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EARLY INTERVENTION IN PRETERM INFANTS:

EFFECTS ON NUTRITION AND NEURODEVELOPMENT

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Content

Chapter 1 – Introduction

1.1 The preterm infant

1.1.1 Epidemiology

Preterm birth is a major pediatric public health problem. Rates of preterm birth are rising and prematurity is associated with a considerable risk to develop cognitive, behavioral, neurosensory, and motor disabilities: the lower the gestational age, the higher the risk of neurodevelopmental impairment[1]

Preterm birth is defined by the World Health Organization (WHO) as all births occurring at less than 37 weeks of gestational age (GA)[2]. It may be further subdivided, based on GA, into extremely preterm (<28 weeks), very preterm (28-32 weeks) and moderate preterm (33-37 weeks) - within this category late preterm birth can be identified (34-36 weeks)[2].

The classification of preterm birth is also based on birth weight (BW) as follow: Extremely Low Birth Weight (< 1000 g; ELBW), Very Low Birth Weight (1001-1500 g; VLBW) and Low Birth Weight (1501-2500 g; LBW).

It is also important to relate BW to GA at birth to evaluate fetal growth and identify those born Small for Gestational Age (SGA) defined as an infant born with a BW ≤ the 10th percentile or 2 standard deviations (SD) below the mean BW for GA[3].

The WHO estimates that 14.9 million infants were born preterm in 2010, representing the 11.1% of all births.[4] However the incidence of preterm delivery around the world varies: with rates of preterm birth around 11.8% in low-income countries versus a 9.3% of preterm delivery in high-income countries[1].

Moreover, despite advancing knowledge of risk factors and the introduction of many public health and medical interventions, in recent decades rates of preterm delivery have risen in developed countries.[5,6] This is mainly because of the availability of assistive reproductive technologies and the increased number of medically indicated labor as a consequence of maternal or fetal problem[7,8].

Preterm delivery is a syndrome with a variety of causes related both to the mother and the fetus[9].

Many maternal factors have been associated with preterm labor and in particular: young or advanced age, previous history of preterm delivery, multiple pregnancies, infections, stress, smoking and excessive alcohol consumption.[10,11]

Males and infants with congenital abnormalities are more likely to be born preterm while role of ethnicity is still debated.[12,13]

1.1.2 Mortality and Morbidities

Preterm birth is estimated to be a risk factor in at least 50% of all neonatal deaths[14]. Mortality rates increase with the decrease of GA and infants born SGA present a greater risk.[15] Complications related to premature birth represent one of the leading cause of death in children under 5 years of age worldwide.[16]

However, survival rates have raised up to 95%, in high-income countries, for those born between 28 and 32 weeks of GA[17] with infants born less then 32 weeks representing about the 16% of all preterm birth.[4] This increase is related to continuous research in perinatal care and innovative technologies that are primarily associated with earlier use of antenatal corticosteroids, surfactant and changing in attitude towards intensive care.[18,19]

Premature birth is associated with a wide range of complications, whose frequency and severity increase with the reduction of GA and quality of care[1].

Premature birth is associated both with short-term morbidities, that occur during NICU stay, and long term morbidities that become evident during childhood and adolescence.[20,21]

Neonatal morbidities associated with prematurity affect several organs and systems and are mainly represented by: Germinal Matrix Hemorrhage-Intraventricular Hemorrhage (GMH – IVH), cystic Periventricular Leukomalacia (cPVL), Necrotizing Enterocolitis (NEC), Retinopathy of Prematurity (ROP), infectious diseases, Respiratory Distress Syndrome (RDS), persistence of Patent Ductus Arteriosus (PDA).

A study conducted by the Eunice Kennedy Shriver National Institute of Child Health and Human Development in Bethesda (USA) in 2010 reveals the epidemiological significance of such complications. According to this report, in a group of 9575 infants born between 22 and 28 weeks of GA and with a BW between 400 and 1500 g, enrolled in a five-year period from 2003 to 2007, RDS occurred in 93% of infants, persistence of PDA in 46%, GMH-IVH in 16%, NEC in 11% and late sepsis in 36%.[22]

Long-term complications include Bronchopulmonary Dysplasia (BPD) and both major and minor neurodevelopmental delay.[21,23]

The prevalence of neurodevelopmental impairment is significantly associated with length of gestation and a greater impairment is observed at decreasing of GA.[21]

Major neurodevelopmental delays are represented by: Cerebral Palsy (CP), mental retardation, deafness and blindness.

