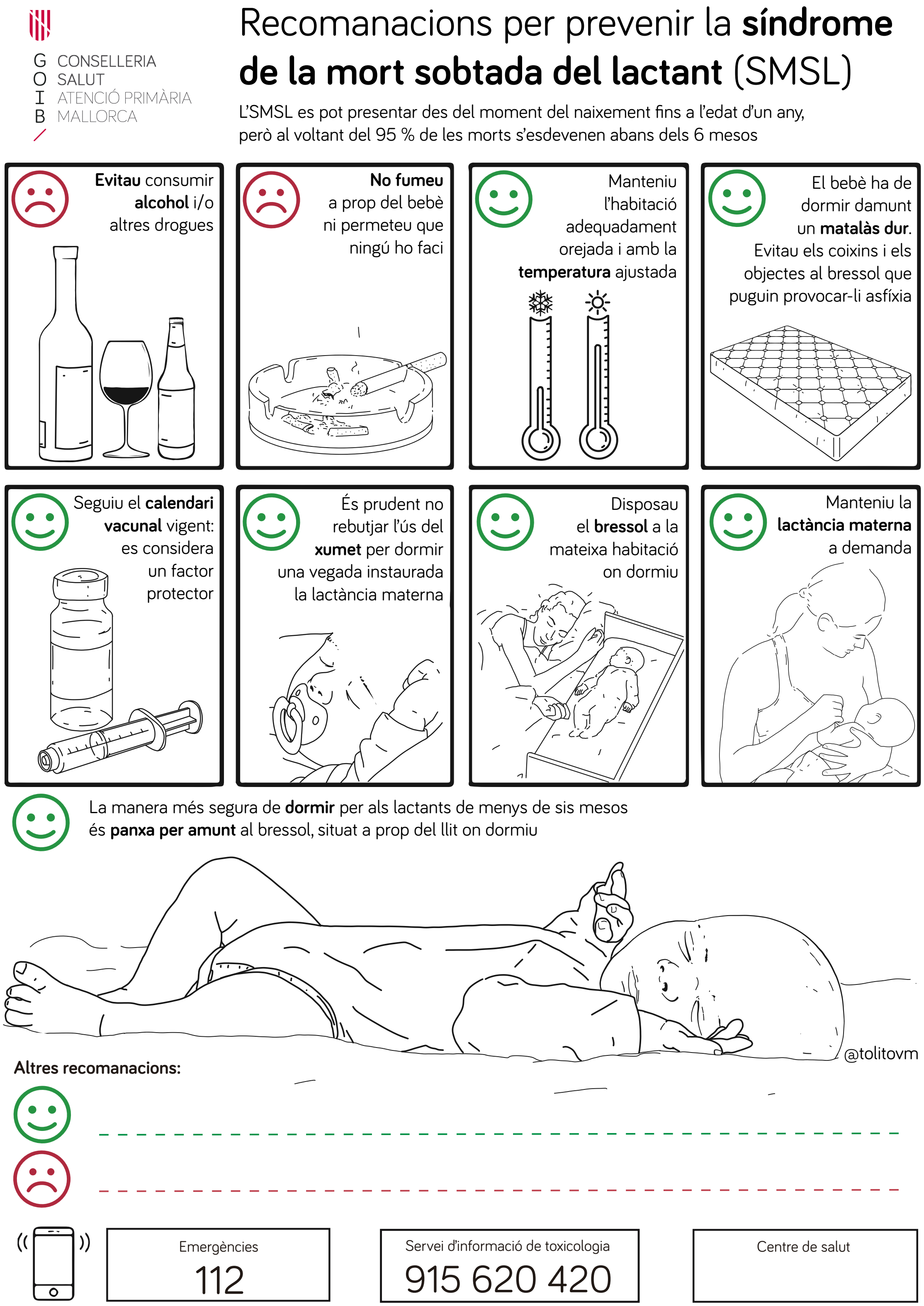
12. ANNEXES

Annex 1 – SIDS prevention measures



Annex 2 – Epidemiological survey

ENCUESTA EPIDEMIOLÓGICA

Juzgado: ..... Asunto: ..... Necro n.º: .....

A. Datos de filiación

Nombre y apellidos:
Nº Historia:
Domicilio:
Tfno. de los padres:
Fecha nacimiento:.....
Fecha de muerte:..... Edad (fallecimiento): .....
Fecha de autopsia:.....

