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Tesi di Specializzazione in Pediatria:

"Nociceptive Experiences in Preterm Infants and Early Clinical Markers of Neurodevelopmental Abnormalities"

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A mio nonno Domenico: sei sempre qui con me.

“Dio è il bene che facciamo, nulla di più.”

F. Arminio

Ringrazia,
vattene via quando serve,
non portare rancore,
ringrazia ancora, ricorda il male
che hai trasformato in bene,
libera la tua tenerezza,
ma studia il nero del mondo,
goditi quello che sei diventato,
niente di meglio era possibile,
non nascondere il tuo sconforto,
ringrazialo, intervistalo,
ma non dare retta
a tutto quello che ti dice,
inventati la gioia del giorno,
se ne trova sempre qualcuna
se ti guardi bene intorno.

Franco Arminio, Canti della Gratitudine

ABSTRACT

Introduction: Newborns, especially in neonatal intensive care units (NICUs), are frequently exposed to painful procedures. Preterm infants often undergo multiple interventions, which may affect neurological and visual development. Early pain experiences, assessed by the Neonatal Infant Stress Scale (NISS), have been linked to long-term neurobehavioral and neuro-visual impairments. This study evaluates the impact of early painful experiences on preterm infants, focusing on neurological and visual outcomes at term-equivalent age (TEA) (179).

Methods: This prospective observational study included 29 preterm neonates (birth weight <1500g) admitted to the NICU within their first 30 days of life. Standardized neurological assessments using the Hammersmith Neonatal Neurological Examination (HNNE) and visual evaluations were performed at TEA (40 weeks). Painful procedures were quantified using the NISS. Pearson's correlation and multiple regression analyses were used to examine relationships between NISS, VISIVO score, and Dubowitz score, adjusting for gestational age, birth weight, and Apgar scores. Neonates were also grouped by brain lesions (no lesion, minor lesion, moderate lesion) (198).

Results: Painful experiences significantly correlated with both neuro-visual and neurobehavioral outcomes. A positive correlation was found between NISS and VISIVO scores ($r = 0.812$; $p < 0.001$), and a negative correlation between NISS and Dubowitz scores ($r = -0.712$; $p < 0.001$). Painful procedures impact the neurological and neurovisual development independently of the presence of minor brain lesions . In regression models, NISS significantly predicted VISIVO ($\beta = 0.829$, CI 95% 0.019, 0.057 p=0.049) and Dubowitz scores ($\beta = -0.859$, CI 95% -0.102, -0.031 p=0.008).

Conclusions: Early painful experiences, particularly in neonates with brain lesions, significantly affect neurological and neuro-visual development. These findings highlight the importance of managing pain in NICUs to prevent long-term impairments, especially in infants exposed to high levels of stress (200, 201).

INTRODUCTION

Newborns inevitably encounter pain as part of routine neonatal care, which includes essential procedures such as vitamin K injections and heel sticks to obtain blood samples for screening tests. These painful interventions are not only routine but are often intensified for infants who are either critically ill or born prematurely, resulting in increased exposure to painful experiences in their earliest days of life. Over recent decades, the incidence of preterm births has continued to rise on a global scale, with an estimated 15 million babies now born prematurely each year worldwide **[1]**. Preterm birth, defined as occurring before the 37th week of gestation, is further subcategorized based on gestational age into three groups: extremely preterm (<28 weeks), very preterm (28–<32 weeks), and moderate to late preterm (32–<37 weeks) **[2]**. In 2010, a large-scale study across 184 countries indicated that preterm birth rates, as a percentage of live births, range between 5% and 18%. Interestingly, these rates do not show a straightforward correlation with a country's level of economic development. For instance, countries in northern Europe report a preterm birth rate of 5%, whereas the United States reports a significantly higher rate of 13% **[3] [4]**. In the United States alone, the economic burden associated with preterm births is substantial; in 2005, it was estimated at $26.2 billion, encompassing medical care, productivity loss, maternal delivery costs,

early intervention programs, and special education services **[5]**. Beyond economic impacts, prematurity remains the leading cause of mortality in children under the age of 5 globally **[1]** , solidifying it as a critical issue in contemporary global health.

While rates of preterm birth continue to increase worldwide, advances in neonatal medicine have enabled more preterm infants to survive, leading to significant improvements in survival rates. However, this increase in survival is frequently associated with the need for intensive medical and surgical interventions over extended periods of hospitalization. Newborns delivered at full term (39–41 weeks) have an average stay in neonatal intensive care units (NICUs) of approximately 4.9 days **[6]**. In contrast, when accounting for risk factors such as birth weight, sex, small-for-gestational-age status, ethnicity, fetal distress, and maternal stress, the average NICU stay for extremely preterm infants rises dramatically to around 81 days **[7]**. Throughout this period, these infants may undergo numerous painful procedures daily as part of their medical treatment. Premature infants represent a large proportion of NICU patients; in fact, infants born between 24 and 36 weeks make up 72.3% of the NICU population, compared to 27.7% of term infants (37–42 weeks) **[8]**. This distribution highlights that, while preterm infants are particularly vulnerable to pain due to their frequent exposure to invasive procedures, term

infants are not exempt from painful experiences. In the United States alone, roughly 460,000 newborns are admitted to the NICU each year, where they encounter pain from procedural, medical, or surgical interventions **[9]**.

The neonatal nervous system, characterized by its immaturity and high plasticity, is in a critical phase of development, which makes newborns especially susceptible to the impacts of early painful stimuli on their neurodevelopment **[10]** Painful sensory experiences during this period of neurodevelopment are generally detrimental, as they increase the likelihood of neurodevelopmental issues, which may present as both short- and long-term physical and psychological challenges **[4] [11] [12]**. These adverse effects can include altered brain development and processing patterns **[13] [14] [15]** and somatosensory changes that can heighten pain sensitivity over time **[16] [17]**. Such neurological impacts may have enduring effects that persist into childhood and adulthood, as demonstrated by an increasing body of research supporting these findings **[17] [18]**

Painful Interventions in Neonates During Early Life

Newborns regularly undergo a range of invasive procedures that may cause significant pain. These include blood sampling, vaccinations, vitamin K injections, and occasionally circumcision. The experience of pain is particularly

heightened in preterm infants or full-term newborns requiring admission to neonatal intensive care units (NICUs), where the frequency of painful interventions is significantly increased due to their vulnerable conditions **[8]**, **[18]**. On average, premature infants who require NICU care experience approximately 14 painful procedures each day, though the number may vary depending on the complexity of their care needs **[19] [20]**. These painful interventions span from repetitive heel sticks for blood collection to more intensive procedures, such as minimally invasive and open surgeries **[21] [22] [23] [24]**.

Among these procedures, the heel stick is one of the most performed in NICUs. It involves a quick puncture, often paired with venipuncture or mechanical lancets, to collect blood samples from the heel. Although there is a push to incorporate non-pharmacological analgesic strategies to reduce discomfort **[9]**, heel sticks remain standard in clinical practice due to their accessibility and the volume of blood they yield, even though newborns may experience significant pain with each procedure.

For newborns with severe respiratory distress, a different level of intervention may be necessary. Endotracheal intubation is frequently performed on newborns needing respiratory support, as it secures airway access and facilitates mechanical ventilation. In adult patients, mechanical ventilation has

been linked with pain, discomfort, and feelings of depression **[25]**. Since the procedure is acknowledged as painful **[26] [27]**, guidelines by the International Evidence-Based Group for Neonatal Pain advise that intubation without analgesia or sedation should be limited to emergency resuscitation in the delivery room or situations where intravenous access is unavailable **[22]**. Although research has shown that opioids can effectively reduce pain indicators in ventilated neonates **[28]**, pain management practices in the NICU are still less than optimal **[29]**. In addition, concerns persist regarding potential long-term opioid effects, such as neuromotor impairments **[30] [31]**. In the United States, approximately 35,000 preterm and 20,000 term neonates undergo mechanical ventilation annually, which underscores its role as a major contributor to the early-life pain burden **[32]**.

In some cases, open or minimally invasive surgeries are required to address congenital abnormalities or manage life-threatening conditions. While exact figures are not available, up to 33% of extremely preterm infants undergo surgery to correct congenital anomalies or address serious complications **[17] [33]**. These newborns often undergo multiple surgical interventions, resulting in increased exposure to painful procedures such as repeated venipunctures, endotracheal intubations, anesthetic administrations, as well as the surgery and postoperative pain itself. As the number of procedures rises, so does the

risk of developing chronic pain and heightened pain sensitivity at the injury site **[18] [34] [35]**. This chronic post-surgical pain is believed to result from central sensitization—a process where maladaptive changes in the spinal cord increase neuronal excitability and reduce inhibitory signals, leading to enhanced and prolonged pain responses **[36]**.

Another common painful procedure for neonates is the heel lance, or needle prick, used to draw blood samples for various screenings, including glucose levels, chemistry panels, complete blood counts, and toxicology tests. This procedure involves puncturing the heel, often followed by squeezing it to gather sufficient blood for testing, which is known to cause more pain for newborns than venipuncture **[37]**. Despite the discomfort and recommendations advocating for less invasive options, heel sticks are still commonly performed, given their diagnostic necessity and reliability **[8] [38]**.

Neonatal Pain: historical perspectives and Management

Historically, the inability of the newborn to describe experiences, the lack of memory of early life events, and the immaturity of the nervous system have led many to believe that newborns are incapable of experiencing pain. This belief, until recently, has resulted in inadequate pain management in the neonatal population. In fact, in the early years of anesthetic use, due to the associated risks, surgical procedures on newborns were sometimes performed

without any anesthesia. In other cases, only mild anesthesia was administered, using a combination of muscle relaxants and nitrous oxide.

