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Non-invasive monitoring of stress biomarkers in the newborn period

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A B S T R A C T

The neonatal period is a highly sensitive time span during which stressful experiences may have an influence on later health outcomes. Medical procedures applied to newborn babies during hospitalization are stressors that trigger a physiological and psychological stress response. Stress response has been traditionally evaluated using scores based on behavioural signs such as facial expressions, limb movements, crying, etc., which are subjectively interpreted. Only few studies have employed measurable physiological signs to objectively evaluate the stress response to specific interventions.

The aim of this review is to inform of recently developed biochemical methods that allow clinicians to evaluate the stress response to medical procedures performed in the neonatal period in biological samples non-invasively obtained. Stress biomarkers are based on the physiological stress response mediated by the hypophysis-pituitary-adrenal axis and the sympathetic-adreno-medullary systems. Cortisol is at present the most widely employed laboratory determination to measure stress levels. In recent years, sequentially determined salivary cortisol levels have allowed non-invasive monitoring of newborn infants under stressful conditions in the NICU.

1. Introduction

Stress was first defined by Hans Selye as *"the non-specific response of the body to any demand for change"* [1]. From a biological point of view, it could be interpreted as the unspecific physiological response to certain triggering factors (internal or external stressful stimuli) that imply an activation of endocrinological systems [2]. These neuroendocrine systems include the hypothalamic-pituitary-adrenal (HPA) axis and the efferent systemic sympathetic-adreno-medullary systems (SAM) [2]. The most studied stress biomarkers are cortisol as indicator of HPA axis activity [3] and α-amylase as indicator of Sympathetic-Adreno-Medullary (SAM) axis activity due to its association with catecholamines [4]. Importantly, when stress stimuli are prolonged or very intense, the stress response may adversely affect crucial physiological functions such as growth, metabolism, circulation, immune response, etc. [2].

Assessing the stress response in newborns is difficult. It has been approached from different perspectives such as measurement of clinical variables (e.g.: heart rate, blood pressure, sweating response, etc.), or specifically designed scoring systems based on the interpretation of behavioral response (facial expressions, limb movements, crying, etc.). However, all these methods frequently lack reproducibility, are interfered with by other physiopathological circumstances or are excessively subjective [3]. In recent years, determinations of cortisol and α-amylase in biofluids such as amniotic fluid, plasma, and saliva have been broadly employed to determine the level of stress under specific circumstances or medical interventions [5]. In the newborn period, the tendency is to avoid using invasive methods for research purposes. Under these circumstances, both urinary and saliva analytical methods to reliably determine stress biomarkers have been validated and increasingly employed [6].

Accumulated evidence has shown that repeated stressful input during a sensible period such as the neonatal period may have profound and long-lasting effects upon brain development [7]. Survival of preterm infants has significantly increased in the last decade [8]; however, this is at the expense of being subjected to by a multitude of stressors that act during a period of extreme vulnerability upon the central nervous system and may lead to unwanted consequences [9].

This review article describes the physiology of the stress response and underscores its importance in the neonatal period. Moreover, clinical scoring systems employed to measure pain-derived stress response and analytical methods employed to assess stress biomarkers using non-invasive matrices are described.

2. Physiology and pathophysiology of the stress response

2.1. Chronic dysregulation of the stress physiological system

The physiological systems involved in the stress response (Fig. 1) are constituted by: (i) central components represented by different neuronal types responsible for the secretion of corticotropin releasing