B. Factores epidemiológicos

1. Edad gestacional: .....
2. Peso nacimiento: ..... Talla: ..... P. craneal:.....
3. Apgar 1':..... Apgar 5':..... Reanimación: ☐ sí ☐ no
4. Grupo sanguíneo:.....Rh:.....
5. Parto: a. ☐ Domicilio b. ☐ Hospital c. ☐ Único ☐ Múltiple
   d. Presentación: ☐ cefálica ☐ podálica ☐ nalgas
   e. Cesárea: ☐ sí ☐ no
   f. Amniorrexis: ☐ espontánea ☐ provocada
   g. Amnionitis: ☐ sí ☐ no
6. Periodos neonatal y postnatal:
   a. Lactancia: ☐ natural tiempo:
      ☐ artificial
   b. Vacunaciones y tipo .....
   c. Comportamiento: ☐ tranquilo ☐ nervioso
   d. Llanto: ☐ fuerte ☐ normal ☐ débil
   e. Posición normal del niño: ☐ decúbito supino ☐ decúbito prono
   f. Sueño: ☐ tranquilo ☐ excitado ☐ llanto
   g. Sudoración durante el sueño: ☐ no ☐ poca ☐ importante
7. Situación social familiar:
   a. Tipo de domicilio: ☐ bueno ☐ normal ☐ malo
   b. Nivel social: ☐ alto ☐ medio ☐ bajo
   c. Población/Distrito .....
   d. Raza o etnia: ☐ padre ☐ madre
   e. Edad: padre ..... madre .....
   f. Profesión: padre ..... madre .....
8. Madre: a. Estado civil: ☐ casada o con pareja estable ☐ sin pareja
   b. Nivel de instrucción: ☐ elemental ☐ medio ☐ universitario
   c. Hábitos tóxicos habitualmente: ☐ tabaco ☐ alcohol ☐ drogas
   d. Hábitos tóxicos durante embarazo: ☐ tabaco ☐ cantidad/día .....
      ☐ alcohol ☐ cantidad/día .....
      ☐ drogas ☐ dosis/día .....
   e. Ant. patológicos: ☐ HIV+ ☐ HIV– ☐ Otras infecciones .....
   f. Control de embarazo: ☐ sí (a partir de qué mes) ..... ☐ no
   g. Grupo sanguíneo Rh .....
   h. Embarazo: ☐ normal ☐ patológico (causa).....
   i. Número de gestaciones previas: .....
   j. Tiempo entre gestaciones sucesivas: .....
   k. Abortos anteriores: ☐ sí ☐ no
   l. Antecedentes de M. súbita: ☐ sí ☐ no

9. Padre: a . Nivel de instrucción: ☐ elemental ☐ medio ☐ universitario
   b. Hábitos tóxicos habitualmente: ☐ tabaco ☐ alcohol ☐ drogas
10. Otros antecedentes familiares:
   a. Muerte súbita infantil: ☐ sí ☐ no
   b. Enfermedades congénitas y hereditarias (espec.) .....

C. Antecedentes patológicos

1. Periodo neonatal (enfermedades, infecciones).
2. Semana anterior a la MS:
   a. Infecciones: .....
   b. Otra patología: .....
   c. Tratamiento realizado: .....
3. Ingresos hospitalarios (motivo, duración, diagnóstico y tratamiento efectuado).
4. Antecedentes de apnea o dificultad respiratoria.
5. Antecedentes de malos tratos: ☐ sí ☐ no

D. Datos referidos a la muerte súbita

1. Edad:
2. Mes: ..... Día: ..... Hora: .....
3. Lugar: a. Domicilio
   b. Hospital, urgencias: Ingresa cadáver: ☐ sí ☐ no
   c. Reanimación: ☐ sí ☐ no Planta: .....
   d. Otros (guardería, calle)
4. Breve explicación acerca del acontecimiento:

.....
.....
.....
.....

E. Levantamiento del cadáver o inspección ocular posterior

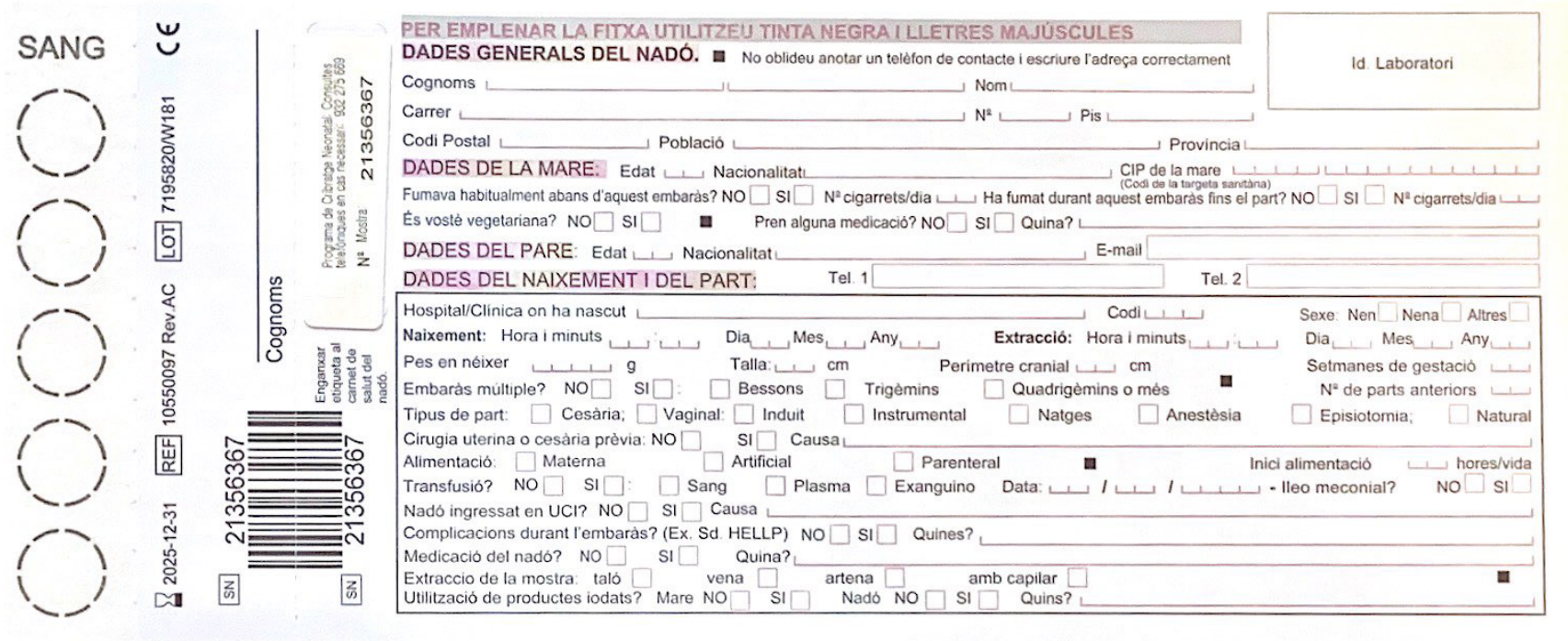
1. Día y hora: .....
2. Situación del cadáver:
   a. Cuna
   b. Cama padres: ☐ solo ☐ compartiéndola*
   c. Cochecito