Even if the incidence of major disabilities is fairly stable, there is a growing awareness that a high percentage of nondisabled survivors encounter minor neurodevelopmental problems. In fact around 25-50% of preterm infants born < 32 weeks of GA suffer from minor neurodevelopmental delay[23], which include: behavioral problems (i.e. attention-deficit/hyperactivity disorder), executive functions' deficit, academic underachievement, visual processing problems.[23–25] These minor neurological disorders occur in the absence of overt brain lesions and are most likely related to brain micro-structural maturation.[26,27]

In this context the availability of accurate developmental assessments for the early detection of infants at high risk of adverse neurodevelopmental outcomes has become a major issue. Indeed, early confirmation of developmental impairment is important so that early referral for intervention can be made to maximize children's abilities and to assist in their transition to school.

Several neurodevelopmental tests are available, however, concerns have arisen about the interpretation of tests scores and the subsequent classification of neurodevelopmental impairment.

In order to establish, in a cohort of Extremely Low Birth Weight Infants, the agreement in developmental scores between the two versions of the most widely used neurodevelopmental test (Bayley-II and Bayley-III) we compared it to the Griffiths Mental Developmental Scales Revised.

Our study suggested that the Bayley-III, although having a higher agreement with the Griffiths, slightly tends to underestimate neurodevelopmental impairment, whereas the Bayley-II tends to overestimate it. Therefore in follow-up settings the use of multiple measures to assess neurodevelopment is needed to ensure the reliability of diagnosed delays and to determine subsequent qualification for early intervention services.

These results have been published in BMC Pediatrics and are reported in Appendix 1.

Even though broadly used, most of the current neurodevelopmental assessment tools are impairment-based models of disability and do not account for the relevant contribution of contextual factors.

Conversely, the importance of these factors is recognized by the biopsychosocial model endorsed by the International Classification of Functioning, Disability, and Health – Children and Youth version (ICF-CY) proposed by the World Health Organization in 2007. We performed a study to evaluate the longitudinal trend of neurodevelopmental outcomes in a cohort of Very Low Birth Weight Infants using an ICF-CY-based approach. Our study highlighted the feasibility to extend the ICF-CY to follow-up assessment of preterm infants to capture information connected to social situations that would not be addressed otherwise.

These results have been published in the International Journal of Rehabilitation Research and are reported in Appendix 2.

1.2 Brain Development

The last trimester of pregnancy, which corresponds to preterm birth, is an important period of brain development. It is a stage of rapid neuronal proliferation and cell differentiation including oligodendroglial maturation, differentiation of subplate neurons, formation of synapses, cerebellar

neuronal proliferation and migration, and major axonal development in the cerebrum[28,29] with an accelerated maturation of the cortical surfaces (Figure 1).[30]

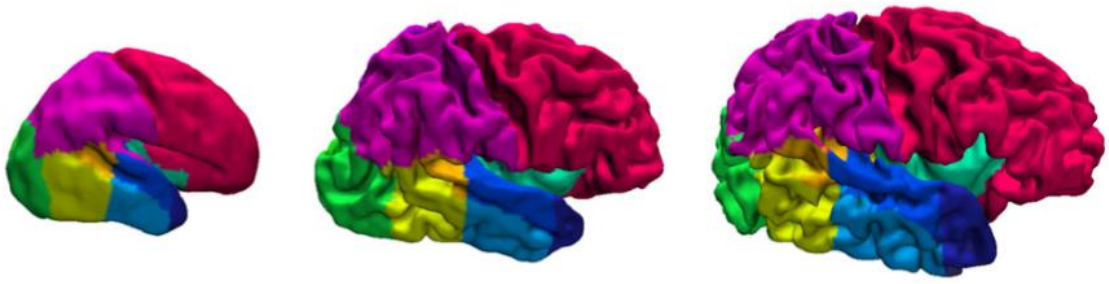

Figure 1 - Example of cortical surfaces at 28, 36 and 44 weeks of GA

Brain development is tailored by a continuous interaction of genetically coded processes that are first influenced by the intrauterine environment and then from several stimuli from the extrauterine environment. [28]

The incomplete development of the Central Nervous System (CNS) makes the premature infant more vulnerable to brain damage.