In the 1980s, a randomized controlled trial (RCT) conducted by Anand et al. demonstrated that the addition of fentanyl or halothane to the standard anesthesia of that period led to a reduction in surgical stress, with a lower hormonal and metabolic response [39] [40]. These effects were observed both during and after surgery, resulting in a decrease in complications and mortality [41]. Anand's studies were pivotal in changing the perception of neonatal pain, affecting both term and preterm infants. It is important to note that it has only been in the last 30 to 40 years that nociceptive procedures in neonates have become a routine practice. For instance, since the introduction of metabolic disease screening, all newborns undergo at least one heel prick.

The age of the newborn, and particularly cerebral immaturity, raises concerns that such nociceptive experiences may influence an immature brain. These concerns are particularly heightened in the case of preterm infants, who present a high degree of anatomical and functional immaturity. With advancements in neonatal care techniques, there has been a progressive increase in the survival rate of preterm infants. During their stay in the neonatal intensive care unit (NICU), these infants are exposed to the highest number of stressful procedures [42]. Furthermore, they undergo atypical and

abnormal sensory exposure in an environment that is unnatural not only due to its potential invasiveness but also because of physical separation from the mother. This hostile environment is characterized by invasive procedures such as tracheal intubation, insertion of vascular access, thoracic drainage, and repeated arterial, venous, and capillary blood draws, which, along with surgical interventions, represent the most significant events. In contrast, the impact of medical procedures like nasogastric tube insertion and various dressings is less clear [22].

Many of these procedures are considered only mildly painful and, therefore, are routinely repeated numerous times during the infant's stay in intensive care with relative ease, with an average frequency of 1 to 14 procedures per day, often without the use of specific measures to mitigate discomfort [43] [44] [45]. However, the high frequency of these interventions, despite their relative harmlessness, makes them particularly stressful for the newborn.

To minimize pain, a range of technical measures can be employed, both pharmacological and non-pharmacological. The choice of analgesia depends on the invasiveness of the procedure. For interventions that are not excessively stressful (e.g., venous access insertion, blood sampling, nasogastric tube changes), the oral administration of glucose solution [46]

[47], skin-to-skin contact [48], pacifier use, and breastfeeding [49] are commonly utilized and effective in clinical practice for calming the infant.

For mechanical ventilation, surgical interventions, or situations where the newborn experiences significant distress, pharmacological analgesic treatments are used, sometimes involving the administration of opioids. However, the long-term effects of opioid use on brain development remain unclear [50].

In some situations, the pain perceived by the infant may be underestimated by healthcare professionals, leading to no pain relief measures being undertaken. An example of this is the execution of capillary blood gas analysis, which is performed in newborns through heel pricking and subsequent squeezing. This procedure, often considered harmless, is in fact painful and distressing for the patient [13] [51].

The long-term effect of all these stressful events on the brain is still not completely understood [52] [53].

The Neurophysiology of Pain

The limb withdrawal reflex in response to a painful stimulus is one of the earliest to develop, present even before cortical connections are formed. This reflex exemplifies spinal-level processing of pain, persisting even in cases of decortication. The first nociceptive responses are observed in the fetal stage, when needles used for cordocentesis puncture the fetus at 20-22 weeks of gestation. However, for the nociceptive stimulus to acquire the emotional and affective component typical of pain perception, cortical processing across various brain areas is required [54].

Around the 20th week of gestation, thalamic axons form connections with the subplate zone, a transient population of neurons that exists during intrauterine development and undergoes programmed cell death by the end of gestation or shortly thereafter [55] [56] [57]. These subplate neurons connect with developing cortical neurons, enabling the reception of external stimuli through indirect thalamic connections [56] . The subplate is crucial for brain development, as animal studies have shown that its ablation results in weak and abnormal thalamo-cortical and cortico-cortical connections [56] [58] [59].

After a period in which thalamic neurons remain connected to the cortex via the subplate, thalamo-cortical connections begin to form directly around the 31st to 35th week of gestation, depending on the brain region [56]. The subplate starts to disappear around this time [57]. Based on this evidence, it can be speculated that sensory perceptions, including painful stimuli, begin to reach the cerebral cortex as early as the 20th week of gestation and become gradually more effective [60] [61].

Several EEG studies have demonstrated that sensory and nociceptive inputs are processed in both term and preterm neonates [62] [63] [64]. Further investigations using Near-Infrared Spectroscopy (NIRS) have revealed that following painful clinical procedures, such as heel sticks for capillary blood gas analysis and venous blood draws, the concentration of oxyhemoglobin increases in the sensorimotor cortical areas [60] [61]. NIRS has also registered significant hemodynamic changes in the brain following prolonged and painful medical procedures, such as endotracheal tube placement [65].

In recent years, more studies have examined nociceptive processing in the neonatal brain using EEG and functional magnetic resonance imaging (fMRI). Through EEG, Slater, Fitzgerald, and colleagues demonstrated that term-born infants show a specific cerebral pattern for nociception in response to acute painful stimuli. In their study, they compared the brain's response to a heel

prick with a lancet to a simulated procedure where the heel was not punctured. The results showed that while an initial EEG potential (around 250 ms) was recorded for both stimuli, a delayed potential (around 500 ms) was observed only in infants who were pricked [66]. This led to the conclusion that the second potential is specific to nociception, demonstrating a neural differentiation between tactile and painful stimuli.

When analyzing the responses to heel pricks in neonates between 28 and 45 weeks of gestation, brain activity was found to be more pronounced after the 35th week [67]. Before this time, non-specific neuronal discharges, known as delta-brushes, are more likely to occur in response to both painful and non-painful stimuli. The transition from delta-brushes to specific evoked potentials happens around the 35th week [68], coinciding with the development of visual and auditory evoked potentials [63] [64] and is likely explained by the maturation of direct thalamo-cortical connections and the disappearance of the subplate [56] [67].

The studies mentioned above have generally focused on nociceptive stimuli experienced during clinical procedures. To further investigate the neonatal brain's response to painful stimuli, Slater et al. introduced alternative stimulators known to activate Aδ fibers [68] [69]. Adults subjected to this stimulation describe a quick, moderately painful pricking sensation [70]. In

term neonates, Hartley et al. observed EEG activation of nociception-specific areas on the heels at three levels of stimulation force, even in the absence of observable behavioral changes. The activity recorded in these areas was proportional to the intensity of the stimuli, though it was never as high as that evoked by a heel prick [71]. The stimulator could also be used during fMRI, making it useful for conducting fMRI studies [72].

Goksan's group replicated this study, analyzing the brain's response to stimuli via fMRI. The authors compared neonates to a group of adults receiving the same stimuli. The brain activity of the adults matched previous studies, showing activation in the precentral and postcentral gyri, insula, thalamus, and other brain regions associated with pain experiences. In neonates, 18 of the 20 areas active in adults were also active. The two regions not involved in neonatal pain processing were the orbitofrontal cortex and the amygdala. These areas are known to be involved in reward processing and fear responses, respectively [73] [74], suggesting that neonates might be too immature to contextualize stimuli in this manner [70]. However, increased activity was observed in the anterior cingulate cortex, which is activated in adults in response to unpleasant stimuli, suggesting that neonates may be capable of attributing an emotional component to pain beyond mere sensory perception.

These studies have demonstrated both the spatial and temporal aspects of the neonatal brain's response to even low levels of pain.

Neurobiological Mechanisms Underlying Enhanced Pain Responses to Subsequent Injury

Pain is a complex phenomenon with multidimensional and multisensory aspects, requiring the integration of numerous intact systems to produce a final emotional and sensory response. To perceive pain, a noxious stimulus must be transmitted through various levels of the neuroaxis and processed by higher brain centers. Pain responses, or nociception, can be regulated at the spinal cord level through the descending inhibitory pain pathway. This descending pathway includes key structures like the periaqueductal gray matter in the upper brainstem, the locus coeruleus, the nucleus raphe magnus, and the nucleus reticularis gigantocellularis. Under normal circumstances, this modulatory system maintains a balance between facilitating and inhibiting pain signals, thus preserving a baseline of sensory processing [75] . When disrupted, however, this system can lead to an increase in nociceptive sensitivity, facilitating the promotion and persistence of chronic pain [76].

The pain transmission system becomes even more intricate due to interactions between the central and peripheral nervous systems and the immune system,

known as neuroimmune interactions. These interactions serve multiple roles: they help recruit local neuronal components to fine-tune immune responses, contribute to synaptic plasticity during both development and adulthood, and coordinate the body's response to infection by pathogens [77]. This established bidirectional communication between the neuronal and immune systems is crucial for pain modulation [78].

During the neonatal period, infants exhibit a high degree of neuroplasticity, making them particularly vulnerable to the modulating effects of noxious stimuli [79] [78], especially if such stimuli are repetitive. Repeated painful experiences during this critical developmental phase can lead to both structural and functional changes within the nervous system, affecting peripheral, spinal, and supraspinal levels of pain processing, as well as neuroendocrine functions and overall neurological development [78] [80]. Evidence from human studies supports that prolonged and repetitive pain early in life can alter the way pain is processed later, increasing pain sensitivity in subsequent experiences [81] and affecting overall pain responses in the long term [82]. Although it is not fully determined whether these changes contribute to the onset of chronic pain, structural and functional reorganizations resulting from early pain exposure are believed to play a role in heightened responses to later painful stimuli.

Development of Peripheral Nociceptive Fibers

It was once thought that neonates were incapable of perceiving pain due to the immaturity of their sensory nervous systems [83]. However, both neuroanatomical and behavioral studies have provided substantial evidence to refute this belief [80]. During the neonatal period, significant maturation of pain transmission and modulation pathways is actively occurring. Although the peripheral nervous system becomes mature and functional by 24 weeks of gestation, notable neuroanatomical changes in the distribution of unmyelinated (C) and myelinated (A) ascending fibers continue into the postnatal period [84].

At birth, neonates have a higher density of myelinated Aδ sensory fibers, which are primarily responsible for the initial perception of pain, but a lower density of C fibers, which contribute to the intensity of pain perception [85]. This distribution may make neonates more susceptible to hypersensitivity due to an imbalance between the number of afferent sensory fibers and the inhibitory (descending) influences in the pain pathway [82]. Such structural and functional development in the sensory nervous system during this early life stage underscores the capacity for pain perception in neonates and the potential for lasting impacts on pain sensitivity.

