STUDY PROTOCOL PLAN AND STADISTICAL ANALYSIS PLAN (SAP)

UNIVERSITY CEU CARDENAL HERRERA

Principal investigator: Dr. Jose Miguel Soria

TITLE: Parental stress, child development and attachment in premature children during their first 24 months of life cared for in Child Development and Early Care Centers.

Research and Ethics Committee of CEU Cardenal Herrera University Number: CEEI24/544

NCT ID:

DATE: 12-20-2024

STUDY PROTOCOL PLAN

Objectives:

The objectives of our study have been the following:

To analyze the degree of perceived and physiological stress in mothers of premature children, as well as the possible effects that it can generate in the care of the child and, therefore, in the interaction and attachment patterns at 24 months of age. In such a way that preventive intervention programs can be designed that guarantee optimal interactive patterns between the neonate and its family.

Specific objectives

• Specifically analyze how parenting stress affects the development and formation of attachment in children born prematurely.
• Examine how socioeconomic differences influence parents' stress level and, therefore, their interaction and attachment with their children.
• Assess how parenting stress over time affects the perceived quality of life of families who attend Early Care, with a particular focus on how this influences the relationship and development of the child.
• Analyze the influence on stress, depression and anxiety in mothers and fathers of premature children through biometric records and thermographic records in real time.
• Determine by quantifying cortisol in hair as a biochemical marker that determines the level of chronic stress.
• Determine the acute impact of stress on mothers and fathers of premature children, through the quantification of the level of cortisol and oxytocin in saliva.
• Explore the association between child development and attachment at 24 months of age.

Protocol

Design, population and sample

A prospective population-based cohort study will be conducted on premature births referred, after hospital discharge, to the Child Development and Early Care Centres of the Fundación Salud Infantil de Elche (Alicante, Spain). All mothers and fathers who give birth to a premature child and who are referred to the Child Development and Early Care Centre in the period between June 2024 and December 2025 will be invited to participate in the study. All participants who meet the following inclusion criteria will be invited to participate:

• Children born under 37 weeks of gestation, admitted to the Neonatal Unit and who begin treatment at the Child Development and Early Care Centre of the Fundación Salud Infantil de Elche (Alicante, Spain).

• Parents of legal age and who have sufficient cognitive capacity to understand the informed consent.

Group 1: formed by mothers who had premature births before 37 weeks of gestation

Group 2: formed by mothers who had births after 37 weeks of gestation

Procedure

After the child is admitted to the CDIAT, the parents will be informed about the study by the principal investigator of the study (Annex 1). If the parents decide to participate in the study, they will be called for a first interview (Annex 2) in which their participation in the study will be explained in more detail and they will be asked to sign the informed consent (Annex 3) to participate in the study. During this first visit, the parents provide information about their reproductive and sociodemographic history, data on the birth and the newborn (through the ad hoc interview generated for the study and the clinical report on admission). At the same time, information on stress is collected through the Spanish version of the Parenting Stress Index, PSI (Abidin, 1995; Spanish adaptation Brito de la Nuez, 2001) (Annex 4) and exosomatic records will be taken with the BIOPAC system and saliva and hair samples will be taken to determine oxytocin, serotonin and cortisol in both the father and the mother. On the other hand, information on family quality of life will be collected through the online Questionnaire on Family Quality of Life in Early Intervention (CVFAT) (Annex 5). Regarding the child, neonatal behavior will be assessed through the neonatal behavior assessment test (Brazelton and Nugent, 1997; Spanish adaptation Costa, Fornieles, Botet, Boatella and De Cáceres, 2014) (Annex 6) whenever

the child is at a corrected age of between 0 and 2 months and information on the child's development will be collected through the Bayley-III Infant Development Assessment Scales, BSID-III (Bayley, 2015) (Annex 7). Finally, the risk estimate will be taken through the Perinatal Risk Index, PERI (Scheiner and Sexton, 1991) (Annex 8). The estimated population n=30 and the control population will be made up of fathers and mothers (n=30) who had full-term births and gave birth to normotypical children (General University Hospital of Elche.

Later, at 3, 6, 9, 12, 18 and 24 months of corrected age, the parents will be called again to collect information on the child's development, through the Bayley-III Child Development Assessment Scales, BSID-III (Bayley, 2015) (Annex 7) and on the parents' stress through the questionnaire of the Spanish version of the Parenting Stress Index, (PSI) (Annex 4), as well as exosomatic records will be taken with the BIOPAC system and saliva and hair samples for the determination of oxytocin, serotonin and cortisol in both the father and the mother. On the other hand, Information on family quality of life will be collected through the Family Quality of Life Questionnaire in Early Care (CVFAT) (Annex 5).