Recent scientific evidences support the theory of a multifactorial origin of brain damage in preterm infants. The so-called encephalopathy of prematurity is a complex amalgam of primary destructive disease and secondary maturational and trophic disturbances.[29]

Different pathophysiological mechanisms are involved in injuring the developing brain, in particular infection-inflammation, pre- and/or postnatal malnutrition, and abnormalities in systemic and cerebral haemodynamics and oxygen supply.

Other factors such as biological influences (i.e. infections and BPD) and environmental influences such as altered auditory and visual stimuli, along with physical separation from parents. [31–33] seems to play a role in brain development.

The mechanism underlying these modifications is early brain plasticity. Neuroplasticity reflects the capability of the brain to modify throughout life by adapting, at different levels, to environmental exposition. It underlies the processes of learning and memorizing and the damage-induced processes of brain recovery and reorganization. Neuroplastic mechanisms appear to be greatest during infancy, the so-called critical period, at a time when brain maturation has a faster pace.[34]

1.2.1 Nutrition and brain development

Early nutrition is one of the crucial factors for brain development. In preterm infants, inadequate nutritional support leads to delayed cortical maturation as measured by fractional anisotropy (FA) using diffusion tensor imaging (DTI).[35] Anatomical structures that are most susceptible to postnatal nutritional deficiency seem to be the cerebellum and the hippocampus.[36]

Several papers have reported on the impact of early nutrition on postnatal head growth and later neurodevelopment in preterm infants.[37,38] Tan et al. reported a correlation between energy deficit during the first month after birth and total brain volume at TEA, and between protein-energy deficit and neurodevelopmental outcome at three months post-term in infants born before 29 weeks gestation.[39] Non-optimal nutrition may have reversible effects, but may also have negative consequences on cognitive and psychic development at a distance. Hence, in preterm infants, especially in the neonatal period, there is a crucial need to ensure adequate nutrition and try to reproduce fetal growth.[40,41]

Moreover, research has shown that nutritional components might influence gut microbiota and this, in turn, may impact brain development and plasticity, through immunological, endocrine, and neural pathways.[42]

In this framework breast milk plays a crucial role in preterm infants nutrition strategies.

The American Academy of Pediatrics recommends exclusive breastfeeding and human milk as the reference normative standards for infant feeding and nutrition in the first six month of life for both term and preterm infants.[43]

There are several significant short- and long-term beneficial effects of feeding preterm infants human milk. The main benefits are: reduction in incidence of NEC, reduction of neonatal sepsis, lower rates of ROP, fewer hospital re-admissions for illness in the year after NICU discharge.[44–46]

Long-term studies suggest that extremely preterm infants receiving the greatest proportion of human milk in the NICU also had significantly better neurodevelopmental outcome.[47,48] More specifically Vohr et al. reported beneficial effects of breast milk on motor, cognitive and behavioral outcomes; the reported positive outcomes were closely related to rates of breast milk ingested.[47] It is also important to take into account the positive effect of parental participation on breastfeeding and its influence in promoting mother-infant relationship.[49]

1.3 Early Neurodevelopmental Intervention

The developing brain is particularly vulnerable to adverse insults, but its rapid growth and the brain plasticity suggests that early experiences may also positively influence brain development. Early intervention is a recently proposed strategy to positively modulate brain maturation and child neurodevelopment.[50]

Early intervention has no unique definition but it is broadly defined as "multidisciplinary services provided to children from birth to 5 years of age to promote child health and well-being, enhance emerging competences, minimize developmental delays, remediate existing or emerging disabilities, prevent functional deterioration and promote adaptive parenting and overall family function".[51] Moreover, the first year is a unique period for both the nature of parent-infant relationship and the interaction of the infant with the environment; therefore, in this time span, interventions are more likely to have a maximal impact.[52,53]

The principle underling early intervention arise from both animal and human studies showing that an early strategy favors a reactive synaptic plasticity resulting in brain structures reorganization and hence improved outcomes.[54,55]

In preterm infants, early developmental intervention aims to improve brain connections during key periods of brain development, rather then waiting for an impairment to occur once altered brain connection have developed;[56] this highlights the preventive role of Early Intervention.[50,57]

1.3.1 Key aspects of Early intervention

The theoretical base of Early interventions is the Environmental Enrichment (EE) first defined by Rosenzweig as the "combination of complex inanimate and social stimulation".[58] Both animal and human studies described the positive effect of EE on brain development and subsequent neurodevelopmental outcomes.[59,60]

A crucial factor of EE is the positive active experience that produces a functional reorganization through which the infant could learn.[50,61]

However, there are two key aspects of EE in preterm infants that should be emphasized: parents involvement and multisensory stimulation.