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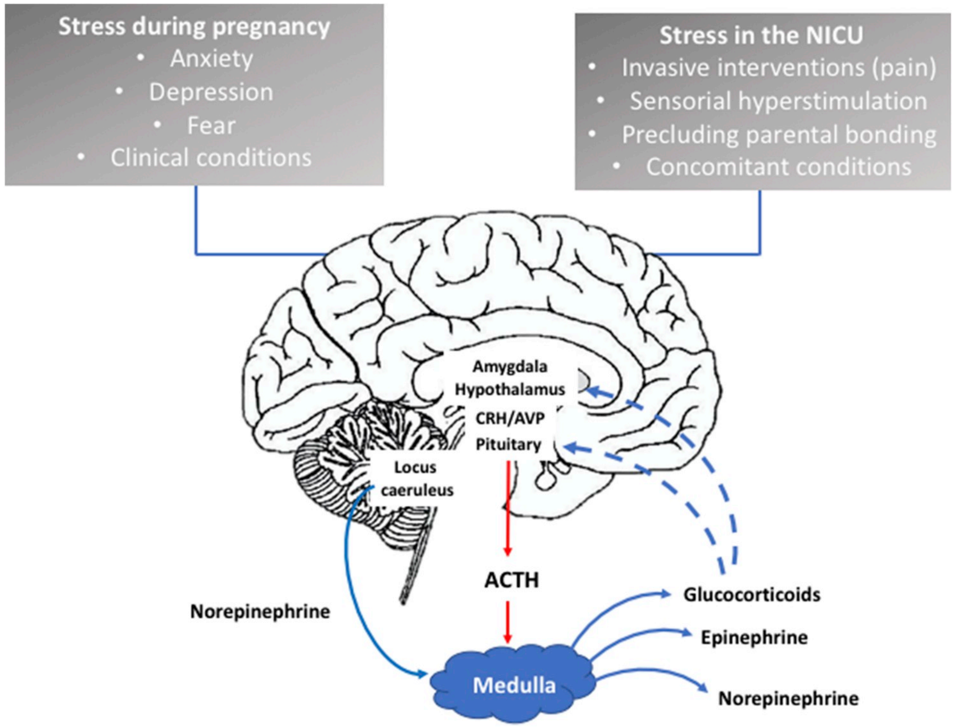

Fig. 1. Diagram representing the mechanisms involved in acute and chronic stress procedures.

hormone (CRH), arginine-vasopressin, or norepinephrine; and (iii) peripheral components represented by the HPA-axis, the efferent systemic SAM systems, and components dependent on the parasympathetic system [2]. The SAM axis is involved in preparing the body for sustained physical effort and decision making by means of the activation of the sympathetic branch of the Autonomic Nerve System in the brain, which in turn activates the adrenal medulla provoking the release of catecholamines that subsequently stimulate different target organs including brain and gastrointestinal tract among others [2] (Fig. 1). On the other hand, the HPA axis is involved in maintaining the parameters of effort and attention by means of the activation of the pituitary gland in the central nervous system which, in turn, activates the adrenal cortex leading to the release of glucocorticoids (GCs) into the systemic circulation (Fig. 1). GCs regulate the basal activity of the HPA axis. Of note, high plasma GCs concentrations act upon the hypothalamus and the pituitary gland forming negative feedback loops on the secretion of CRH and ACTH. GCs receptors that are widely distributed throughout the brain and peripheral tissues [10]. GCs control the resting and stress-related homeostasis, maintain the cardiovascular tone, regulate hepatic, muscular, and adipose tissue catabolism, and influence the inflammatory and immune responses. Moreover, GCs also influence other relevant functions such as reproduction, growth, behaviour, cognition, and electrolyte and water homeostasis [11]. In this sense, acute stress situations trigger a bundle of behavioural responses including alertness, increased arousal, improved cognition, focused attention among others [12]. In addition, physical adaptation to stress requires an increased oxygen supply to brain, myocardium, and the stressed body site, an increased cardiovascular tone and a shift towards catabolism to supply energy producing metabolites to the organs [13].

Repeated or very intense stress stimuli may have long-term somatic consequences. Chronic overproduction of cortisol and catecholamines lead to alterations in the secretion of insulin, growth, and sex steroid hormones [12]. The consequences are adiposity, sarcopenia, and osteoporosis and metabolic syndrome with carbohydrate intolerance, dyslipidemia, and arterial hypertension [12,14]. Moreover, chronic stress during this vulnerable period may impact growth and specific brain structures with life-long consequences on health [13]. Experimental studies have shown that chronic stress reduces the volume of the hippocampus, amygdala, and frontal cortex, brain structures involved in high-level reasoning process, decision-making process, reward processing, emotional regulation, and frustration tolerance. As a consequence, learning, memory, control of the stress system, and behavioural problems may arise [15].