At 24 months, information on attachment will also be collected through the Strange Situation (Ainsworth, Bleahr, Waters and Wall, 1978) (Annex 9).

Families with their children will go to the Child Development and Early Care Center of the Children's Health Foundation of Elche (located at Calle Masamagrell, 4) for the entire process described above. For the collection of samples, health professionals from the Cardenal Herrera University (CEU, Elche) will take measurements and keep the samples, respecting the cold chains, in their laboratory.

Primary outcomes

During the study, data come from several sources of information such as:

- Data derived from the interview (sociodemographic and neonatal and delivery status)
- Data derived from the clinical report of hospital discharge (diagnosis, somatometry at discharge and estimation of the prognostic value)
- Data derived from evaluation tests (questionnaires and observational tests for

stress, development and neonatal behavior)

- Data derived from medical tests (Saliva and hair test for cortisol in saliva and per, serotonin and oxytocin)

- Data derived from biometric records using the BIOPAC system

Description of the scales to be used:

- Parenting Stress Index (PSI) created by Abidin, 1995 and adapted to Spanish by Brito de la Nuez, 2001. It evaluates the stress experienced in parenthood, through several subscales such as parental distress, dysfunctional parent-child interaction and whether the child is difficult based on how the parents perceive their children's behavior.

- The Perinatal Risk Inventory designed by Scheiner and Sexton, 1991. It is a measurement instrument that offers information on the neonatal situation prior to discharge and that facilitates an estimate of the biological risk that the child may present from a very early age. It is made up of a total of 18 risk factors such as 1) Apgar score; 2) electroencephalogram; 3) nonmetabolic seizures; 4) intraventricular hemorrhage; 5) hydrocephalus; 6) neurologic findings (not due to hydrocephalus or HIV); 7) gestational age at birth; 8) weight for gestational age; 9) dysmorphia; 10) assisted ventilation; 11) cranial growth (hospitalized children 6 weeks or more); 12) cranial growth (hospitalized children less than 3 weeks); 13) polycythemia; 14) meningitis; 15) hypoglycemia; 16) congenital infections; 17) hyperbilirubinemia; and 18) associated medical problems (not central nervous system). These factors are evaluated with a score of 0 to 3 for each of the aforementioned factors, and can range from a minimum of 0 points to a maximum of 51. Taking into account the cohort criteria established by the authors (Muller-Nix et al., 2004; Pierrehumbert, et al., 2003; Zaramella et al., 1996), the risk levels were determined as:

a. Low Neonatal Risk: 0 to 6 points.

b. Moderate Neonatal Risk: 7 to 9 points.

c. High Neonatal Risk: 10 or more points.

- Neonatal Behavioral Assessment Score (NBAS) designed by Brazelton, 2014 is an assessment technique that provides a score profile that allows us to detect a possible alteration or pathology, but at the same time, within normal parameters, detect

both the potential or "strong points" and the problems or "weak points" of the newborn, as well as their peculiar ways of acting and reacting to environmental variables. Therefore, a profile of the child's behavioral characteristics is obtained, which can be considered as a first outline of their temperamental traits. It evaluates the newborn's repertoire of behaviors in 28 behavioral items that are valued according to a 9-point scale. The scale also includes an assessment of the neurological status in 18 reflex items, each with a 4-point graduation. In the second edition of the NBAS (Brazelton, 1984) a series of 7 supplementary items were added with the intention of better capturing the degree of fragility and the quality of the behavior of high-risk children. These seven items attempt to summarize the quality of the child's response and the amount of stimulation that he/she needs from the examiner to organize his/her responses. The NBAS can be used without any type of adaptation in full-term children and can be applied until the end of the second month of life. By adding the supplementary items it can also be used in apparently healthy premature children (less than 37 weeks of gestation) and for them, depending on the degree of immaturity, the application is possible up to 48 weeks post-conceptional age.

- Bayley-III Infant Development Assessment Scales (Bayley, 2015). They are a set of three standardized assessment scales that evaluate cognitive, motor and language development in children aged 1 to 42 months. For our study, we will only use the motor scale, made up of two subscales, the gross motor scale and the fine motor scale. This motor scale is made up of 138 items (the gross motor scale is made up of 72 items and the fine motor scale is made up of 66 items) through which it evaluates the degree of body control, the coordination of large muscle masses and the manipulative ability of hands and fingers. The total administration time of the motor area for children aged 12 months, as is the case in the sample, is between 15 and 20 minutes. - The Strange Situation is a standardized observational procedure, in which the child is exposed to two moments of separation from his/her attachment figure, two moments of reunion with the attachment figure and moments in which he/she interacts with an unknown person. Specifically, there are 8 episodes (3 minutes long) in which the level of stress caused in the child increases in intensity as the situation progresses (28). Its duration is estimated to be around 20 minutes. It is evaluated on a 7-point Likert-type scale, so that low scores indicate low frequency and low intensity.