Within an ecological framework parents have the strongest, most proximal, and enduring influence on child development.[62] Sensitive parenting and a positive family environment can have a protective effect on the development of preterm infants, even after accounting for the influence of medical risk factors such as brain injury.[63,64] Thus the parent-infant relationship is considered one of the primary mechanisms through which early intervention may favor brain maturation and subsequent neurodevelopment.[65,66]

For these reasons Early intervention strategies that favor parental involvement should aim to: decrease stress and anxiety, promote parental self-efficacy and sensitivity in interactions with their infants, favor parent direct delivery of therapeutic developmental support for the child.[52]

Several parental-training program have been suggested: among these, the Premiestart focuses on sensitive maternal involvement to reduce infant stress and promote dyadic interactions.[67]

The second crucial aspect of early intervention, closely related to parental involvement, includes multisensory stimulation. It relies on the neurobiological process known as multisensory integration

"by which information from different sensory systems is combined to enhance and accelerate detection, localization, and reaction to biologically significant events".[68] This integration offers an enhanced, immediate, uniform and thus complete representation of the environment which is crucial for early perceptual, cognitive and social development.[61,69]

Early multisensory intervention in preterm infants may include visual and tactile stimulation through infant massage.[70] In preterm infant massage therapy consists in a slow tactile stimulation of the back giving moderate pressure stroking with both hands. Recent studies have shown how specific interventions, such as infant massage, can favor brain plasticity in infants at a neurodevelopmental risk.[71] Also visual function can be positively influenced by targeted early visual interventions because it has a strong connection with brain development as its maturation is described to be related to subcortical and cortical mechanisms.[61,72]

In the field of Early Interventions, different neurodevelopmental approaches coexist; between those the Newborn Individualized Developmental Care and Assessment Program (NIDCAP) is based on preterm infant's observation during hospitalization and considers infant's behavior as the key to evaluate the level of neurobehavioral maturation. We performed a non-Randomized Controlled Trial to evaluate the effectiveness of NIDCAP on mother's support and infant development. Our study provided evidence of the capability of the NIDCAP to support mothers of preterm infants in the NICU. Moreover it confirmed that NIDCAP is effective in promoting infant's neurofunctional development in the short term.

These results have been published in Early Human Development and are reported in Appendix 3.

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Chapter 2 - Effects of nutrition on body composition and brain growth: preliminary results

Adequate nutritional support to preterm infants after birth is crucial to ensure optimal quantitative and qualitative postnatal body growth, as well as brain maturation and neurodevelopment.[1,2]

Postnatal growth can be assessed through anthropometric parameters but the evaluation of body composition is more sensitive as it reflects qualitative growth measurements (i.e. fat-free mass [FFM] represents organ's growth and protein status).[3]

Despite the nutritional recommendations of the American Academy of Pediatrics,[4] preterm infants show a different growth pattern compared to term infants (reduction of length/height /FFM and increased of fat mass [FM]).[5–7]

Nutritional strategies in the early stages of the preterm life affect the quality of body growth resulting in differences in body composition at term corrected age (TEA);[5,8,9] our hypothesis to be tested is that the body composition at TEA, depending on early nutritional support, is associated with the quantitative development of specific structures of the immature brain.

To test our hypothesis we designed a retrospective study to assess the correlation between body composition and brain volume in preterm infants at TEA.

The study is still ongoing and it is conducted in collaboration with Professor Manon Benders Department of Neonatology, University Medical Centre Utrecht, The Netherlands.

The study included all the infants born before 32 weeks gestation that undergo both brain MRI and assessment of body composition at TEA. Infants with major brain lesions were excluded.

Brain growth was calculated using T2 coronal MRI images identifying 6 different structures (cerebellum [CB], cortical gray matter [GM], unmyelinated white matter [UWM], ventricles [VL], external cerebral spinal fluid [ECSF] and basal ganglia [BG]) through semi-automatic segmentation. Tissue brain volumes (BVs) were calculated and corrected for the intracranial brain

volume (TBV) defined as the sum of all tissues volumes except VL and ECSF. Figure 2 shows an example of tissue's segmentation on MRI (coronal T2-WI).