2.2. Stress and inflammation

The association between the immune system and the SAM and HPA axis may explain the damage of stress on health. The SAM and HPA axis activate the immune system by means of the secretion of proinflammatory cytokines [16]. Specific proinflammatory cytokines such as interleukin 1 or tumour necrosis factor α, enhance the biological response to infection, inflammation, and trauma and even block the anti-inflammatory response contributing to fever, inflammation or even tissue destruction leading to shock, death or chronic conditions such as rheumatoid arthritis or inflammatory bowel disease among others [17]. Additionally, a prolonged secretion of proinflammatory cytokines contributes to neurotoxicity and neurodegeneration altering the noradrenergic, serotonergic and dopaminergic systems leading to impaired neurodevelopment [18–20].

2.3. Pain and stress

The HPA axis activation following an acute stress causes an elevation of CRH subsequently eliciting analgesic rather than pronociceptive effects [21]. Contrarily, chronic stress has been associated with allodynia and hyperalgesia. Rodents treated for long periods of time with cortisone exhibit intense pain reactions to cold, heat, or mechanical stimuli [22,23]. Moreover, further studies in rodents have also shown that increased HPA axis activation in response to early-life psychological stress may alter pain mechanisms in later life and elicit intense pain responses or compromised stress-induced analgesia even under acutely stressful conditions (for a review reference # [24]).

The central nervous system of newborn infants, and especially preterm infants is vulnerable to excitoxicity, oxidative stress, and inflammation. Hence, repeated exposure to neonatal pain-related stressful stimuli has been associated with altered brain microstructure and stress hormone levels, and neurodevelopment alterations which involve cognitive and motor disabilities such as behavioral problems [25].

3. The neonatal intensive care unit environment

3.1. Stress in the NICU

Undoubtedly, the NICU is a stressful environment for preterm infants. Survival of preterm infants with increasingly lower gestational ages entails prolonged NICU stays with the subsequent long-term exposure to stressful stimuli due to both medical procedures and the NICU environment. Firstly, hundreds of medical procedures are performed in such vulnerable infants as extremely low birth weight (ELBW) neonates in the first days after birth [26]. A substantial number of these interventions are highly stressful and can be perceived as painful such as heel pricks or vein, lumbar or thoracic punctures. Despite the introduction in recent years of protocols fostering the use of non-invasive procedures and the implementation of local and general analgesia and sedation, the amount of pain derived from stressful stimuli is still substantial [27]. In addition, other interventions such as clustered care, position changing, care of the airway, intravenous drug administration, etc., prompt stress responses in very preterm infants [28]. Invasive and non-invasive interventions trigger clinically-perceived responses in preterm infants such as tachycardia, hypotension, hypoxemia, hypothermia, or increased intracranial pressure, but also increases in stress hormones [29]. Finally, relevant aspects of the NICU environment which include sensorial hyperstimulation especially noise and light also provoke stress responses in the preterm infant [9]. The cumulative effect of the long-lasting stay in the NICU, submitted to stressful interventions/environment puts the ELBW infant at high risk of chronic stress with long-term consequences such as postnatal alterations in growth, neurobehavioral, cortical activation, and brain development outcomes [30,31].

3.2. Stressor scales for the newborn infant

In recent years, new protocols aiming to reduce stress in the NICU have been put forward. Interventions such as reducing noise and light, minimal and gentle care handling, prolonged periods of skin to skin contact, avoidance of invasive interventions with increased use of non-invasive ventilation, or point-of-care ultrasound have become standard of care. However, the use of effective interventions to reduce stress in the neonatal patient in the NICU requires reliable means of evaluating the level of stress as well as the efficacy of the intervention. In this regard, most of the clinical scores developed are based on clinical parameters that can be monitored (desaturations, tachycardia, hypotension) and evaluation scores based on behaviour such as the facial expressions (grimace), crying, agitation, alertness or muscle tone. However, these scales are highly dependent on the caregiver's knowledge of pain physiology, attitude towards pain and its expression, sensitivities to the patient, and attitudes or biases regarding pain [27]. Therefore, the Achilles heel of these assessments is an excessive individual-dependent scoring system. In addition, preterm infants' behavioral and physiological responses to stress stimuli are not always identifiable. Moreover, the stress response can be modulated by accumulated experiences, gestational age, and postconceptional age, gender, position and prenatal exposure to betamethasone among other factors [9]. Multimodal assessments usually employed combine both observable behavioral reactions and clinical autonomic indicators of stress responses. However, behavioral responses are subjectively biased [32] and the measurement of clinical autonomic indicators is usually carried out under patient sedation [27].