   * *Existen indicios de que la persona que compartía la cama con el niño en el momento de la muerte pudiera estar bajo los efectos de algún hipnótico: ☐ sí ☐ no*
3. Posición del cadáver: ☐ decúbito supino ☐ prono ☐ lateral
4. Temperatura rectal: .....
5. Cantidad y tipo de ropa del cadáver:
6. Cantidad y tipo de ropa de abrigo en la cuna o cama:
7. Existencia de medicamentos o tóxicos en la habitación:
8. Focos de calor próximos (estufas, radiadores, braseros).
9. Condiciones ambientales del domicilio:

.....
.....
.....

10. Sospecha de malos tratos: ☐ sí ☐ no
11. Sospecha de sofocación o asfixia: ☐ sí ☐ no

Annex 3 – "Heel prick test"



SANG

CE

LOT 7195820/W181

REF 10550097 Rev.AC

2025-12-31

Cognoms

Programa de Cribratge Neonatal. Consultes telefòniques en cas necessari: 902 275 669

Nº Mostra 21356367

Enganxar etiqueta al carnet de salut del nadó.

21356367

21356367

SN

SN

PER EMPLENAR LA FITXA UTILITZEU TINTA NEGRA I LLETRES MAJÚSCULES

DADES GENERALS DEL NADÓ. ■ No oblideu anotar un telèfon de contacte i escriure l'adreça correctament

Id. Laboratori

Cognoms ____ Nom ____

Carrer ____ Nº ____ Pis ____

Codi Postal ____ Població ____ Província ____

DADES DE LA MARE: Edat ____ Nacionalitat ____ CIP de la mare (Codi de la targeta sanitària) ____

Fumava habitualment abans d'aquest embaràs? NO ☐ SI ☐ Nº cigarrets/dia ____ Ha fumat durant aquest embaràs fins el part? NO ☐ SI ☐ Nº cigarrets/dia ____

És vostè vegetariana? NO ☐ SI ☐ ■ Pren alguna medicació? NO ☐ SI ☐ Quina? ____

DADES DEL PARE: Edat ____ Nacionalitat ____ E-mail ____

DADES DEL NAIXEMENT I DEL PART: Tel. 1 ____ Tel. 2 ____

Hospital/Clínica on ha nascut ____ Codi ____ Sexe: Nen ☐ Nena ☐ Altres ☐

Naixement: Hora i minuts ____ Dia ____ Mes ____ Any ____ **Extracció:** Hora i minuts ____ Dia ____ Mes ____ Any ____

Pes en néixer ____ g Talla: ____ cm Perímetre cranial ____ cm Setmanes de gestació ____

Embaràs múltiple? NO ☐ SI ☐: ☐ Bessons ☐ Trigèmins ☐ Quadrigèmins o més ■ Nº de parts anteriors ____

Tipus de part: ☐ Cesària; ☐ Vaginal: ☐ Induit ☐ Instrumental ☐ Natges ☐ Anestèsia ☐ Episiotomia; ☐ Natural

Cirugia uterina o cesària prèvia: NO ☐ SI ☐ Causa ____

Alimentació: ☐ Materna ☐ Artificial ☐ Parenteral ■ Inici alimentació ____ hores/vida

Transfusió? NO ☐ SI ☐: ☐ Sang ☐ Plasma ☐ Exanguino Data: ____ / ____ / ____ - Ileo meconial? NO ☐ SI ☐