Spinal Cord Mechanisms

The spinal cord undergoes neuronal and synaptic changes in response to peripheral inputs, a phenomenon known as activity-dependent plasticity. This plasticity shapes spinal cord function during postnatal development and continues to play a role throughout life. [78] Activity in peripheral C-fibers drives cellular "wind-up" in the spinal cord, triggering widespread changes in the neuronal network. These changes can result in clinical symptoms such as spontaneous pain, abnormal sensitivity to painful stimuli, or sensitivity to normally non-painful stimuli, as well as referred pain that often follows peripheral tissue injury [86]. The intensity, duration, and timing of these peripheral inputs are critical factors influencing the resulting modifications within the spinal cord.

At the spinal cord level, the balance between excitatory and inhibitory neurotransmitters evolves with maturation. In mature individuals, excitatory neurotransmitters involved in pain transmission include substance P, calcitonin gene-related peptide, and glutamate, while inhibitory neurotransmitters include γ-aminobutyric acid (GABA), norepinephrine, glycine, adenosine, endogenous cannabinoids, and opioid peptides [78]. During early neurodevelopment, GABA acts in an excitatory manner in certain regions, such as the hippocampus, due to an inverted chloride gradient that causes

depolarization [78]. Similarly, in the immature dorsal horn, GABA initially has an excitatory effect; however, this shifts to an inhibitory function as development progresses, typically by the end of the first postnatal week [78].

Research suggests that, in the spinal cord, GABAergic inhibitory transmission to dorsal horn neurons becomes functional early in life, and low chloride extrusion capacity does not prevent GABA from exerting its typical inhibitory effects. GABA can activate voltage-gated sodium and calcium channels, enhancing the activity of N-methyl-D-aspartate (NMDA) receptors by reducing the voltage-dependent magnesium block on these receptors. NMDA receptors, which are associated with central sensitization, contribute to the expansion of receptive fields in the dorsal horn. This expansion persists until approximately 42 weeks of gestation, gradually narrowing to an adult configuration by 44 gestational weeks in humans. The simultaneous upregulation of NMDA receptor expression and shifts in GABA function create a highly excitable neuronal environment during the early stages of life. In such a state, repeated exposure to painful and non-painful stimuli can lead to hypersensitivity, which may become exacerbated with additional stimuli. [78]

Descending Modulatory Systems

In the mature nervous system, descending pathways play a key role in inhibiting noxious signals at the spinal cord level, helping to modulate pain.

During infancy, however, the descending modulatory system operates primarily in a facilitatory mode, which is influenced by mu-opioid receptor pathways located in the rostroventral medulla (RVM) [78]. As this system matures, descending control over spinal nociceptive circuits gradually shifts from facilitation to inhibition, achieving a more balanced modulation of pain by adulthood [87]. Until these inhibitory mechanisms are fully developed, typically later in postnatal life, the endogenous suppression of noxious peripheral stimuli remains incomplete, leaving the neonatal nervous system more susceptible to the effects of painful stimuli [72] [88]. Early exposure to noxious stimuli can therefore lead to long-term or even permanent changes in RVM circuits and other inhibitory pathways.

The neonatal period represents a stage of increased vulnerability to long-term modifications induced by painful experiences, especially before the descending inhibitory pathways are fully established. Such early-life noxious stimuli can lead to enduring alterations in pain modulation circuits, including those of the RVM, which may affect pain sensitivity throughout life. Early painful experiences are associated with both immediate hyperalgesia and prolonged hypoalgesia, indicating that the impact of these stimuli can vary over time depending on developmental factors within the pain modulatory system. These findings underscore the importance of understanding the

distinct properties and timing of descending modulatory mechanisms in relation to early-life pain exposure. [78]

Brain Development

Pruning, the selective refinement of active neuronal circuits, occurs throughout life; however, during the late second trimester and the neonatal period, this and other processes that shape neural architecture are particularly active in the human brain [89]. During the neonatal stage, rapid neuronal proliferation and differentiation take place. These include maturation of oligodendrocytes, distribution and activation of microglia, differentiation and migration of cortical neurons, and the development of subplate neurons, cerebral cortex, deep nuclear structures, and axons. Additionally, this period involves the formation of synaptic connections, an increase in cortical surface area, and the onset of gyral folding [90]. As mentioned earlier, procedural pain commonly experienced in the NICU can have significant effects on brain development and function [78].

Studies have linked neonatal pain with reduced brain microstructure and volumes in humans [91]. Proposed mechanisms responsible for these reductions in brain volume and structure include excitotoxicity and disrupted axonal development. Pain experienced early in life may lead to alterations in neuronal growth and structure due to excitotoxic damage, with potential

downstream effects on neuronal function. In human imaging studies, such as diffusion tensor imaging (DTI) and MRI, pain-related impairments in axonal development have been observed, providing a structural basis for abnormal brain development [13]. For example, repeated procedural pain in preterm infants has been associated with damaged subcortical neurons, which may result in secondary axonal abnormalities in the white matter (13).

Brain regions most affected by early-life pain and noxious stimuli are those connected to the limbic system (e.g., hippocampus, amygdala, and thalamus) and the basal ganglia [92]. The thalamus, a critical relay center for sensory and motor signals to the cerebral cortex, shows decreased volumes and disrupted metabolic growth in infants who have experienced pain, as well as alterations in the maturation of thalamocortical pathways [14]. Given that thalamocortical connections undergo rapid formation during gestation and early postnatal life, these structures are particularly vulnerable to excitotoxic damage [93].

In human development, NMDA and GABA receptors play essential roles in synaptic plasticity, especially in areas such as the amygdala, where they support neuronal responsiveness and structural reorganization. Early life exposure to painful stimuli may disrupt the normal development of these receptors, affecting processes like neuronal cytoskeleton development and

myelination, with potential long-term impacts on cognitive functions and learning [94]. These structural and functional alterations within the limbic system are believed to influence lifelong memory and emotional regulation, contributing to persistent changes in memory processing and fear responses [78].

These factors and mechanisms influencing brain development continue to be an area of research, and understanding the cellular and molecular mechanisms involved in pain-related developmental disruptions remains crucial.

The Long-Term Effects of Early Nociceptive Experiences

Several lines of evidence suggest that exposure to pain early in life may have long-term impacts on both subsequent pain processing and the functioning of neurological structures. In addition to genetic factors, the development of the nervous system is activity dependent **[95] [96]**. While a lack of activity during critical developmental windows can disrupt normal nervous system development, excessive activity during early development can also lead to non-physiological or even maladaptive adjustments. As previously noted, neonates requiring neonatal care may undergo multiple nociceptive or stressful procedures daily as part of their essential medical treatment. These procedures are performed during a period of rapid neurological development when the neonatal nervous system is particularly vulnerable. The threshold for evoking reflexes in neonates is lower than in adults and increases with age **[97] [98] [99]**. Particularly in preterm infants, repeated stimulation induces sensitization **[97] [98] [100]** and heightened responses to tactile stimulation following painful procedures **[101]**. The increased sensitivity to nociceptive stimuli in the neonatal period, compared to adulthood, may exacerbate the effect of early life pain on nervous system development, impacting affective and behavioral domains.

Preterm infants exhibit greater perceptual sensitization to tonic heat and decreased thermal sensitivity in school age compared to full-term infants, but they do not show altered responses to mechanical stimuli **[102] [103]**. Thermal sensitivity alterations are more frequently observed in preterm infants who underwent surgical interventions early in life **[102]**. Early nociceptive experiences, which some define from an evolutionary perspective as "recent and unexpected," can lead to long-lasting local and global alterations in sensory processing **[104] [105]**. For instance, children who underwent cardiac surgery during infancy exhibit altered sensory processing in the scarred thoracic area compared to the contralateral region. **[105]**

Using EEG, Slater et al. **[106]**demonstrated that preterm infants studied at term-equivalent age exhibit more pronounced nociceptive-specific brain activity compared to full-term infants, who are relatively naïve to pain. Hohmeister et al. **[107]** evaluated fMRI responses to a heat stimulus (adjusted for each child to be slightly painful) in former preterm and full-term children aged 11 to 16 years. The former preterm children showed higher levels of brain activity (in the thalamus, anterior cingulate cortex, cerebellum, basal ganglia, and periaqueductal gray) in response to the painful thermal stimulus compared to full-term children who had not required neonatal care.

Consequences of Early Life Pain in Humans

Despite the high frequency of painful procedures that neonates endure in NICUs, the majority do not receive pharmacological pain relief **[108] [84] [109]**or receive insufficient dosages of analgesics **[110] [111]**. Contributing factors include underestimation of the infants' pain perception and concerns regarding potential side effects, leading clinicians to either withhold or administer suboptimal doses. Nonetheless, studies have increasingly shown that untreated pain in early life can have lasting negative effects, influencing sensorimotor and cognitive development, mood, pain responses, medication needs, and overall health by the time children reach adolescence.

Long-Term Neurosensory and Cognitive Impairments

Early painful experiences appear to shape the somatosensory foundation that underpins subsequent perceptual, cognitive, and social development **[112]**. Research supports this view, as recent studies have identified associations between early painful experiences and diminished neural responses to non-painful touch **[112]**. The risk of neurosensory impairments is heightened in premature infants, and outcomes can include sensorimotor difficulties such as visual and auditory impairments, cerebral palsy, delayed development, and reduced intellectual functioning throughout childhood and into adulthood **[113]**. For instance, neonatal surgeries have been linked to significant, lasting

neurosensory disabilities, with effects observable even at eight years old, and males appear to be at a greater risk for these disabilities **[114]**.