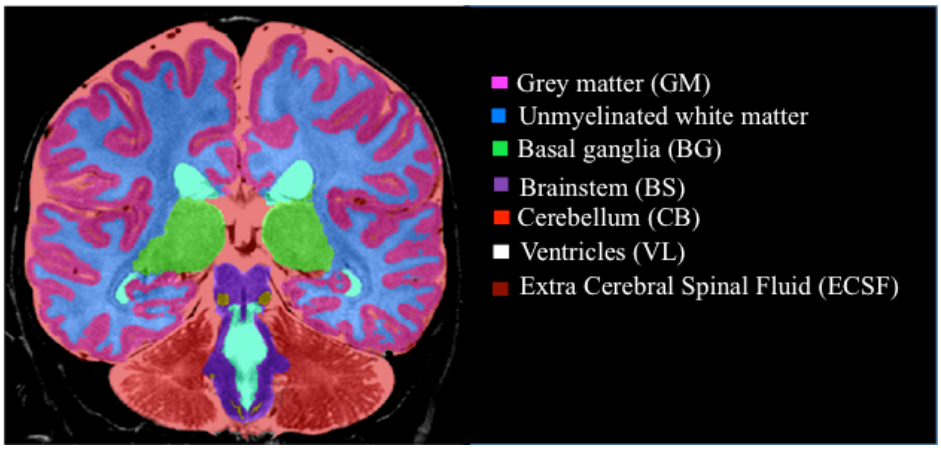

Figure 2 – Example of tissue's segmentation on MRI

Body Composition (BC) was assessed using an air displacement plethysmography (Pea Pod Infant BC System) and data on fat-free mass (FFE) and fat mass (FE) were obtained. The association between BC and BV were assessed using linear regression (univariate and multivariate models).

We currently enrolled 34 preterm infants (mean weeks of GA at birth= 29±1.8 and mean birth weight=1115±249 g). The interim analysis suggests a positive association between FFM (mean= 2629±285 g) and Grey Matter volume (p=0.03). When considering a sub-group of 24 preterm infants without mild brain abnormalities, FFM was associated also with the TBV (p=0.003). No association between BVs and FM (mean= 599±157 g) was found. These results were confirmed in a multivariate model including potential confounders (GA, postnatal age at MRI, twins, gender).

These preliminary results suggest that an association between FFM and brain growth, in particular with GM development: the more the FFM the larger the brain. However, these results are far to be conclusive as more data are necessary to further explore the direct effect of nutrition on preterm brain growth and later neurodevelopmental outcome.

Furthermore a specific analysis on mother milk assumption is needed given its key beneficial effects on preterm infants, both on brain growth and neurodevelopmental outcome.[4,10,11]

Considering the beneficial impact of human milk feeding and, at the same time, the potential detrimental effects of the NICU environment for the preterms' development, we designed a parallel prospective study to assess the effectiveness of an early intervention program (based on parental involvement together with a multisensory stimulation) in enhancing infant-mother relationship, infant's human milk assumption, brain growth and long-term neurodevelopment.

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Chapter 3 - Aim of the Study

The purpose of the present thesis is to evaluate the effectiveness of an Early Intervention Program on different aspects of infant's feeding behavior and neurodevelopment in a cohort of very preterm infants.

The thesis is based on the results of a parallel-group, randomized controlled trial including preterm infants born between 25^{+0} and 29^{+6} weeks GA without severe morbidities and their families aimed to compare the effects of an Early Intervention (EI) program, based on parental involvement together with a multisensory stimulation (both tactile – through infant massage - and visual stimulation) with the Standard Care (SC), delivered according to NICU protocols, included Kangaroo Mother Care and minimal handling.

Primary outcome is the assessment of visual function at term equivalent age (TEA) as an early emerging cognitive function.

Secondary end-points include assessment of:

- The effects of promoting mother-infant interaction on the infant's feeding behavior and in particular on breast milk assumption;
- Epigenetic changes in methylation status at NICU discharge;
- Brain development measured by advanced Magnetic Resonance Imaging (MRI) at TEA as a function of both early EI strategies and human milk assumption.