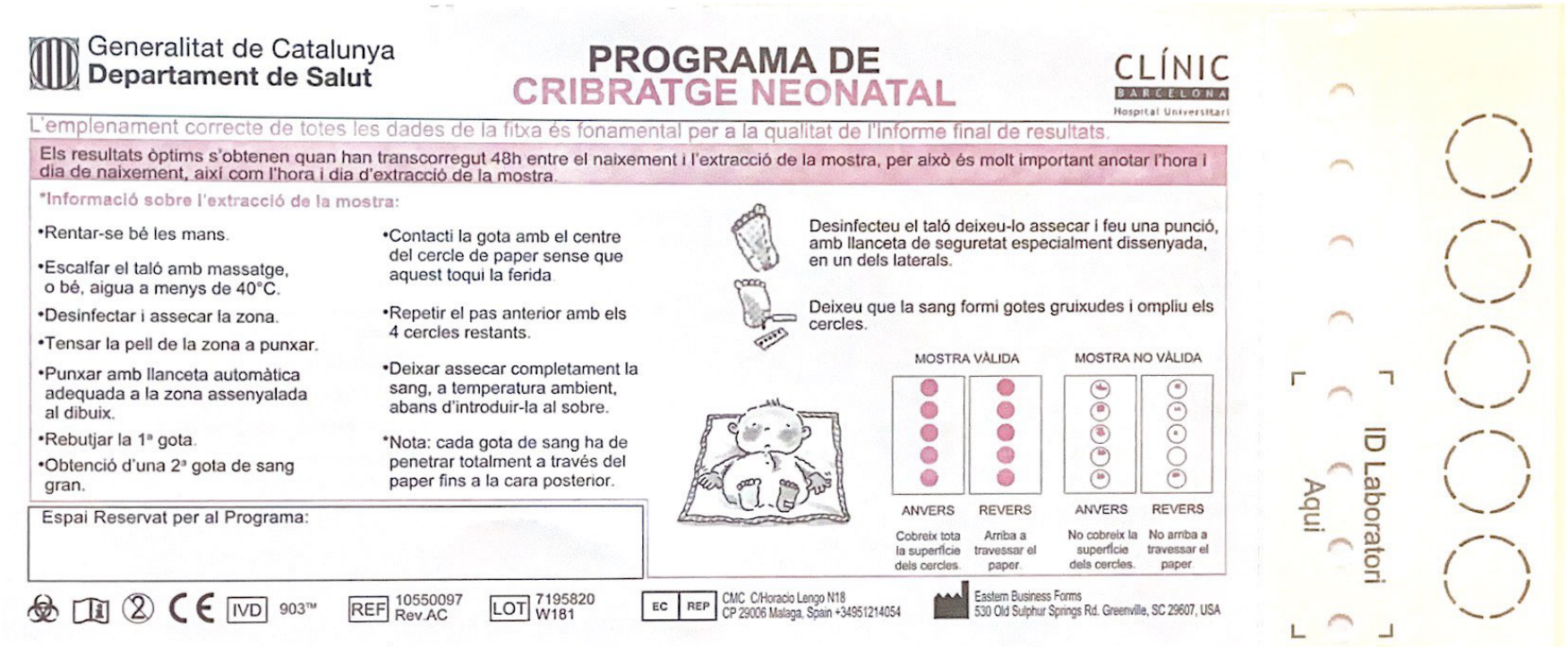
Nadó ingressat en UCI? NO ☐ SI ☐ Causa ____

Complicacions durant l'embaràs? (Ex. Sd. HELLP) NO ☐ SI ☐ Quines? ____

Medicació del nadó? NO ☐ SI ☐ Quina? ____

Extraccio de la mostra: taló ☐ vena ☐ artena ☐ amb capilar ☐ ■

Utilització de productes iodats? Mare NO ☐ SI ☐ Nadó NO ☐ SI ☐ Quins? ____



Generalitat de Catalunya
Departament de Salut

PROGRAMA DE CRIBRATGE NEONATAL

CLÍNIC BARCELONA Hospital Universitari

L'emplenament correcte de totes les dades de la fitxa és fonamental per a la qualitat de l'informe final de resultats.

Els resultats òptims s'obtenen quan han transcorregut 48h entre el naixement i l'extracció de la mostra, per això és molt important anotar l'hora i dia de naixement, així com l'hora i dia d'extracció de la mostra.

*Informació sobre l'extracció de la mostra:

- Rentar-se bé les mans.
- Escalfar el taló amb massatge, o bé, aigua a menys de 40°C.
- Desinfectar i assecar la zona.
- Tensar la pell de la zona a punxar.
- Punxar amb llanceta automàtica adequada a la zona assenyalada al dibuix.
- Rebutjar la 1ª gota.
- Obtenció d'una 2ª gota de sang gran.
- Contacti la gota amb el centre del cercle de paper sense que aquest toqui la ferida.
- Repetir el pas anterior amb els 4 cercles restants.
- Deixar assecar completament la sang, a temperatura ambient, abans d'introduir-la al sobre.
- *Nota: cada gota de sang ha de penetrar totalment a través del paper fins a la cara posterior.

Espai Reservat per al Programa:

Desinfecteu el taló deixeu-lo assecar i feu una punció, amb llanceta de seguretat especialment dissenyada, en un dels laterals.