Exposure to pain in early life can negatively impact neurodevelopment, including brain growth, which has implications for cognitive abilities. For example, a study found an inverse relationship between the frequency of invasive procedures in the NICU and the volumes of the amygdala and thalamus in eight-year-old children who were born very preterm **[92]**. Lower brain volumes in these children have also been correlated with poorer cognitive outcomes, including lower IQ, language and attention difficulties, deficits in visual-motor skills, and behavioral challenge **[92] [78]**. Notably, these cognitive and behavioral deficits often persist into adolescence and young adulthood **[115] [116] [117]**. Research suggests that preterm boys may be more susceptible to adverse neurodevelopmental outcomes compared to girls **[116] [78]**. Meanwhile, early pain exposure in females has been associated with slower growth in the thalamus, basal ganglia, and total brain volume **[118] [91]**, though these sex-based differences may vary depending on study design, environmental factors, and other individual characteristics.

Studies in rodents reveal similar patterns of cognitive and brain development impairment resulting from early life pain. In rodent models mimicking NICU-like conditions, exposure to neonatal pain has led to long-term brain

development changes **[119]**. For example, rats subjected to frequent needle pricks in early life (4 times daily during the first two weeks) demonstrated lasting memory impairments **[120]**, while those exposed to repeated pricks every other day over eight weeks developed short-term memory deficits **[121]**. Similarly, Ranger et al. reported that adult mice exposed to repetitive pain in their first week of life exhibited poorer memory **[122]**.

Negative Impacts on Psychosocial Behaviors

There is a strong link between early exposure to painful procedures and altered behavioral development. Individuals who experience repetitive pain in infancy may develop attention-deficit disorders, heightened vigilance, exaggerated startle responses, and other long-term stress-related psychosocial difficulties **[123] [124]**. Internalizing behaviors, characterized by actions directed inward—such as withdrawal, anxiety, and depression—are more common among these individuals **[125]**. Higher levels of internalizing behaviors are associated with lower social competence in children, contributing to social challenges and reduced peer acceptance **[126]**. These behavioral impacts have been observed as early as 18 months of age and often continue into childhood and adulthood **[78]**. Notably, children born preterm and exposed to early neonatal pain show a higher rate of internalizing behaviors than those born full-term **[127] [128]**. For instance, young adults

born extremely preterm who underwent neonatal surgery exhibit higher levels of anxiety and pain catastrophizing compared to term-born peers **[129]**. Such internalizing tendencies can lead to other serious health risks later in life, including an increased likelihood of substance use disorders, such as drug addiction **[130]**, alcoholism **[131]**, and obesity **[132]**.

Varied Behavioral Pain Responses

The recognition of early-life pain can be challenging due to variability in pain responses, which may be influenced by age and cumulative painful experiences. Facial expressions and withdrawal reflexes are among the key indicators of pain response in neonates, often associated with nociceptive activity **[78]**. Facial grimacing following a heel stick procedure has been observed as early as 28 weeks' gestation, with expressions becoming more pronounced as gestational age increases. Recent studies demonstrate that discriminative facial expressions in response to painful versus non-painful stimuli emerge at around 33 weeks' gestation, coinciding with brain maturation **[133]**.

Interestingly, some research suggests an inverse relationship between the degree of facial grimacing and the number of invasive procedures experienced **[110]**. Additionally, infants who undergo numerous painful procedures in early life tend to display reduced behavioral pain responses (e.g., facial grimace,

crying, state of arousal) and lower pain scores in response to subsequent painful stimuli within the first month of life **[134] [135]**. Although behavioral changes may not always be apparent following pain exposure, noxious stimuli like heel sticks in infants between 25- and 43-weeks' gestation are processed at the somatosensory cortical level **[136]**. Evidence of cortical activation—measured by increased hemodynamic responses—without accompanying facial motor responses suggests that pain perception involves emotional processing requiring cortical engagement **[136] [137]**. Sex differences are also evident, with preterm female neonates exhibiting more pronounced facial responses to acute pain compared to males **[138]**.

Reorganization of Pain Processing

Painful interventions during neonatal development have been shown to influence sensitivity to painful stimuli later in life. Individuals with a history of neonatal pain have reported adverse hemodynamic effects, such as increased heart rate and decreased oxygen saturation, in response to subsequent painful experiences **[78]**. In these cases, males show a more pronounced response than females, with differences particularly evident in hemoglobin oxygenation levels after a secondary venipuncture **[139]**.

Experiencing procedural pain early in life appears to lead to alterations in sensory function, resulting in initial hyposensitivity to acute pain during the

neonatal period, which can shift to hypersensitivity as the individual ages **[79]**. However, the direction of these changes in sensitivity is not entirely consistent. Studies show that children born preterm or full term who underwent neonatal surgery may exhibit generalized thermal hyposensitivity, as well as mechanical and thermal hyposensitivity in areas near previous tissue injury, between the ages of 10 and 12 **[105]**. On the other hand, adolescents born preterm (12–18 years old) demonstrate greater mechanical hypersensitivity compared to full-term peers, with females generally reporting higher sensitivity than males **[140]**. Furthermore, young adults (18–20 years old) who were born extremely preterm and underwent surgery as neonates report higher pain intensity levels and moderate to severe persistent pain more often than those born at term **[17]**.

Research has also explored whether painful experiences in early life can influence pain responses following later acute injuries. Hypersensitivity and allodynia after secondary injuries have been observed, with studies indicating heightened pain responses, such as longer crying times and elevated pain scores, during routine vaccinations among infants who had undergone neonatal circumcision compared to those who had not **[141] [142]**. Additionally, infants aged 4–21 weeks requiring repeat surgeries on scarred areas from previous neonatal procedures experienced higher postoperative

pain scores and required more analgesics than control groups **[143]**. A positive association has been identified between the number of invasive procedures experienced in the neonatal period and higher pain intensity ratings during venipuncture at 7.5 years old **[144]**. Variability in pain responses, manifesting as either hypersensitivity or hyposensitivity with or without secondary injuries, likely stems from factors such as differences in the intensity and frequency of painful stimuli, age at follow-up, as well as individual and environmental variables across study populations and outcomes.

Increased Risk of Poor Health Outcomes

Painful experiences early in life can lead to biological changes that increase the likelihood of poor health outcomes later. Premature infants exposed to repeated painful stimuli face a greater risk of developing non-communicable diseases, such as diabetes and hypertension, as well as other chronic health issues as they grow older **[145]**, thereby creating an intergenerational cycle of health risks. Adjusting for other factors, infants who undergo repeated procedural pain in the neonatal period show reduced postnatal growth **[146]**. Infants who are smaller at birth or experience restricted growth in early life are more likely to encounter cognitive challenges as previously discussed.

While some of these infants experience rapid catch-up growth during the first two years, this growth pattern is associated with an increased risk of adiposity

and reduced insulin sensitivity in later life **[147]**. These metabolic outcomes can predispose individuals to obesity and other metabolic conditions, such as type II diabetes, as they mature **[148]**. Additionally, studies indicate that adults born prematurely may have higher blood pressure than those born at term, linking prematurity with an elevated risk of hypertension, cardiovascular disease, and stroke **[149]**. Individuals with these health conditions are more likely to experience chronic pain later in life.

There is a substantial body of research examining the long-term health effects of early life injury, with detailed reviews available elsewhere. Further clinical studies continue to investigate the lasting impacts of early life pain that persist beyond infancy **[78]**.

Neurological Development of the At-Risk Neonate

Even in the absence of evident neurological consequences, very preterm infants are more likely to develop future cognitive, behavioral, and social problems compared to their full-term peers **[150] [151]**. They are also reported to have an increased risk of psychiatric disorders, including attention deficits and autism spectrum disorders **[152]**. Numerous studies have examined structural neurological abnormalities in preterm infants, reporting various differences, including decreased brain volumes, alterations in gray and white matter, and specific regions of vulnerability, such as the hippocampal

and frontotemporal regions. Moreover, these structural changes have been associated with reduced cognitive development scores **[153] [154]**.

Exposure to the extrauterine environment may also play a negative role in the neurological development of preterm infants. Indeed, neonatal intensive care units (NICUs) are now attempting to minimize visual, auditory, and sensory stimuli to which preterm infants may be exposed. This has led to the development of a care science known as NIDCAP (Newborn Individualized Developmental Care Assessment Program), which focuses on these aspects.

Numerous studies have investigated the correlation between neurological development measures and the number of painful procedures experienced during the preterm period. Smith et al. **[155]** studied a group of infants born at less than 30 weeks of gestation, recording all stressful procedures they underwent between birth and term-equivalent age. Examining MRI scans conducted at term-equivalent age, they found that a higher number of stressful procedures was associated with reduced brain volumes in the frontal and parietal regions, as well as alterations in brain diffusion and functional connectivity in the temporal lobes. **[155]**

Zwicker et al. **[156]**examined corticospinal tract development in preterm infants born before 33 weeks of gestation using Diffusion Tensor Imaging (DTI), with scans acquired both at birth and at term-equivalent age. They found a

significant interaction between the number of painful procedures during the neonatal period and corticospinal tract development, with slower increases in fractional anisotropy between the two scans associated with more painful procedures.

Brummelte et al. **[13]**showed that a higher number of painful procedures during the neonatal period until term-equivalent age was significantly associated with reduced maturation of white matter (as indicated by lower fractional anisotropy observed in DTI scans) and subcortical gray matter (as indicated by a reduced N-acetylaspartate/choline ratio measured using magnetic resonance spectroscopy).

Grunau and colleagues have also demonstrated associations between the number of painful procedures during the neonatal period and neurological maturation in older children. In 7-year-old children born before 32 weeks of gestation, a higher number of invasive procedures during the neonatal period was associated with reduced cortical thickness **[157]**, smaller cerebellar volumes **[158]**, and lower white matter integrity (as indicated by lower fractional anisotropy values) **[159]**. Furthermore, the combination of a higher number of invasive procedures and lower white matter fractional anisotropy was significantly associated with lower IQ scores. In these studies, Grunau et al. adjusted the number of painful procedures for clinical factors such as

gestational age at birth, severity of illness on the first day of life, number of days on mechanical ventilation, infections, and cumulative exposure to morphine **[13] [160] [158] [159]**. Functional alterations in brain activity have also been linked to exposure to painful procedures during the preterm period. Doesburg et al. **[161]**demonstrated that altered brain functional activity (increased alpha-gamma ratio measured by EEG) was correlated with pain exposure in very preterm infants and negatively correlated with visuoperceptual abilities at 7 years of age.