Determination and analysis of biomarkers: Cortisol and oxytocin

- Determination of cortisol in hair, as a biomarker of chronic stress: To extract cortisol

from hair, the sample is carefully sectioned into segment lengths that approximate the time period of interest (from the outermost part, if the hair were 3 cm long, the cortisol secreted proportionally by the individual in the last 3 months of life will be extracted). Next, an approximate average weight per sample of 150 mgrs will be selected. The samples will be individualized and placed in eppendorfs (one for each patient) and pulverized with scissors. After incubation in methanol for 36 hours, the supernatant portion that will already have the cortisol dissolved will be extracted. This solution will be evaporated to dryness, and then the pellet will be reconstituted with phosphate (PBS). A commercial kit will be used for the quantification of cortisol using the immunoassay technique (ELISA Victor X5). The ELISA test kit for the detection of cortisol in saliva (ELISA Kits › Cortisol ELISA Kits Human Cortisol Competitive ELISA Kit ,Invitrogen), the sensitivity of the test kit was 0.024 ng/ml, with a detection range of 0-30 ng/ml. The concentration of the hormone (picograms of cortisol/milligram of hair) in each of the samples was calculated from the standard curve.

- Determination of cortisol in saliva, as an indicator of punctual stress. Three saliva samples will be collected from each subject, at 8:00, 15:00 and 23:00 hours. Before collecting the saliva, they will be told to rinse their mouth with cold water, without brushing their teeth. They should not eat or drink in the hour prior to taking the sample. Approximately 1 ml of saliva will be obtained, collected by direct expectoration into an ependor tube. The samples will be stored for 24 hours at 4ºC. The samples will then be centrifuged at 2,500 rpm/10 min and the supernatant will be stored at -20 °C until processed using an ELISA kit, as per the protocol indicated above.

- Determination of oxytocin in saliva. The treatment and obtaining of saliva as a biological sample will be identical to the protocol described for the determination of cortisol. The determination of oxytocin will be carried out using an ELISA kit for immunoassay (Oxytocin ELISA Kit (ab133050 ABCAM)

Recording of biometric variables.

The BIOPAC system is a device used to measure electrical signals in the human body. It is designed for research purposes, offering practicality and versatility by incorporating specialized software. This equipment allows understanding the generation and analysis of various medical conditions and their respective diagnoses.

The system can measure different electrical signals from the body, such as electromyograms (EMG), electrocardiograms (ECG) and electroencephalograms (EEG).

It is also capable of monitoring pulse, blood pressure, lung flow and volume, providing accurate measurements. It is useful for acquiring and analyzing electrical signals produced by the body. It is designed to record differential signals, which are then processed and analyzed with specialized software.

The BIOPAC system can be used to analyze physiological signals related to mood changes. The system can measure various electrical signals in the body which can provide information about the body's response to different emotional states.

For this study, the BIOPAC MP36R acquisition system has been used. It is a research tool used in a variety of areas of physiology and science. This system features a four-channel data acquisition unit with built-in universal amplifiers, capable of recording a wide range of physiological signals. The system also includes a stimulator, audio output, digital input and output lines, and supports air and gas analysis. The MP36R comes with AcKnowledge 5 software, which includes powerful automation and scoring tools. The software supports automated analysis for ECG, EEG, EMG, and many more. The system supports a wide range of signal-specific wired and wireless amplifiers. The MP36R is quick and easy to set up and is approved for human and animal research. When used in conjunction with AcKnowledge software, electrodes, transducers, and other system components, the MP36R forms part of a complete data acquisition and analysis system.

The BIOPAC MP36R System will be used to collect data:

The BIOPAC SS3LA Galvanic Response Transducer measures the galvanic response of the skin, also known as electrodermal activity (EDA). This transducer is used to record the electrical behavior of the skin, which varies according to the activity of the sweat glands. This measurement of electrodermal activity is essential for studying the activity of the autonomic nervous system, which controls involuntary body functions such as sweating and heart rate.

The BIOPAC SS2LB 3-Lead ECG Cables are used for electrode connection in the context of electrocardiography, a technique that records the electrical activity of the heart. These cables allow the connection of electrodes in a three-lead format, which can improve the accuracy and quality of the recorded signals. Proper use of these cables can contribute to the diagnosis and monitoring of various cardiovascular conditions.