Deixeu que la sang formi gotes gruixudes i ompliu els cercles.

MOSTRA VÀLIDA		MOSTRA NO VÀLIDA	
ANVERS	REVERS	ANVERS	REVERS
Cobreix tota la superfície dels cercles.	Arriba a travessar el paper.	No cobreix la superfície dels cercles.	No arriba a travessar el paper.

IVD 903™ REF 10550097 Rev.AC LOT 7195820 W181

EC REP CMC C/Horacio Lengo N18 CP 29006 Malaga, Spain +34951214054

Eastern Business Forms 530 Old Sulphur Springs Rd. Greenville, SC 29607, USA

ID Laboratori
Aquí

Annex 4 - Algorithm of the Neonatal Screening Program of Catalunya

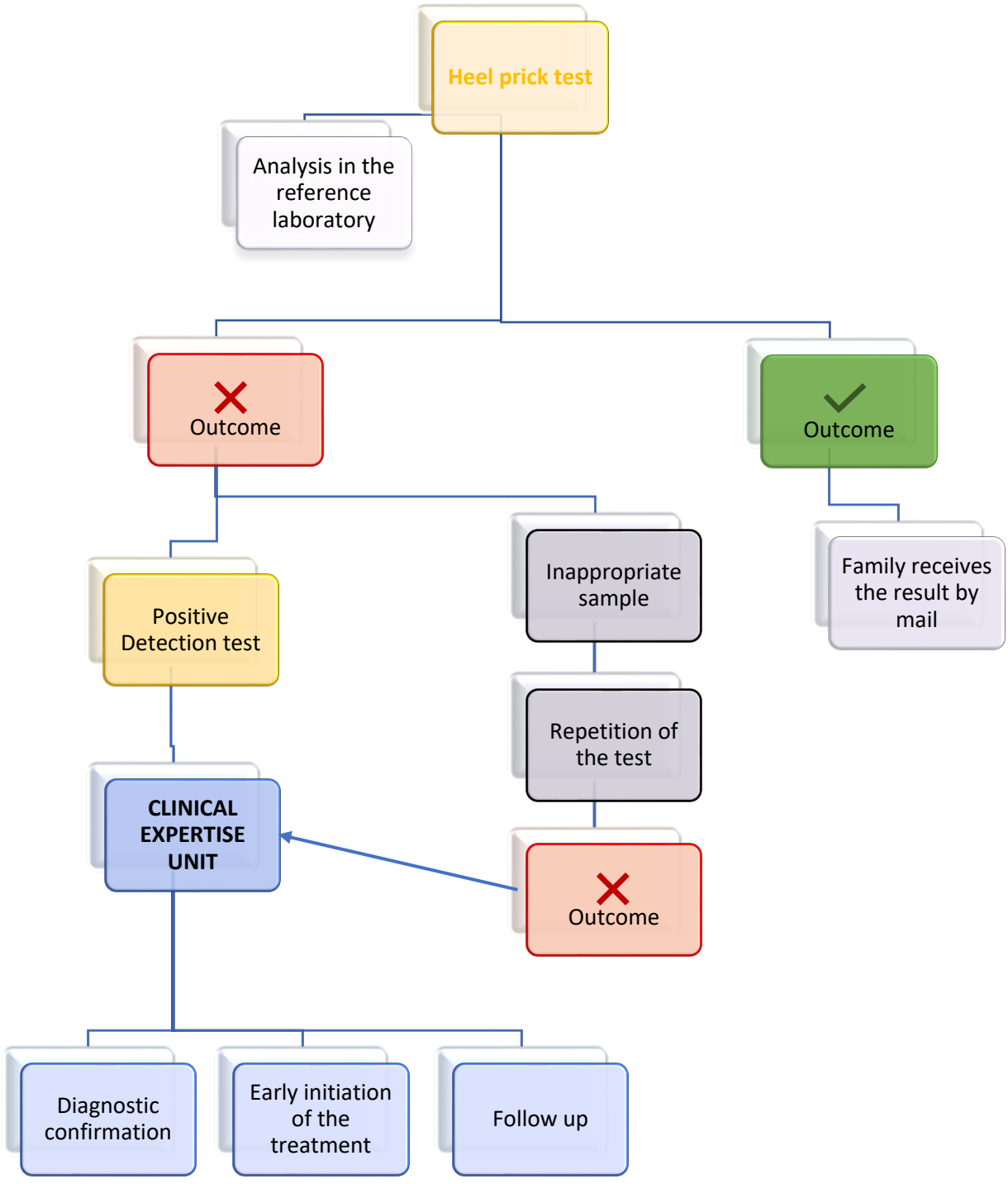


Figure 14. Newborn screening procedure. Adapted from (28)

Annex 5 – Metabolopathies detected in the neonatal screening program

Summary of the detected metabolopathies in recent years (24)