Interestingly, two randomized controlled trials aimed at evaluating the effectiveness of developmental care interventions, involving interactions with parents and nurses, have shown that these interventions may improve structural and functional neurological outcomes **[162] [163]**. In the first study, infants born between 28 and 33 weeks of gestation were randomly assigned to receive NIDCAP or standard care. NIDCAP involves an individualized approach to assess each infant's stress signals and behaviors, adjusting medical care accordingly. At 42 weeks corrected age, the NIDCAP group showed greater coherence in alpha and beta bands between frontal, occipital, and parietal regions, as well as higher relative anisotropy in the left internal capsule. Furthermore, at 9 months of age, behavioral functions were improved. **[83]** In the second study, parents in the NIDCAP group were trained

to recognize signs of discomfort in their infants and optimize their interactions. At term-equivalent age, this group showed lower apparent diffusion coefficients, suggesting improved white matter microstructure. Future neuroimaging studies may further enhance our understanding of these interventions in this vulnerable population **[163]**.

Pain Assessment Tools

Over 30 pain assessment scales have been developed to objectively measure neonatal pain experiences [164]. These scales evaluate vital signs, such as changes in heart rate, oxygen saturation, respiratory rate, and blood pressure, as well as behavioral factors like facial expression changes, body movements, or the duration of crying [164].

The **Premature Infant Pain Profile (PIPP)**, a widely used tool for acute procedural pain, assesses behavioral state, gestational age, heart rate, oxygen saturation, and facial expressions (frowning, nasolabial furrowing, and eye squeezing) [165] [166] [167]. Another commonly used scale, the **Neonatal Facial Coding System (NFCS)**, assesses 10 facial components [168], while the **Neonatal Infant Pain Scale (NIPS)** includes facial expressions, crying, breathing patterns, and movement [169].

For chronic or post-operative pain, specific scales such as the **Échelle Douleur Inconfort Nouveau-né (EDIN)**, evaluate facial expression, movement, sleep,

and consolability, specifically in preterm infants [170]. However, the correlation between behavioral and physiological indicators of pain, such as heart rate or oxygen saturation, is often weak [171]. Behavioral responses, particularly facial expressions, are more selectively associated with painful procedures, while physiological changes frequently respond to non-painful stimuli as well [171] [172].

It has been shown that not all neonates exhibit expressive responses to painful procedures, and these responses are influenced by factors such as gestational age, previous painful experiences, sleep state, gender and pre-natal exposure to betamethasone [172] [173] [174] [110]. A lack of concordance has also been noted between brain activity and pain scales. For instance, cortical hemodynamic responses may be correlated with PIPP scores, yet 40% of neonates show no facial expression changes despite brain activity alterations [136].

Studies have demonstrated that sweet solutions, such as sucrose, can effectively reduce pain scores in response to procedures like heel pricks [175]. However, Slater et al. showed that while sucrose lowers PIPP scores compared to placebo, it does not reduce the magnitude of nociceptive-specific brain activity recorded by EEG or the amplitude of the limb withdrawal reflex [176].

Since pain is a subjective experience, neonatal pain assessments are surrogate scales derived from those used in adults, where it remains difficult to objectively measure pain. Often, these scales are limited to observing responses to nociception. Given that cortical processing is required for the conscious perception of nociceptive stimuli [177], evaluating brain activity may provide the best surrogate for neonatal pain assessment, though this remains challenging at such an early developmental stage.

Current clinical tools for assessing brain activity have limited use, as they should ideally be easy and quick to apply. Measuring neonatal brain activity alongside physiological and behavioral changes provides a more detailed understanding of neonatal nociceptive processing [178]. For instance, the limb withdrawal reflex is visually observable, and its amplitude correlates with nociceptive-specific brain activity [71]. Thus, the withdrawal reflex could serve as a useful element in neonatal pain assessment. However, it is important to note that this reflex is not nociceptive-specific and may also be observed in response to non-painful tactile stimuli [97].

A pivotal contribution in this field came from the work of Newnham et al. [179], which was later expanded and personalized by other researchers, such as Xiaomei Cong et al. [180], to adapt it to the specific needs of their NICUs. This work focused on identifying potential stressors and compiling a list of

procedures suspected to be sources of stress, with the goal of developing a cumulative measure of painful events experienced by preterm infants during the neonatal period. The authors developed an extensive list of procedures considered by doctors and nurses with many years of NICU experience to be stressful and assigned each procedure a score that varies depending on gestational age. By summing the total number of procedures, the patient undergoes each day, a score is obtained, called the NISS (Neonatal Infant Stressor Scale) score, which is useful for quantifying the infants' painful experiences. According to the authors, this score can be used in linear regression models to investigate the neurological outcomes of these vulnerable patients [179].

Early Visual Abilities and Cognitive Development

The development of early visual abilities plays a fundamental role in facilitating and enhancing early cognitive development, as well as in the interaction between the infant and their physical and social environment [181]. It is well-documented in the literature that infants are not passive receivers of early visual experiences from birth; rather, these visual inputs are crucial in guiding the exploration of their surrounding environment from the moment they are born [182]. Attention, memory, and even problem-solving appear to be

influenced by early abilities such as visually tracking an object, maintaining attention, and rapidly shifting focus when a new stimulus enters the visual field. All these abilities seem to develop within the first six months of life [183].

Three specific abilities develop as early as the first month of life: the ability to maintain attention on a target (fixation); the capacity to follow a moving object in the visual field (following); and the ability to shift attention to a new stimulus that replaces the previous one in the visual field (fixation shift). Attentional skills typically emerge shortly after birth (alertness) [184], while slow eye movements, useful for following an object, begin to appear around two months of age [185]. In the following weeks, infants gradually learn to disengage attention from one target to focus on another object that appears in their visual field; this skill acquisition generally occurs within the first six months of life [186].

Several scientific studies have shown that early visual abilities may play a role in long-term cognitive development, suggesting a continuity between the early months of life and subsequent years [187] [188] [189]. In one study, Sigman and colleagues demonstrated that an estimation of visual attention in infants (specifically by measuring the duration of visual fixation on a target) was correlated with selective attention observed at 12 years of age [188]. Another study found that at three and a half months, ocular reaction time to

a target in a visual expectation context was correlated with total IQ at 4.5 years [189] . In another study examining infants at seven months, information processing abilities in three domains (attention, processing speed, and memory) predicted executive functions at 11 years [187]. These findings support the idea that distinct abilities observed in infancy represent early manifestations of their full development as complex cognitive skills later in life [187] [188] [189].

It is important to note, however, that studies on infant visuomotor behavior are usually conducted in controlled experimental environments, often using computerized methodologies in complex laboratories. Limited research has been conducted in clinical practice settings, focusing on visual abilities as part of follow-up programs. This has undoubtedly limited the understanding of the potential role of visuomotor function as a predictive tool for future cognitive development in at-risk infants [190]. In a recent review, Morgan and colleagues examined all available cognitive assessments for infants under two years of age to provide recommendations on the most appropriate tools for discriminating, predicting, and assessing cognitive outcomes in infants at risk of neuromotor development issues [191]. Overall, the tools identified as useful for predicting and discriminating future cognitive problems in these infants

were generally outdated, not widely used in modern clinical practice, and primarily targeted infants aged seven months or older.

In recent years, scientific interest in this area has increased, and the number of studies investigating the clinical utility of behavioral assessments of visual function in at-risk infants has grown [192] [193] [194] [195]. In some cases, these tools have been specifically designed to evaluate visual functions in early infancy, such as the scale by Ricci and colleagues [192], or the "Atkinson Battery of Child Development for Examining Functional Vision" [193]. In other cases, the assessment of visual behavior is part of a broader neurological or neurodevelopmental examination, as in the Hammersmith Newborn Neurological Examination (HNNE) [194]or the Einstein Neonatal Neurobehavioral Assessment Scale (ENNAS) [195] [196].

Numerous studies have assessed visual function in newborns within the first days of life, focusing particularly on eye movements, visual attention in response to a stimulus, and the ability to fixate and track it horizontally, vertically, and along a full arc. In 2008, Ricci et al. [192] expanded this analysis by developing a battery of tests consisting of 9 items, and they applied the finalized version to a cohort of full-term newborns without risk factors. This study showed that the items in the battery could be reliably tested as early as 48 hours after birth. For 3 out of the 9 items (fixation on a black-and-white or

colored target and horizontal tracking), nearly all newborns tested achieved consistent results, whereas the remaining 6 items exhibited a wider range of outcomes.

This study also evaluated discrimination of black-and-white stripes with progressively smaller spatial frequencies and assessed distant visual attention, which had never been systematically evaluated in neonates immediately after birth. All newborns in the study were able to fixate on at least the first 4 cards (those with the widest spatial frequencies) and could maintain visual attention for at least 30 cm, with a maximum distance between 30cm and 70 cm. In this study, neonates were evaluated at both 48 and 72 hours of life, revealing that certain abilities remained unchanged at 72 hours, while others showed lower capabilities at 48 hours compared to the assessments conducted after an additional 24 hours. The responses related to vertical and arc tracking, stripe discrimination, and distant attention were significantly more mature at 72 hours. This study provided extended insights into the frequency of visual responses in a low-risk population, and the test battery was subsequently applied to a cohort of preterm neonates, offering valuable information on the maturation of visual function at lower gestational ages. According to our literature review, these tests have not been used as screening tools to predict cognitive development in preterm newborns.