4. Non-invasive biochemical stress biomarkers

4.1. Cortisol under stressful situations

The physiological response to high stressful stimuli in newborns has been evaluated using different biomarkers [33,34]. At present, cortisol is the most employed biomarker [6,35]. For instance, during perinatal adverse events, such as asphyxia, HPA axis is activated and consequently, increased serum cortisol levels are found [36]. Cortisol levels are measured as stress biomarker in plasma, saliva, and urine samples using methods based on enzyme linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) with great accuracy, limits of quantification of less than 1 μg/nL, and less than 20% of coefficient of variation [6,33–36]. It should be highlighted that HPLC allows the simultaneous determination in the same assay of different compounds apart from cortisol that include catecholamines, providing more information with the same sample size [34].

4.2. Conundrum of alpha amylase in the perinatal period

The most common serological measures of autonomic activation are both plasma catecholamines, epinephrine and norepinephrine. However, the measurement of catecholamines in non-invasively obtained biofluids by HPLC or immunoassay has proven extremely difficult, and does not reflect the activity of the central nervous system. As a consequence, non-invasive surrogate markers have been actively sought. The action of norepinephrine upon the salivary glands rich in β-adrenoreceptors promoting the protein-to-fluid ratio, and among these the α-amylase in the saliva has been increasingly employed to indirectly measure the activity of NE as response to stress stimuli [37–39]. Seemingly, important as it would be to include α-amylase in the screening for stressful situations in the newborn period, however, its applicability is limited because the maturation of the salivary glands is not achieved until later in infancy and therefore changes in its composition in response to norepinephrine are lacking [40]. In a recent study, no differences in the levels of α-amylase were detected in the newborn and at 3 months in response to stress [41].

4.3. Other stress biomarkers

Enzyme immune analysis (EIA) allows to determine a series of additional stress biomarkers such as Copeptin which reflects perinatal stress associated with delivery mode [42], cytokines highly expressed as a consequence of stressful stimuli [43], and plasma oxytocin, associated with lower infant social engagement, or impaired social behaviour [44].

Also, to investigate if oxidative stress is involved in stressful medical procedures, some oxidation protein products and hydroperoxides have been determined in blood samples from full-term newborns during heel prick, observing a relationship between crying behavioural parameters and oxidative stress biomarkers levels [45].

Among other potential stress biomarkers, it was observed that levels of 17-hydroxyprogesterone in newborns' blood are highly sensitive to stressful stimuli [46]. This biomarker has been determined using immunoassay with satisfactory analytical performance [45,46]. However, most of these studies are based on biomarkers determined in blood samples with the consequent stress response associated to sampling that may invalidate the results [47].

5. Non-invasive biomarkers for stress monitoring

To accurately evaluate the level of stress, it seems reasonable to avoid the concurrence of a stressful intervention such as venepuncture or heel prick with analysis of a sample obtained. Therefore, non-invasively obtained samples have become generalized and a promising approach (Table 1). In this sense, saliva sampling is the most employed

Table 1
Non-invasive biomarkers for newborns stress monitoring.