24 malalties objecte de CN	Any d'inici	Total de diagnosticats 1969-2008	Any 2009	Any 2010	Any 2011	Any 2012	Any 2013	Any 2014	Any 2015	Any 2016	Any 2017	Any 2018 (*)	Total de diagnosticats 2009-2018	Total de diagnosticats 1969-2018	Total nounats cribrats 1969-2018
Fenilcetonúria	1969-1982	189	6	17	14	14	14	19	6	9	19	8	126	315	2.788.179
Hipotiroïdisme congènit	1982	674	34	42	46	38	54	33	30	27	33	32	369	1043	2.411.462
Fibrosi quística	2000	125	8	5	8	11	10	13	6	8	7	4	80	205	1.458.271
Grup de 19 malalties metabòliques	2013						14	12	14	13	9	14	76	76	404.099
Anèmia de cèl·lules falciformes	2015								21	17	29	19	86	86	271.593
Immunodeficiencia combinada greu (SCID)	2017										0	1	1	1	131.096
Total malalties metabòliques objecte del CN		988	48	64	68	63	92	77	77	74	97	78	738	1726	2.788.179

(*) Dades any 2018 provisionals

		Total de diagnosticats 1969-2008	Any 2009	Any 2010	Any 2011	Any 2012	Any 2013	Any 2014	Any 2015	Any 2016	Any 2017	Any 2018 (*)	Total de diagnosticats 2009-2018	Total de diagnosticats 1969-2018	Total de nounats cribrats 1969-2018
Total de malalties detectades en el CN (malalties objecte de CN + troballes col·laterals)		**988**	**48**	**64**	**68**	**63**	**110**	**103**	**110**	**115**	**154**	**132**	**967**	**1955**	**2.788.179**
Total de nascuts		**2.046.141**	**85.347**	**84.071**	**81.137**	**77.548**	**71.771**	**71.634**	**70.461**	**68.902**	**66882**	**64.285**			**2.788.179**

Malaties no objecte de CN, però trobades col·lateralment	Any d'inici	Total de diagnosticats 1969-2008	Any 2009	Any 2010	Any 2011	Any 2012	Any 2013	Any 2014	Any 2015	Any 2016	Any 2017	Any 2018 (*)	Total de diagnosticats 2009-2018	TOTALS diagnosticats 1969-2018	Total de nounats cribrats 1969-2018
Altres malalties metabòliques	2013	0					5	8	6	7	4	11	41	41	404.099
Carència de vitamina B_{12}	2013	0					13	18	21	31	41	34	158	158	404.099
Altres hemoglobinopaties	2015	0							6	3	3	4	16	16	271.593
Altres immunodeficiències	2017	0									9	5	14	14	131.096
Total de malalties metabòliques col·laterals		0	0	0	0	0	18	26	33	41	57	54	229	229	404.099

Annex 6 – Protocol Information Sheet

ENGLISH

PROTOCOL INFORMATIVE SHEET

TITLE OF THE STUDY: Is butyrylcholinesterase a potential biomarker for sudden infant death syndrome's prevention?

PRINCIPAL INVESTIGATOR: Bet Solé Roqueta

REFERENCE CENTER: Fundació Salut Empordà

1. INTRODUCTION

We are contacting you to invite you to participate, on a completely voluntary basis, in a research study on sudden infant death syndrome. The study has been approved by the ethics committee of the Hospital Doctor Josep Trueta, in accordance with current legislation, and with respect to the principles enunciated in the declaration of Helsinki and the guidelines for good clinical practice. The intention of this document is to provide you with all the necessary information about the study so that you can evaluate and judge whether you want to participate in it completely freely. Please read this information sheet carefully and contact us if you have any questions or concerns.

2. STUDY DESCRIPTION

What is the aim of the study?

The aim of this study is to determine if the absence of butyrylcholinesterase's monomers in heel's blood is a good biomarker for the screening of sudden infant death syndrome.

Screening is a set of diagnostic tests that allow diseases to be detected in the apparently healthy population early and in asymptomatic phases.

Currently, in Catalunya, there is already a neonatal screening program for some metabolic diseases that can be life-threatening for the newborn. If it is determined that this biomarker is effective, more studies would be done to consider introducing it as a new determination in neonatal screening.

What characteristics must the patients meet to participate in the study?

To be able to participate in the study, the child must have died during the first year of life from an unexplained cause after a thorough forensic study and must have been cataloged as sudden infant death syndrome (SIDS).

What does my participation consist of?

Once the participant's parents agree to join the study, the sample from the neonatal screening test will be retrieved to determine this new analyte.

What is the financial compensation?

The study does not offer any financial compensation to its participants, nor will it entail any additional costs for the patient.

3. RISKS AND BENEFITS

What risks do I assume if I participate in the study?

As this is a retrospective study, we declare that you will not be at any risk.

What benefits will I get from my participation in the study?

Your participation will contribute to a better understanding of sudden infant death syndrome and to know what impact its screening could have on the prognosis of the disease.

This knowledge will contribute to provide future benefits to children like your son and to consider whether it would be useful to implement this measure at a population level.

4. VOLUNTARY PARTICIPATION

If you agree to participate in the study, you will be given a copy of this document and the Consent Form, which you must sign in accordance with current regulations.

Is participation mandatory?