Background of the Study

The background of this study is based on evidence showing that, while many studies investigate the effect of nociceptive experiences on cognitive development in children older than 40 weeks, our literature search highlights a significant lack of research on the clinical evaluation of preterm infants exposed to early nociceptive experiences, assessed already at the corrected term age. The results of this study could open new avenues for designing tailored rehabilitation programs for these young patients.

Aim of the Study

The aim of this study is to quantify the effect of early nociceptive experiences in preterm infants, specifically in the neurological and visual domains, tested already at the corrected term age. The goal is to identify potential markers for timely and personalized treatment in those patients who have undergone multiple painful experiences during the first 30 days of life.

Materials and Methods

Study Design

This is a prospective observational study conducted to clinically assess the neurological and visual effects of early nociceptive experiences in preterm infants at the corrected term age.

Participants

The study included a sample of 32 preterm infants, born after January 1st, 2024, who reached 40 weeks of corrected gestational age (GA) by October 15th, 2024, and who were admitted for the first 30 days of life to the neonatal intensive care unit (NICU) of the Giannina Gaslini Institute in Genoa. Infants met the following inclusion criteria:

- Gestational age greater than or equal to 25+0 weeks;
- Gestational age less than or equal to 32+0 weeks;
- Preterm infants (<37 weeks GA) with low birth weight (BW <1500g);
- Availability for follow-up at the corrected term age (40 weeks of GA).

The following exclusion criteria were applied:

- Congenital brain malformations and/or complex genetic defects;

- Presence of severe acquired neurological injuries visible on routine MRI performed at 40 weeks corrected GA (e.g., Grade IV intraventricular hemorrhage with destructive infarct; ischemic damage; stroke);
- Infants with retinopathy of prematurity (ROP) stage >II at the time of clinical evaluation.

The study included patients with anatomically detectable brain lesions on magnetic resonance imaging (MRI) performed at term-equivalent age (TEA). Brain lesions were classified based on severity as intraventricular hemorrhage (IVH) or cerebellar hemorrhage (CBH) graded as mild, moderate, or severe, or as white matter lesions (WML) graded as mild, moderate, or severe. For details regarding the MRI protocol and lesion classification, please refer to our previous study [197]. For the purposes of this study, only patients with minor lesions (IVH grade I or II) and moderate lesions (IVH grade III or IV with or without post-hemorrhagic ventricular distention- PHVD) were included.

Methodology

Each infant underwent standardized neurological and visual assessments at the corrected term age (40 weeks). These assessments included:

- **Neurological Examination**: A comprehensive neurological evaluation using the Hammersmith Infant Neurological Examination (HINE) to

assess motor function, reflexes, and overall neurological status. The raw scores of the analyzed patients were converted into optimality scores.

- **Visual Function Assessment**: Evaluation of visual function, including visual acuity, eye movement, and response to visual stimuli, conducted by expert neonatologists using the 9-Item Neuro-Visual Test, validated by Ricci et al.

The Hammersmith Neonatal Neurological Examination (HNNE) is a well-established tool for assessing the neurological status of both term and preterm infants during the neonatal period. It evaluates six key domains—muscle tone, tone patterns, reflexes, spontaneous movements, abnormal neurological signs, and behavior—using a five-point Likert scale. This method was structured around an optimality score introduced by **Dubowitz and Mercuri** in 1998, with a maximum score of 34 points that reflects neurological development (Dubowitz et al., 1998).

Historically, the HNNE has been validated for use at term or term-equivalent age (TEA), and has demonstrated predictive capability for motor outcomes (Mercuri et al., 2003; Ricci, Romeo, et al., 2008). More recently, the tool has been adapted for the evaluation of preterm infants at earlier postmenstrual ages (PMA), demonstrating high predictive value in this population as well (Howard et al., 2023). Despite newer normative data for earlier assessments

(Spittle et al., 2016), this study follows the original optimality score framework for evaluations conducted around 37-38 weeks PMA, in line with previous research (Howard et al., 2023).

The **HNNE** was selected for its reliability in assessing neurological outcomes in preterm infants, and also due to its demonstrated correlation with later cognitive outcomes (Huf et al., 2023).

The Neonatal Visual Assessment Battery, developed by **Ricci et al. (2008)**, consists of 9 items designed to assess various aspects of neonatal visual function. These items evaluate ocular movements, both spontaneous and in response to a visual target, the ability to fixate on and follow a black-and-white target in horizontal, vertical, and arc trajectories, the reaction to colored targets, the ability to discriminate black and white stripes with increasing spatial frequency, and the capacity to maintain attention on a target moved slowly away from the infant (Ricci, Cesarini, et al., 2008). The total score is derived by summing the individual item scores, where **lower scores indicate better visual performance** (Ricci et al., 2008).

This visual assessment tool was selected due to its practicality and feasibility for early administration, starting as early as 31 weeks postmenstrual age (PMA). Most preterm infants are able to complete the test at this stage of development (Ricci et al., 2010, p. 201).

Quantification of Pain Exposure

To quantify the painful experiences of the infants enrolled in the study, patient medical records were retrospectively analyzed. To maintain the standard workflow in our NICU, no additional data collection forms were introduced, and nursing activities were not modified for the purposes of the study. Data were extracted from the regular nursing charts and medical diaries. The following events were identified as the most stressful and painful: intubation, insertion and removal of central venous catheters (CVC) and arterial lines (AL), PICC line insertion, heel prick, venipuncture, lumbar puncture, chest drainage, and surgery. To classify and quantify the level of stress and pain caused by these events, the scores from Newnham et al.'s work were applied, adjusted for gestational age, and using the Neonatal Infant Stress Scale (NISS).

Nociceptive experiences for each infant were quantified for each procedure performed during the first 30 days of hospitalization in the NICU. The total number of selected painful procedures performed on each infant was recorded.

Data Collection

Demographic data, clinical history, and medical interventions during NICU hospitalization were collected from the infants' medical records. Additional information on gestational age, mode of delivery, birth weight, and Apgar scores was also recorded.

Outcome Measures

The primary outcome measures were:

1. **Neurological Function**: Assessed through standardized neurological evaluations at the corrected term age. (Doubowitz 39-40 weeks EG)
2. **Visual Function**: Assessed using the validated visual tests as described above (VISIVO score).

Outcomes included correlations between the total number of nociceptive experiences and specific neurological and visual outcomes, with the aim of identifying early markers for future developmental interventions.

Statistical methods

Statistical analysis was conducted using SPSS v.26 for Macintosh. Normality of the variables was tested using the Shapiro-Wilk test. Quantitative variables were expressed as means and standard deviations, while qualitative variables were expressed as frequencies and percentages. Bivariate correlations

between NISS scores and visual and neurological outcomes were calculated using Pearson's correlation test. To determine whether the NISS score significantly moderates visual skills and neurological outcomes at term-equivalent age, while controlling for gestational age and birth weight, a multiple regression analysis was performed. This analysis tested the association between visual skills and neurological outcomes, considering gestational age, birth weight, and Apgar scores as moderating variables.

Results

A total of 29 neonates admitted to the Neonatal Intensive Care Unit during the study period (January 2024 – October 2024) were included in the study. The descriptive analysis of the sample showed that the average birth weight of the neonates was 1259.8 grams, with a standard deviation of 390.66 g and a 95% confidence interval (CI) ranging from 1098.54 to 1421.06 g. The mean gestational age (GA) was 29.68 weeks, with a standard deviation of 2.73 weeks and a 95% CI between 28.55 and 30.81 weeks. The mean APGAR score at one minute was 4.96, with a standard deviation of 2.64 and a 95% CI between 3.87 and 6.05. At five minutes, the mean APGAR score increased to 7.80, with a standard deviation of 1.38 and a 95% CI between 7.23 and 8.37. The sex distribution indicated a female prevalence, with 58.1% females and 41.9% males in the studied sample. The overall sample was considered homogeneous.

Table 1: Demographic Characteristics of the Sample

Characteristic	**Mean ± SD (or %)**	**95% CI**	**Range**
Gender	Male: 41.9% (n = 13)	Female: 58.1% (n = 18)	-
Gestational Age	29.68±2.7329.68±2.73 weeks	[28.55, 30.81]	25.57 – 36.14 weeks
Birth Weight (g)	1259.80±390.661259.80±390.66	[1098.54, 1421.06]	560 – 1955 g
SGA	Yes: 16.7% (n = 5)	No: 83.3% (n = 25)	-

Characteristic	Mean ± SD (or %)	95% CI	Range
APGAR 1'	4.96±2.644.96±2.64	[3.87, 6.05]	1 – 9
APGAR 5'	7.80±1.387.80±1.38	[7.23, 8.37]	5 – 10

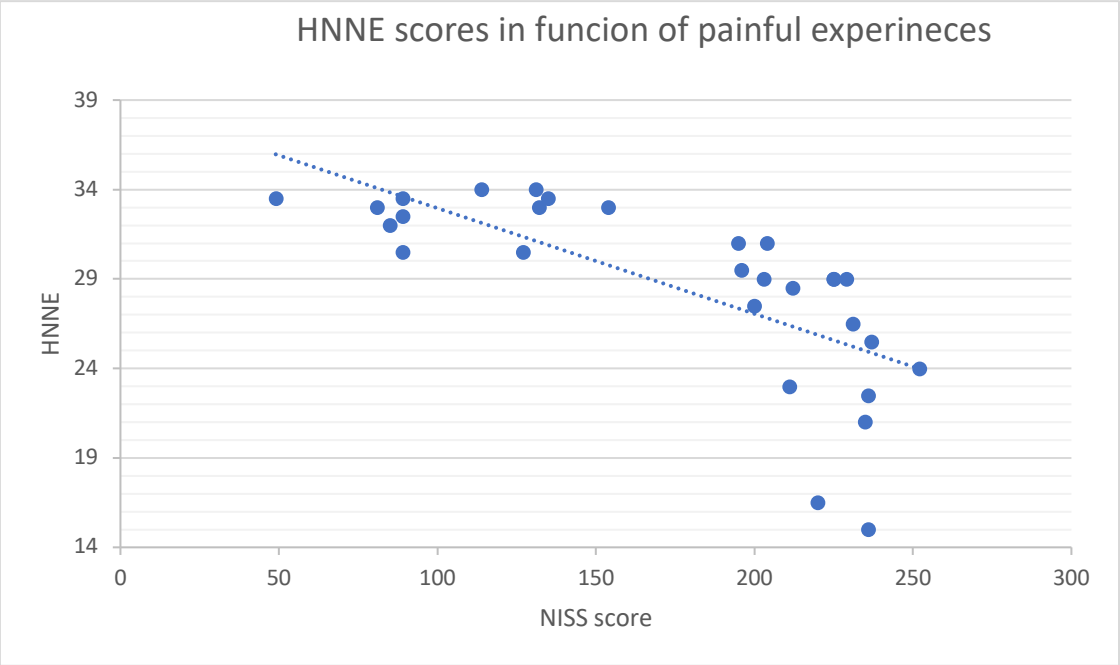
Table 2: Number of Painful Procedures in the First 30 Days