Chapter 4

Effects of Early Intervention on visual function in preterm infants: a Randomized Controlled Trial

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Submitted

Abstract

Objectives: To determine the effectiveness of an early intervention program in enhancing visual function in very preterm infants.
Methods: We conducted a parallel-group, randomized controlled trial to assess the effect of a multisensory intervention on visual function. We included preterm infants born between 25^{+0} and 29^{+6} weeks of gestational age (GA) without severe morbidities and their families. Infants were recruited and randomized to either receiving Early intervention (EI) or Standard Care (SC). EI included PremieStart and parental training to promote infant massage and visual attention according to a detailed protocol. SC, according to NICU protocols, included Kangaroo Mother Care and minimal handling. Visual function, such as ocular spontaneous motility, ability to fix and follow a target, reaction to color, visual acuity and visual attention at distance, was assessed at term equivalent age (TEA).
Results: Seventy preterm (EI n=34, SC n=36) infants were enrolled. Thirteen were excluded according to protocol. Fifty-seven infants (EI=27, SC=30) were assessed at TEA. The two groups were comparable for parent and infant characteristics. In total, 59% of infants in the EI group achieved the highest score possible in all 9 items compared to 17% in the SC group (p=0.001): all infants in both groups showed complete maturation in four items, but EI infants showed more mature findings in the other 5 items (ocular motility both spontaneous and with target, tracking arc, visual acuity and attention at distance).
Conclusions: Our results suggest that EI has a positive effect on visual function maturation in preterm infants at TEA.

Introduction

During their stay in the NICU, preterm infants face a period of stressful environments, as determined by intensive care, excessive sensory stimulation and painful procedures[1], which may negatively impact early brain development[2,3], even in the absence of overt brain lesions, and may be implicated in impaired neurobehavioral outcomes[4]. Neuro-anatomical correlates, which are represented by micro-structural brain abnormalities, have been documented by advanced neuroimaging studies in preterms at term equivalent age (TEA)[5–9]. These abnormalities are most likely related to the increased risk of neurodevelopmental, cognitive, attentional or visuo-perceptual difficulties that preterm children can present at preschool and school age[10].

Safeguarding brain development and maturation in preterms is therefore crucial for their neurodevelopment, and research has addressed new beneficial neuroprotective strategies.

Early intervention programs based on the concept of "individualized care" have effectively promoted brain maturation and neurodevelopmental outcomes[11,12].

In this context, parents' role in the NICU has been recently emphasized because it is well known that early parenting plays a central role in the promotion of early neurodevelopment[13]. However, the relationship between parents and their preterm infant during the neonatal period is "NICU mediated"[14,15], which can lead to a paucity of parent-infant interaction[16,17]. In this framework, constructing a dyadic relationship is challenging[18,19] but potentially beneficial in reducing the effects of the NICU stressor environment[20].

Early interventions to improve mother-infant interaction, such as the Mother Infant Transaction Program[21] and its modified version, PremieStart[22], both of which target parental training to facilitate their infant's well-being, seem to have the greatest potential to support child development[12,22].

Recent studies have shown how early and specific interventions, such as infant massage, can accelerate the development of visual competences in preterms in the first year and can favor plasticity in infants at a neurodevelopmental risk[23–25]. This observation supports the findings of Ricci et al., who suggested that some features of visual function are more mature in preterm infants

at TEA than they are in term-born infants and highlighted the role of early visual experience in visual function maturation[26].

Other authors demonstrated the positive impact of infant massage on different aspects of neurodevelopment[27,28], including a reduction of stress behaviors, even in infants who are at a high neurological risk[24].

Despite a strong evidence supporting the relationship among brain development, neurodevelopment and visual function[29,30], the early detection of functional correlates of altered brain maturation is still challenging.

The positive effect of an enriched environment on the brain and visual system development has been confirmed by animal studies in mouse models[31–33].

However, the effects of early intervention strategies, based on mother-infant interactions combined with an enriched environment, on visual function have not yet been investigated.

Objective: To assess the effectiveness of an early intervention program in enhancing visual function in low-risk very preterm infants.

Patients and Methods

We designed a randomized controlled trial (Trial Registration Number: NCT02983513).

All preterm infants, consecutively born between 25^{+0} and 29^{+6} weeks of gestational age (GA) from April 2014 to January 2017 at the NICU, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milano, were eligible for the study.

The exclusion criteria were as follows: multiple pregnancy (triplets or higher); genetic syndromes and/or major congenital malformations; surgical Necrotizing Enterocolitis (NEC); major brain lesions, including Germinal Matrix Intraventricular Hemorrhage (GMH-IVH) > 2° grade according to Papile[34], documented by early cranial ultrasound (cUS). The infants who, during their postnatal course, developed cystic Periventricular Leukomalacia (cPVL), detected by sequential cUS scans

up to TEA, or retinopathy of Prematurity (ROP) > stage 2 were excluded from analysis related to visual function.