Reference	Sample	Analysis technique	Biomarker	Results
[6]	Saliva	UPLC-MS/MS Kinetic enzyme assay	Cortisol α-amylase	Reference levels for cortisol and alpha-amylase were stablished. It could be applied to further clinical research on determination of perinatal and postnatal stress
[33]	Saliva	luminescence immunoassay	Cortisol	The trajectory of maternal cortisol across gestation was associated with the infant cortisol response to the heel-stick stressor.
[35]	Saliva	Enzyme Immunoassay kit	Cortisol	Newborns that received full rooming-in care (24 h) show lower cortisol levels compared to partial (14 h) rooming-in care.
[48]	Saliva	Enzyme-linked immunosorbent assay (ELISA)	Cortisol	Higher pain-related stress exposure during NICU stay was linearly associated with lower salivary cortisol reactivity to the Face-to-Face Still-Face procedure at 3-month age
[49]	Saliva	Enzyme Immunoassay kit	Cortisol	Salivary cortisol concentration was not significantly altered in infants after applied a noxious test.
[51]	Saliva	Luminescence enzyme assay Kinetic reaction assay	Cortisol α-amylase	α-amylase increases after an exam/inoculation stress protocol at 6 and 12 months, but not at 2 or 24 months of age. Cortisol increases at 2 and 6 months, but not at older ages.
[52]	Saliva	Radioimmunoassay	Cortisol	Elevated in newborns in a NICU compared to newborns that were sent to their own homes
[53]	Saliva	ELISA-Kit	Cortisol	Decrease in mothers after skin-skin contact mother-infant, while in infants there were found variable cortisol levels that do not indicate stress reduction.
[54]	Saliva	Enzyme immunoassay kit	Cortisol	Comparing changes in stress reactivity they found that tactile-only group infants had highest cortisol levels, followed by control group infants and infants who received a multisensory intervention.
[55]	Saliva	Enzyme immunoassay Enzyme immunoassay	Cortisol Oxytocin	Salivary Oxytocin levels increased significantly during SSC for parents and infants. Infant cortisol levels decreased significantly during SSC.
[57]	Saliva	Multiplexed bead immunoassay	Cytokines	There were a positive association between IL-6 and stress. The cytokine levels were different between preterm and fullterm children and appear to be influenced by stress situation and/or antigenic microbial challenge.
[61]	Saliva Urine Umbilical blood cord	Enzyme immunoassay Enzyme immunoassay Radio immunoassay	Cortisol	Usability rates of cortisol as biomarker were 80.6% (cord blood), 85.9% (saliva), and 93.5% (urine).
[34]	Urine	HPLC	Dopamine, epinephrine, norepinephrine	Urinary norepinephrine levels were slightly higher in the group of children born small for gestational age compared to control group after a stress test.
[60]	Urine	Enzyme immunoassay kit	Oxytocin	Plasma oxytocin decreased with age, at a rate of 15% per week, and exhibited strong stability within infants, Urine oxytocin was not correlated with plasma oxytocin and did not show a significant trend over time.
[68]	Umbilical cord plasma	Immunofluorescent assay	Copeptin	Higher levels in intrauterine growth restriction infants than in appropriate gestational age controls
[70]	Umbilical cord plasma	Enzyme immunoassays	Atrial natriuretic peptide, brain-type natriuretic peptide	High levels were associated with prematurity at birth, uteral contraction and antenatal stress.
[62]	Hair	Enzyme immunoassay kit	Cortisol	Hair cortisol shows intraindividual stability that can be used as chronic stress biomarker.
[63]	Hair	LC-MS/MS	Cortisol	Mild perinatal adversity may act as a susceptibility factor, moderating environmental effects. Elevated cortisol stress reactivity has been suggested to be a marker of enhanced susceptibility to the environment.
[65]	Nails	LC-MS-MS	DHEA or DHEA sulfate	DHEA, but not DHEA sulfate, was increased in infants of mothers with stressful life events during pregnancy.

matrix in the evaluation of several physiological mechanisms related to newborns [48]. Specifically, it is useful in the measurement of both basal levels of stress and response to stressful stimuli, since its simple sampling does not lead to increased stress, while blood sampling could do so. For this reason, serial salivary determinations have been a simple and useful solution for monitoring stress response. Among salivary stress biomarkers, cortisol, as a regulator of the autonomic nervous system [35,49] is the most assayed [50]. In general, salivary cortisol is measured by immunoassay and only a few studies have used analytical methods based on ultra-performance liquid chromatography (UPLC) [33,35,48,49,51–56]. The sensitivity achieved to quantify salivary cortisol using immunoassay is satisfactory; however, it shows poor specificity and high cross-reactivity with other compounds such as cortisone or corticosterone [53]. On the other hand, UPLC-based methods show high specificity, but the expensive equipment required constitutes an important drawback for its routine clinical use.