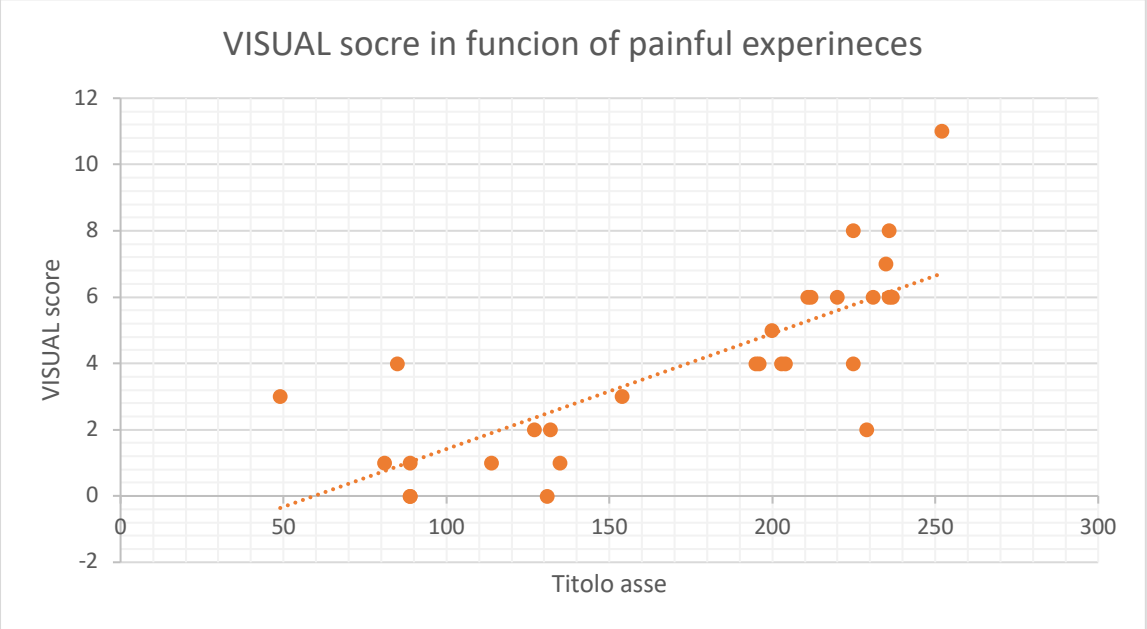
Variable and NISS Coeff.	Number of Procedures in the First 30 Days (n)	Mean
Intubation (5)	28	0.93
Lumbar Puncture (5)	21	0.7
Chest Drain (5)	3	0.03
Venous Punctures (4)	89	2.9
Heel Prick (4)	1016	33
PICC (4)	45	1.5
Arterial Line Placement (4)	1	0.03
Surgery (4)	4	0.13
CVO (Central Venous Catheter) (3)	29	1
CAO (Central Arterial Catheter) (3)	16	0.5
Arterial Line Removal (3)	1	0.03
CVO Removal (2)	29	1
CAO Removal (2)	16	0.5

Painful experiences were found to impact neurological and neurovisual functioning at term-equivalent age in preterm neonates weighing less than 1500 g. Pearson's correlation analysis revealed a significant positive correlation between the total Neonatal Infant Stress Scale (NISS) score and the Visual Score ($r = 0.812$; $p < 0.01$ with a 95% CI of 0.630–0.907), showing an association between increased stress and pain scores and worsening visual function. A significant negative correlation was also found between the total NISS score and the Dubowitz score at 39–40 weeks ($r = -0.712$; $p < 0.01$, with a 95% CI of -0.918 to -0.669), indicating that **higher stress levels are associated with lower Dubowitz scores**.

Table 3: Pearson's Correlations

Variables	Pearson Correlation (r)	95% CI	p-value
NISS tot & VISIVO SCORE	0.809	[0.630, 0.907]	*< 0.001*
NISS tot & Dubowitz 39-40 weeks	-0.831	[-0.918, -0.669]	*< 0.001*
VISIVO SCORE & Dubowitz 39-40 weeks	-0.834	[-0.919, -0.673]	*< 0.001*


HNNE scores in funcion of painful experineces
HNNE
39
34
29
24
19
14
0
50
100
150
200
250
300
NISS score


VISUAL socre in funcion of painful experineces
VISUAL score
12
10
8
6
4
2
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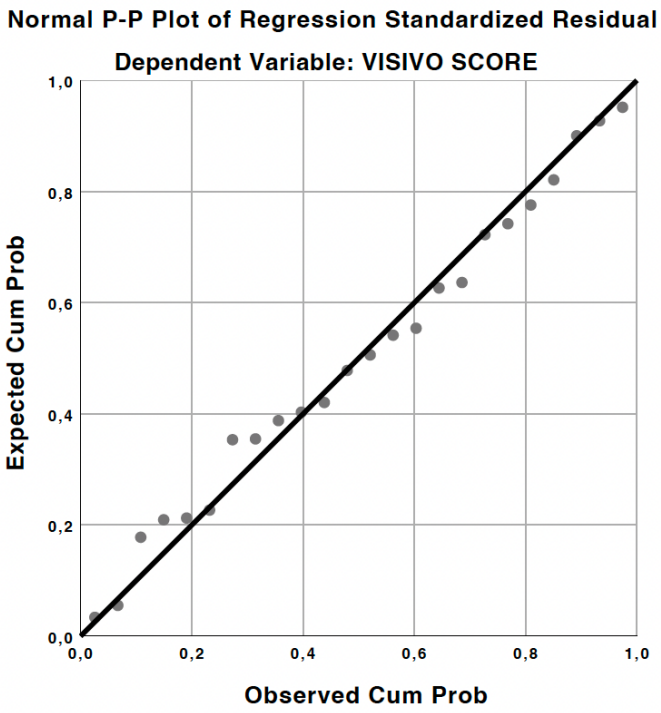
Additionally, a significant negative correlation between the Visual Score and the Dubowitz score was observed ($r = -0.669$; $p < 0.001$, with a 95% CI of -0.919 to -0.673), highlighting a strong association between visual impairment and decreased neurobehavioral outcomes.

Two linear regression models were also performed to analyze the influence of independent variables (Weight, Gestational Age, APGAR at 1 minute, APGAR at 5 minutes, and total NISS) on the Visual Score and Dubowitz score.

In the first model, considering the Visual Score as the dependent variable, the R-squared coefficient was 0.678, suggesting that 67.8% of the variability in the Visual Score was explained by the independent variables considered. Among these, the total NISS emerged as the only significant predictor of Visual Score variability, with a β coefficient of 0.829 and statistical significance at $p < 0.001$, with a 95% CI of [0.019, 0.057]. The other variables (Weight, Gestational Age, APGAR at 1 minute, and APGAR at 5 minutes) did not show significant associations with the Visual Score.

Table 4: Regression Model for VISIVO SCORE

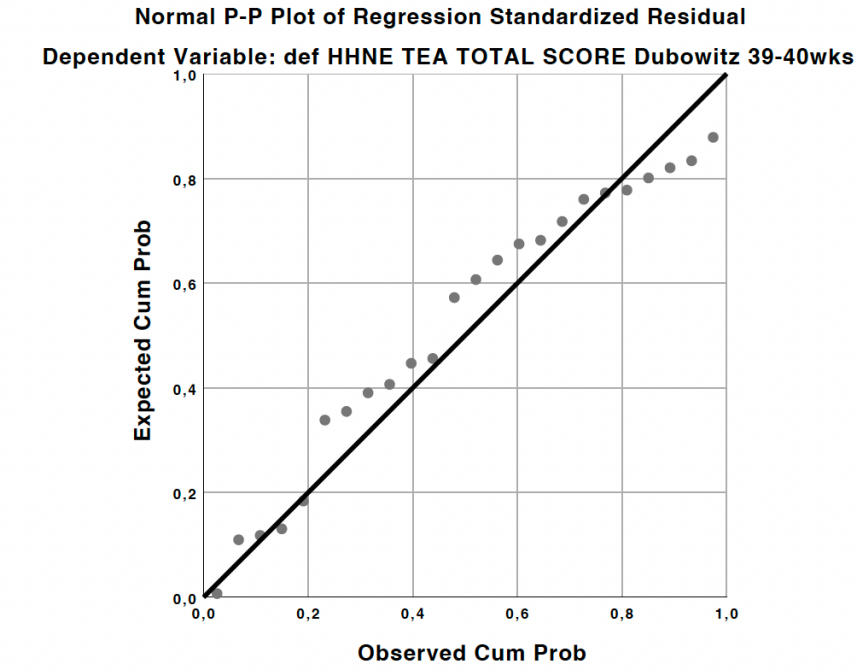
Variable	Coefficient (B)	Std. Error	Beta	t-value	p-value	95% CI Lower	95% CI Upper
Constant	4.691	6.665	-	0.704	0.491	-9.312	18.693
NISS tot	**0.038**	**0.009**	**0.829**	**4.262**	**<0.001**	**0.019**	**0.057**
EG	-0.223	0.241	-0.209	-0.925	0.367	-0.730	0.284
Peso	0.001	0.001	0.151	0.816	0.425	-0.002	0.004
APGAR 1'	0.359	0.226	0.328	1.593	0.128	-0.114	0.833
APGAR 5'	-0.486	0.360	-0.230	-1.350	0.194	-1.243	0.271



In the second model, with the Dubowitz score at 39–40 weeks as the dependent variable, the R-squared coefficient was 0.601, indicating a lower explanatory power of this model compared to the previous one. Again, none of the independent variables showed a statistically significant association with the Dubowitz score, except for the total NISS, which remained statistically significant with $p = 0.001$ and a β coefficient of -0.859, with a 95% CI of -0.102 to -0.031.