The SS5LB Respiratory Band Transducer + Strap is a component of the BIOPAC system used to measure respiratory rate. The transducer attaches to a strap that is placed around the subject's chest, allowing chest movements to be detected during breathing. The strap ensures a secure and comfortable fit for the subject during the measurement process. This transducer is used in conjunction with other BIOPAC components, such as the EDA transducer, to assess physiological responses. Data obtained from the

transducer provides information on respiratory patterns and their relationship to other physiological measurements.

The temperature sensor is the SS6L, which is used to measure temperature at various body locations. The temperature sensor can be used to measure temperature at various locations on the body, such as the skin, mouth, or rectum. The sensor connects to the BIOPAC system via a cable, and temperature data can be viewed and analyzed using BIOPAC software. The sensor is designed to be accurate, reliable, and easy to use, allowing body temperature to be measured in a variety of environments.

To use the BIOPAC System to collect physiological signals from the mother, the following steps will be followed:

- Preparation: Verify that the sensors and transducers are correctly connected to the BIOPAC system. This involves connecting each sensor to the appropriate port on the BIOPAC hardware and ensuring that the sensors and transducers are firmly attached to the participant's body.

- Calibration: Before beginning, the sensors are calibrated to ensure that they are accurately measuring the desired physiological signal. This may involve using a calibration device and following a specific calibration protocol provided by the manufacturer.

- Data acquisition: This involves setting the software to record data at a specific sampling rate and specifying the duration of the recording which will be around 30 minutes per patient.

- Data analysis: The data is visualized, statistical measures are calculated, and patterns or trends in the data are identified.

Statistical Analysis Plan (SAP)

The Kolmogorov-Smirnov test will be used to assess the normality of the sample. Comparative analyses between groups will be performed by analysis of variance (ANOVA) or Kruskal-Wallis test. Associations of categorical variables will be analysed using the Chi-square test and Fisher's exact test. Intra-group assessment will be performed using the Wilcoxon test or Student's t-test for paired samples for continuous variables, and McNemar's test for categorical variables.

The data will also be evaluated using ANOVA for repeated measures in order to simultaneously verify the influence of the two study groups (between-group effect), the two assessments (within-group effect) and to estimate the group × time interaction effect for each of the variables. The results will be analysed by intention-to-treat. The significance level will be 5%. IBM SPSS Statistics for Windows, version 29.0 (SPSS Inc., Chicago, IL, USA) will be used.

Statistical analysis of the data collected will be performed by a researcher blinded for the intervention and for data collection.

GENERAL STUDY INFORMATION AND INFORMED CONSENT

UNIVERSITY CEU CARDENAL HERRERA

Principal investigator: Dr. Jose Miguel Soria

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Research and Ethics Committee of CEU Cardenal Herrera University Number: CEEI24/544

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GENERAL STUDY INFORMATION

TITLE: **Parental stress, child development and attachment in premature children during their first 24 months of life cared for in Child Development and Early Care Centers.**

Mr. Jose Miguel Soria, Neurobiologist, principal investigator and researcher reports that: The study will analyze the association between the level of stress of mothers due to premature birth and attendance at a Child Development and Early Care Center and the development of the child and the patterns of interactions between mother-child

The tests performed are simple and in no case involve difficulty, fatigue, danger, injury, pain or adverse reaction.

They will be carried out by Biologist, Psychologists and physiotherapists.

The general data of the subject will be collected (name, age, sex, physical variables and clinical history). The subject should come with comfortable clothes. The day that you were cited by the researcher, previous notice. Personal data is recognized in this study.

The personal data are confidential, apply to the protection of personal data. The study will be carried out in accordance with the Declaration of Helsinki and in accordance with current Spanish legislation (Royal Decree 223/2004 and the Biomedical Research Act 2007) and any other thing that may be applicable.

This study was approved by the Research and Ethics Committee of CEU Cardenal Herrera University (CEEI24/544).

INFORMED CONSENT

Mr/Mrs..... with Number identification .... freely and voluntarily, I DECLARE:

That I have read the information contained in this document about the general information of the study.

I have been informed that all tests are simple to perform and do not produce harmful effects on health. They will be carried out in appropriate facilities and will be carried out by qualified and specialized personnel.

I have also been informed that, the data collected in this study will be treated confidentially, applying the current legislation on protection of personal data (Royal Decree 223/2004 and the Biomedical Research Act 2007) and any other applicable.

Therefore, I give my consent, and I authorize Mr. Jose Miguel Soria, to carry out the detailed study in this document with the help of the necessary personnel with the appropriate qualification and specialization.

In Elche, to ..... of ..... of 202

SIGNED