Salivary α-amylase is determined by immunoassay, this method shows high sensitivity, requires a previous dilution step of the samples and shows satisfactory precision (coefficients of variation < 15%) [56]. Salivary oxytocin is also measured by a previous validated immunoassay to evaluate the reduction of stress levels produced by skin-to-skin contact between parents and newborns [55]. Moreover, inflammation mediators, such as cytokines were determined in saliva by multiplex immunoassay, showing that interleukin 6 is influenced by the stress situation [57]. However, saliva sampling has some limitations since some factors can influence salivary composition (caries, periodontal disease), so it is important to identify the biomarkers alteration between systemic and oral disease [58]. In addition, the secretion rate influences the metabolites concentration, so sampling should be carried out without stimulation in order not to alter its composition [59].

Among other non-invasive biological samples, it is important to highlight urine. In this matrix, high norepinephrine levels were found in children born small for gestational age after a stress test [34]. This analyte was determined by HPLC with the advantage that other catecholamines could be studied simultaneously. In addition, urinary oxytocin (OT) was determined by immunoassays with great precision in extremely preterm infants as a neurodevelopment biomarker, and authors considered that further research was required in order to explore the dysregulation of the stress response as a potential mechanism contributing to impaired OT levels [60]. Also, cortisol has been determined in urine samples by means of immunoassays showing a higher utility than saliva or spinal cord samples [61]. Nevertheless, since differences in renal function influences the results of the different metabolites in urine samples, creatinine levels should be determined [60].

Finally, hair is also a promising biological sample to evaluate chronic stress in newborns by means of the cortisol levels, since they showed a suitable intra-individual stability [62,63], and cortisol in these samples has been measured by ELISA and HPLC. In the same way, nails can be used to evaluate the effects of stressful life events during pregnancy by means of determination of dehydroepiandrosterone (DHEA) or DHEA sulphate levels [64]. But this type of sample reflects accumulated stress levels and there is a lack of prospective studies about the relationship between cortisol levels in hair and the onset of mental disorders [65]. In addition, these determinations require additional sample treatment steps to remove sample interferences [62] and this is out of the scope of this review.

To summarize, cortisol is the most used stress biomarker but in the last few years new stress biomarkers such as cytokines, oxytocin or catecholamines have appeared, and also new promising non-invasive samples like hair or nails with the advantage of simple sampling to monitor stress without altering biomarker levels.

Practice points

- Diagnosis of acute and especially of chronic stress in the NICU is based on subjective evaluation of newborn infants' reactivity using clinical scoring systems thus lacking of objective measures.
- The use of sedation and analgesia further difficulties the assessment of stressful situations by the caregivers.
- The use of blood biochemical monitoring causes pain and stress to the baby and therefore alters plasma hormone levels rendering inefficacious.
- Saliva is a non-invasive and easily obtained matrix in which progressively more and more biomarkers of stress can be reliably determined, especially cortisol and oxidative stress metabolites.

Future directions

- The development of cot-side easy to interpret and reliable non-invasive methods to evaluate stress hormones especially catecholamine derivatives in urine and stress hormone derivatives in saliva.
- The big data analysis of continuously monitored physiological parameters such as heart rate, temperature, amplitude integrated EEG or arterial blood pressure.
- The development of algorithms integrating clinical, physiological and biochemical parameters with predictive value of the consequences of acute/chronic stress upon patients' health in later stages of life.

Conflicts of interest

The authors report no conflict of interest.