Table 5: Regression Model for Dubowitz Score (39-40 weeks)

Variable	Coefficient (B)	Std. Error	Beta	t-value	p-value	95% CI Lower	95% CI Upper
Constant	41.161	12.552	-	3.279	0.004	14.790	67.531
NISS tot	**-0.067**	**0.017**	**-0.859**	**-3.966**	**0.001**	**-0.102**	**-0.031**
EG	0.181	0.454	0.100	0.398	0.695	-0.774	1.135
Peso	-0.003	0.003	-0.231	-1.119	0.278	-0.008	0.003
APGAR 1'	-0.173	0.425	-0.093	-0.406	0.689	-1.065	0.720
APGAR 5'	-0.244	0.679	-0.068	-0.360	0.723	-1.670	1.182



Painful procedures impact the neurological and neurovisual development of preterm neonates independently of the presence of minor brain lesions detected by MRI performed at term-equivalent age.

Regarding the presence of anatomically detectable lesions on MRI, routinely performed at our center at term-equivalent age (TEA), the sample was divided into three groups: Group 1, comprising 18 subjects, without any detectable lesion ("no lesion"); Group 2, with 8 subjects, having lesions classified as “minor” (“minor lesion”) (Malova et al., 2020); and Group 3, with 3 subjects, having lesions classified as "mild-moderate" (“mild-moderate lesion”).

NISS Score (Neonatal Infant Stressor Scale): This score, which assesses the level of stress and pain in neonates, increase with the severity of the lesions. Neonates without lesions had an average score of 143.17±14.06, while those with moderate lesions had a higher average score of 241.00 ± 5.51, suggesting that neonates with more severe lesions experience greater levels of stress.

Neonates without lesions had an average **Visual Score** of 2.44 ±0,95, while neonates with mild and moderate lesions had scores of 5,88±1,30, and 8,00±6,57, respectively. These differences were statistically significant ($p<0.05$). The suggest that lesion severity may be associated with a deterioration in visual abilities.

Dubowitz Score (39-40 weeks): The Dubowitz score, which assesses neurological maturity, decreases with increasing lesion severity. Neonates without lesions had an average score of 31,306± 1.106, while those with moderate lesions had a score of 20,00± 11,384. These differences were statistically significant ($p<0.05$) and indicate that the presence of moderate lesions is associated with lower neurological maturity.

Table 6: NISS Score, VISIVO Score, and Dubowitz Score by Lesion Category

Lesion Category	NISS Score (Mean ± SD)	VISIVO Score (Mean ± SD)	Dubowitz Score (Mean ± SD)
No Lesions (n=18)	143.17±14.06	2,44±0,95	31,306± 1.106
Minor Lesions (n=8)	215.25 ± 15.04	5,88±1,30	25,875± 4,155
Moderate Lesions (n=3)	241.00 ± 5.51	8,00±6,57	20,00± 11,384

We then excluded neonates with mild and moderate lesions from the sample (n=3 neonates) and conducted a linear regression to assess whether painful experiences independently influenced neurological outcomes, regardless of the presence of lesions.

Regarding the **Visual Score**, the total NISS showed a significant positive impact, with a 95% confidence interval between 0.004 and 0.048 (p = 0.049).

Table 7: Regression for VISIVO Score with Confidence Intervals

Variable	Coefficient (B)	Standard Error (SE)	95% Confidence Interval	t-value	p-value
Constant	2.159	7.558	[-12.654, 16.972]	0.286	0.781
EG	-0.093	0.269	[-0.621, 0.435]	-0.345	0.737
Weight	0.001	0.001	[0.000, 0.003]	0.832	0.425
APGAR 1'	0.169	0.261	[-0.342, 0.680]	0.647	0.532
APGAR 5'	-0.376	0.384	[-1.129, 0.377]	-0.978	0.351
NISS tot	**0.026**	**0.011**	**[0.004, 0.048]**	**2.235**	**0.049**

For the **Dubowitz score** (39-40 weeks), the total NISS had a significant negative effect, with a 95% confidence interval between -0.079 and -0.021 (p = 0.008). (Tab. 6-7-8)

Table 8: Regression for Dubowitz Score (39-40 weeks) with Confidence Intervals

Variable	Coefficient (B)	Standard Error (SE)	95% Confidence Interval	t-value	p-value
Constant	31.750	9.854	[12.436, 51.064]	3.222	0.009
EG	0.405	0.351	[-0.283, 1.093]	1.154	0.275
Weight	-0.002	0.002	[-0.006, 0.002]	-0.994	0.344
APGAR 1'	-0.302	0.340	[-0.968, 0.364]	-0.888	0.395
APGAR 5'	-0.262	0.501	[-1.244, 0.720]	-0.523	0.612
NISS tot	**-0.050**	**0.015**	**[-0.079, -0.021]**	**-3.312**	**0.008**

Discussion

Our study revealed that painful experiences, assessed through the NISS score, an index measuring stress and pain during the first 30 days of life [179], have a significant impact on the neurological and neuro-visual maturation of preterm neonates weighing less than 1500g, even when considering variables such as birth weight, gestational age, and Apgar scores at 1 and 5 minutes, in a sample representative of the VLBW population (Table 1, [198]).

Painful experiences were found to negatively influence neurological maturity at term-equivalent age, as shown by the negative correlation between NISS scores and HNNE scores. This suggests that early stress and pain may hinder neurobehavioral development. These findings are consistent with recent studies showing that exposure to painful events in the early weeks of life, particularly in preterm infants, is associated with reduced functional connectivity between key brain regions, such as the thalamus, somatosensory cortex, insular cortex, and amygdala. These neuro-functional changes, observed through fMRI, have been correlated with poorer neurobehavioral outcomes in early childhood, supporting the notion that early painful events may impair the normal development of brain connectivity, with long-lasting effects on cognitive and motor functions [199].

It is noteworthy that the correlation between NISS tot and the Dubowitz score remained significant even when adjusting for factors such as gestational age (GA), birth weight, and Apgar scores. These factors are traditionally associated with neurological risks but do not fully explain the variability in neurobehavioral outcomes related to stress and pain. Recent literature also supports this observation: stressful experiences in the NICU, including maternal separation and invasive medical procedures, can influence brain maturation independently of perinatal factors such as gestational age and birth weight, increasing the risk of negative neurological and behavioral outcomes [200] .

This study is the first to link early painful experiences with neuro-visual functioning, showing how early pain can impair physiological visual development. This finding aligns with current literature, which documents how early exposure to high levels of stress in the neonatal intensive care unit (NICU) environment, including intense sensory stimuli and painful procedures, can impair neuro-sensory development in preterm infants [201]. To promote visual development and reduce the negative effects of stress, which preterm neonates inevitably experience during NICU hospitalization, positive multisensory stimulation interventions, such as infant massage, have been implemented. These interventions have shown positive results in promoting

visual development and reducing stress-related behaviors in preterm infants, suggesting that stress management could preserve or improve visual function, even in high-risk conditions [202].

Overall, our study suggests that stress evaluation represents a unique risk factor that should be considered in the clinical management of preterm infants to prevent long-term neurological impairments. Given that early stress and pain are associated with compromised visual and neurobehavioral functions, it is crucial to implement pain and stress management strategies in NICU settings. Recent studies have demonstrated that early interventions, such as positive sensory stimulation and support for mother-infant interactions, can promote neuro-sensory development in at-risk neonates [203]. Additionally, such interventions could mitigate the negative effects of painful experiences, protecting the development of the visual system and brain networks involved in cognitive and motor functions.

Finally, the presence of neonates with brain lesions (classified as minor and moderate lesions) within the sample is undoubtedly a limitation of this study. However, while acknowledging that lesions may influence neurological and visual outcomes, it is important to emphasize that this study allowed us to distinguish the effect of painful experiences from anatomical conditions. In fact, despite the presence of moderate lesions in 3 neonates, the regression

models conducted on the 26 patients with no-lesion and minor lesion showed that NISS tot remains a significant predictor of both the VISIVO Score (β = 0.026, 95% CI 0.004,0.0480, p = 0.049) and the Dubowitz Score (β = −0.050, 95% CI −0.079,−0.021, p = 0.008). Further studies are needed, selecting a lesion-free population, to better understand the impact of stress levels and painful experiences on early neurological and neuro-visual clinical outcomes.

Conclusions and Clinical Implications

These findings highlight the importance of monitoring and managing stress and pain in neonates, as these factors not only increase the risk of visual impairments but also represent independent predictors of neurobehavioral alterations. Moreover, neonatal stress management through comfort interventions and family support strategies could be a crucial preventive measure to promote healthy neurological development in high-risk neonates. Further studies could investigate the specific role of the **HNNE** in assessing stress and pain, with the creation of a "PAIN Dubowitz score" applied to the **HNNE**, replacing the **NISS** score as a predictor of long-term outcomes. This approach could be more easily implemented during routine clinical practice, making it possible to assess the impact of stress and painful procedures during NICU discharge using the **HNNE**. This would allow high-risk neonates to immediately receive tailored rehabilitation care at 40 weeks of corrected age.

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