Participation in the study is completely voluntary. In addition, if you agree to participate, you have the right to revoke your consent at any time in the event that you wish to end your participation without giving any explanation and without this causing any harm in your healthcare.

If you decide to withdraw your consent to participate in the study, no new data will be added to the database, however the study managers may continue to use the information collected until then, unless you object.

5. PRIVACY AND CONFIDENTIALITY

How will my confidentiality be protected?

Data obtained will be completely confidential, as established by the Organic Law on the Protection of Personal Data and Guarantee of Digital Rights (3/2018) and Regulation 2016/679 of the European Parliament and Council. It should be added that according to LO15/1999, you can exercise your rights to access, rectification, opposition and cancellation of the data; if you wish, you must contact the Principal Investigator of the study.

Access to your personal information will be restricted to study researchers, but always maintaining confidentiality in accordance with current legislation, and will always be used for research purposes.

What will be done with the information obtained from the study?

Publication of results may be necessary in order for other centers and individuals to benefit from the findings of our study. If the results are published through publications and/or conferences, any personal data will be treated anonymously without the participant being identifiable.

6. QUESTIONS AND APPRECIATIONS

Who can I contact for any questions or problems that arise?

If you need information or to communicate any event that happens during the study, you can contact one of the members of our study through the following email: .....

Whatever your decision is, the research team would like to thank you in advance for your time and attention.

CATALÀ

FULL INFORMATIU DEL PROTOCOL

TÍTOL DE L'ESTUDI: És la butirilcolinesterasa un potencial biomarcador per la prevenció de la síndrome de la mort sobtada del lactant?

INVESTIGADOR PRINCIPAL: Bet Solé Roqueta

CENTRE DE REFERÈNCIA: Fundació Salut Empordà

1. INTRODUCCIÓ

Ens posem en contacte amb vostè per convidar-lo a participar, de manera totalment voluntària, en un estudi de recerca sobre la síndrome de mort sobtada del lactant. L'estudi ha estat aprovat pel comitè d'ètica de l'Hospital Doctor Josep Trueta, d'acord amb la legislació vigent, i amb respecte als principis enunciats a la declaració d'Hèlsinki i a les guies de bona pràctica clínica. La intenció d'aquest document és proporcionar-li tota la informació necessària sobre l'estudi perquè pugui avaluar i jutjar si vol participar-hi de manera totalment lliure. Si us plau, llegeixi atentament aquest full d'informació i posi's en contacte amb nosaltres si té cap pregunta o dubte.

2. DESCRIPCIÓ DE L'ESTUDI

Quin és l'objectiu de l'estudi?

L'objectiu d'aquest estudi és determinar si l'absència del monòmer bàsic de la butirilcolinesterasa en sang del taló és un bon biomarcador per al cribratge de la síndrome de mort sobtada del lactant.

El cribratge engloba un conjunt de proves diagnòstiques que permeten detectar malalties en la població aparentment sana de manera precoç i en fases asimptomàtiques.

Actualment, a Catalunya, ja hi ha un programa de cribratge neonatal d'algunes malalties metabòliques que poden posar en perill la vida del nounat. Si es determina que aquest biomarcador és efectiu, es farien més estudis per considerar la seva introducció com una nova determinació en el cribratge neonatal.

Quines característiques han de tenir els pacients per participar a l'estudi?

Per poder participar en l'estudi, el nen ha d'haver mort durant el primer any de vida per una causa inexplicada després d'un estudi forense exhaustiu, i ha d'haver estat catalogat com a síndrome de mort sobtada del lactant (SMSL).

En que consistirà la meva participació?

Un cop els pares del participant acceptin unir-se a l'estudi, es recuperarà la mostra de la prova de cribratge neonatal per determinar aquest nou analit.

Quina és la compensació econòmica?

L'estudi no ofereix cap compensació econòmica als seus participants, ni tampoc suposarà cap cost addicional per al pacient.

3. RISCOS I BENEFICIS

Quins riscs assumeixo si participo a l'estudi?

Com que es tracta d'un estudi retrospectiu, declarem que no correrà cap risc.

Quins beneficis obtindré amb la meva participació a l'estudi?

La vostra participació contribuirà a una millor comprensió de la síndrome de mort sobtada del lactant i a saber quin impacte podria tenir el seu cribratge en el pronòstic de la malaltia.

Aquest coneixement contribuirà a aportar beneficis futurs a infants com el seu fill i a plantejar-se si seria útil implementar aquesta mesura a nivell de població.

4. PARTICIPACIÓ VOLUNTÀRIA

Si accepteu participar en l'estudi, se us lliurarà una còpia d'aquest document i el Consentiment Informat, que haureu de signar d'acord amb la normativa vigent.

És obligatòria la participació?

La participació en l'estudi és totalment voluntària. A més, si accepteu participar, teniu dret a revocar el consentiment en qualsevol moment en el cas que vulgueu posar fi a la participació, sense donar cap explicació i sense que això suposi cap perjudici en l'atenció sanitària.

Si decideix retirar el consentiment per participar en l'estudi, no s'afegiran dades noves a la base de dades, però els responsables de l'estudi poden continuar utilitzant la informació recollida fins aleshores, tret que s'hi oposi.