Appendix. Analytical methods developed for determination of biomarkers in saliva

Our group has substantial experience in determining non-invasive stress biomarkers, such as cortisol and α-amylase in saliva samples [66–70]. Saliva was collected in sterile bottles between 10 and 12 a.m. (minimum 1h after eating) and aliquoted into 1.5 mL tubes and stored at −80 °C until analysis. The sample treatment was based on previous work [6] (see Fig. 2). First, 50 μL of saliva sample were added to H2O (0.01% (v/v) acetic acid), and 5 μL of internal standard solution (600 nmol L-1 sulfadimethoxine). Then, liquid-liquid extraction (LLE) was carried by means of ethyl acetate and sonication. After that, ethyl acetate layers were evaporated to dryness using the speed vacuum concentrator. The residues were reconstituted in 100 μL of H2O:CH3OH (85:15, v/v, and 0.01% acetic acid) and injected in the chromatographic system coupled to tandem mass spectrometry (UPLC-MS/MS). This system consisted of a Waters Acquity UPLC-Xevo TQD system (Milford, MA, USA). Detection was carried out under positive electrospray ionization and chromatographic separation by using an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm) and a binary mobile phase CH3OH (0.01% v/v CH3COOH):H2O (0.01% v/v CH3COOH) with gradient elution.

In this way, the developed analytical method was validated obtaining satisfactory limits of detection for cortisol (0.2 nmol L^{-1}), as well as high precision (coefficients of variation 12–16%) and accuracy (recoveries 70–109%). Then, the corresponding reference ranges for cortisol were established for newborns and mothers at different perinatal periods [6], observing that the lower levels were obtained at 38 weeks of gestation for mothers and no differences were observed for newborns between birth and 3 months of age.

In addition, other studies applying the previous analytical method have been carried out to evaluate the effect of maternal age over stress-related variables in women [67], to predict preterm birth in pregnant women with threatened preterm labour [66], and to evaluate the influence of in-vitro fertilization over cortisol levels from pregnancy to postpartum period in women [68].

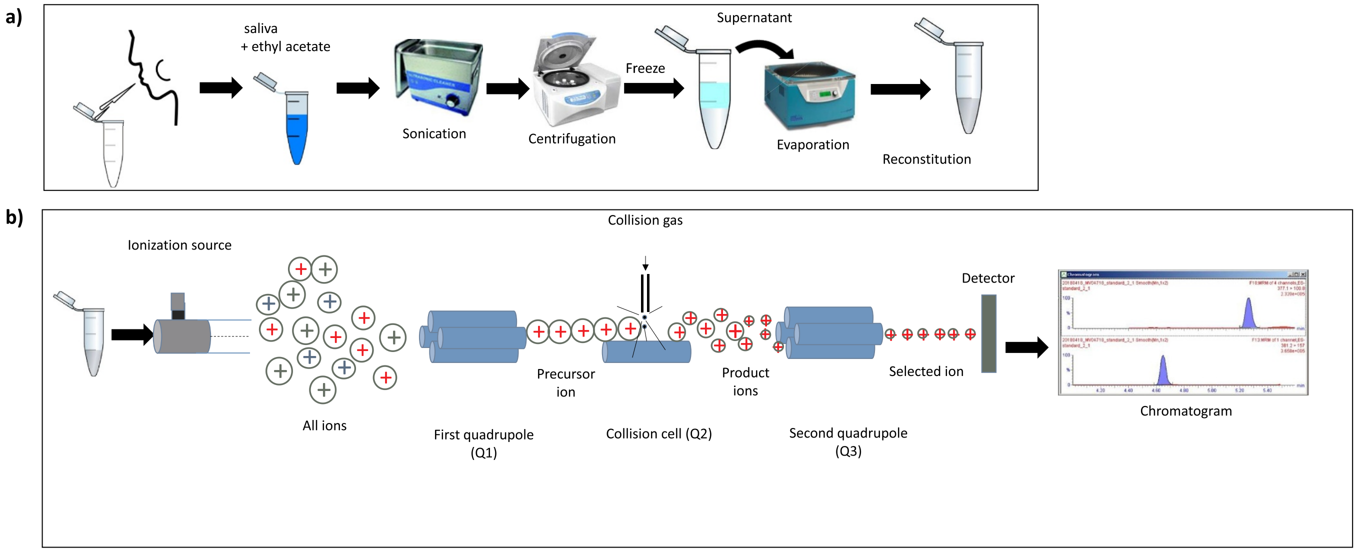

Fig. 2. Diagram representing the methodology to determine salivary stress biomarkers (developed in reference # 8). a) Scheme of saliva sample treatment. b) Scheme of a triple quadrupole mass spectrometer.

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