5. PRIVACITAT I CONFIDENCIALITAT

Com es protegirà la meva confidencialitat?

Les dades obtingudes seran totalment confidencials, tal com estableix la Llei Orgànica de Protecció de Dades Personals i Garantia dels Drets Digitals (3/2018) i el Reglament 2016/679 del Parlament Europeu i del Consell. Cal afegir que segons la LO15/1999, pot exercir els seus drets d'accés, rectificació, oposició i cancel·lació de les dades. Si ho desitja, s'ha de posar en contacte amb l'investigador principal de l'estudi.

L'accés a la seva informació personal estarà restringida als investigadors de l'estudi, però sempre mantenint la confidencialitat d'acord amb la legislació vigent, i s'utilitzarà sempre amb finalitats de recerca.

Què es farà amb la informació que s'obtingui de l'estudi?

La publicació dels resultats pot ser necessària perquè altres centres i persones es beneficiïn dels resultats del nostre estudi. Si els resultats es publiquen a través de publicacions i/o conferències, qualsevol dada personal serà tractada de manera anònima sense que el participant sigui identificable.

6. PREGUNTES I APRECIACIONS

Com puc contactar per possibles dubtes que em sorgeixin?

Si necessiteu informació o voleu comunicar qualsevol esdeveniment que succeeixi durant l'estudi, podeu contactar amb un dels membres del nostre estudi a través del següent correu electrònic: .....

Sigui quina sigui la vostra decisió, l'equip de recerca vol agraïr-vos per endavant el vostre temps i atenció.

Annex 7 – Consent form

CONSENT FORM FOR THE STUDY

Title of the study: Is butyrylcholinesterase a potential biomarker for sudden infant death syndrome's prevention?

Name and surnames of the PATIENT: ……

I …… father/mother of ……. with ID card…… ,
I declare under my responsibility that:

- I have been adequately informed by the responsible physician: ……
- I have read the information sheet and it has been properly explained to me. I will be able to keep a copy of it.
- I have been able to ask all desired questions about the study and my doubts have been answered satisfactorily.
- I have received enough information about the study and its objectives.
- I give permission for my child's data and his medical history to be used by the research team for purposes related to this study. I have been informed about the scientific use that will be made of the personal data.
- I have understood that all data will be kept strictly confidential.
- I have understood that the participation is voluntary.
- I have understood that I can withdraw from the study whenever I want, with no having to give explanations, and that will not affect my health care assistance.

I voluntarily accept my participation in the study and give my consent to the access and use of my child's data, always in accordance with the data protection regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2017 on the protection of individuals with regard to the processing of personal data and on the free movement of such data, and failing that, the organic law on the protection of personal data and guarantee of digital rights of 3/2018.

…… of ……..from 20……

Patient's parents signature: Physician signature:

Name: …… Name: …….

WITHDRAWAL OF INFORMED CONSENT

I, ……. father/mother of ……..revoke informed consent to participate in this study.

…… of ……..from 20……

Patient's parent signature:

Name: ……

Physician signature:

Name: …….

CATALÀ

CONSENTIMENT INFORMAT

Títol de l'estudi: És la butirilcolinesterasa un potencial biomarcador per la prevenció de la síndrome de la mort sobtada del lactant?

Nom i Cognoms del PACIENT: ……

Jo, …… pare/mare de ……. amb DNI …….. , declaro sota la meva responsabilitat que:

- He estat adequadament informat pel metge responsable: ……
- He llegit el full informatiu i me l'han explicat correctament. Podré guardar-ne una còpia.
- He pogut fer totes les preguntes desitjades sobre l'estudi i els meus dubtes han estat resolts satisfactòriament.
- He rebut prou informació sobre l'estudi i els seus objectius.
- Dono permís perquè les dades del meu fill i el seu historial mèdic siguin utilitzats per l'equip de recerca per a finalitats relacionades amb aquest estudi. M'han informat sobre l'ús científic que es farà de les dades personals.
- He entès que totes les dades seran estrictament confidencials.
- He entès que la participació és voluntària.
- He entès que em puc retirar de l'estudi quan vulgui, sense haver de donar explicacions, i això no afectarà la meva assistència sanitària.

Accepto voluntàriament la meva participació en l'estudi i dono el meu consentiment per a l'accés i ús de les dades del meu fill, sempre d'acord amb el Reglament de protecció de dades (UE) 2016/679 del Parlament Europeu i del Consell de 27 d'abril de 2017 sobre la protecció de les persones físiques pel que fa al tractament de dades personals i sobre la lliure circulació d'aquestes dades, i en el seu defecte, la Llei orgànica de protecció de dades de caràcter personal i garantia dels drets digitals de 3/2018.

…… de ……..de 20……

Firma dels pares: Firma del metge:

Nom: …… Nom: ……

RETIRADA DEL CONSENTIMENT INFORMAT

Jo, ……. pare/mare de ……..retiro el consentiment informat per participar en aquest estudi.

…… de ……..de 20……

Firma dels pares:

Nom: ……

Firma del metge:

Nom: …….