Mothers were selected according to the following criteria: age over 18 years, good comprehension of Italian, no single-parent families, no obvious cognitive impairment or psychiatric disorders, and no drug addiction.

Infants were recruited after the first week of life and if they were clinically stable (i.e., no need for invasive mechanical ventilation and no active sepsis).

After obtaining parental written informed consent, infants were randomized to receive either Early Intervention (EI) or Standard Care (SC) using sealed envelopes that were prepared in groups of 10 through computer-generated randomization. The randomization sequence was concealed until the group allocation was assigned, and the examiner remained blinded for the entire study period.

The EI program was delivered in addition to routine care during the NICU stay by the same investigator (CF), according to the PremieStart Protocol[22], to train parents to: recognize signs of infant stress and alert-available behavior to promote mother-infant interaction; adopt principles of graded stimulation and avoid overwhelming infants through facilitation strategies. The program was held in eight main sessions and one additional post-discharge session.

Moreover, parents were trained to promote massage therapy and visual attention when their infants were in an alert behavioral state.[35] A diary was given to parents to register the interventions.

Massage therapy was performed twice per day by parents after they received two training sessions. It started not before the third week from birth and was performed until TEA. Each massage session consisted of 10 minutes of slow tactile stimulation of the back, giving moderate pressure stroking with both hands. During the massage, the infant was placed prone. Each session was performed at least 2 hours after the previous one.

Parents promoted visual attention at least once a day using either a black-and-white toy or the parent's face. This interaction occurred not before 34 weeks of GA and it was performed until TEA.

Infants were in an alert behavioral state, supine, either on a parent's lap or in their crib, and nested with a blanket to avoid excessive stimulation.

SC, according to the NICU protocols, included Kangaroo Mother Care (KMC), nesting and minimal handling.

During the study period, no specific interventions (e.g., Newborn Individualized Developmental Care Assessment Program - NIDCAP) to decrease stress were used.

The baseline characteristics, collected from hospital charts, included: gender, birth weight and GA, Small for Gestational Age (SGA)[36], twin birth, mode of delivery, Apgar score at 1 and 5 minutes, Clinical Risk Index for babies (CRIB)[37], number of days on invasive mechanical ventilation or on nasal continuous positive airway pressure (NCPAP) or High Flow nasocannula, duration of hospital stay and GA at discharge.

The following neonatal morbidities were considered: ROP[38], NEC[39], Bronchopulmonary Displasia (BPD)[40], GMH-IVH[34] and sepsis (increased plasmatic levels of C reactive protein associated with a positive blood culture).

Family socioeconomic status (SES) was calculated and classified according to Hollingshead's criteria[41].

Outcome measure: Visual Assessment

At TEA (40±3 weeks), infants underwent visual assessment according to the protocol developed by Ricci et al.[26,42] that evaluates the following: ocular movements both spontaneous and in reaction to a target, ability to fix and follow a target (horizontally, vertically and in an arc), ability to track a colored stimulus, visual acuity (evaluated using black and white stripes of increasing spatial frequency from 0.24 to 3.2 cycle/degree[43]) and visual attention at a distance.

The best performance, according to the protocol, was defined as: mainly conjugated ocular motility, stable fixation, complete tracking, tracking of colored stimulus, discrimination of a spatial frequency over 2.4 cycles per degree and visual attention beyond 70 cm.

Infants were assessed in a single session (10 minutes) in a quiet environment with low light. The

examination occurred when infants were in an alert behavioral state[35] and in a supine position. Responses for each of the 9 items were recorded.

The examiner (ADC) was experienced in neonatal visual battery and blinded to the group assignment.

The trial was approved by the Ethical Committee Milano Area B study on 14 March 2014. Written parental informed consent was provided for each infant in the study.

Statistical Analysis

This study's sample size was based on clinical feasibility and a power calculation: recruiting 70 infants would provide 80% power to detect a difference equal to 30% or more in visual performance between the groups (based on a 2-sided test with a = .05). We accounted for a 15% drop out.

Baseline characteristics were described as the mean (standard deviation - SD), the median and range, or the number and percentage, as appropriate. Demographic characteristics were compared across infants in the EI and SC groups using Fisher's exact test for categorical variables and Student's t-test or Mann-Whitney U-test for continuous variables, after assessing the normality assumption with the Shapiro–Wilk test.

Logistic regression models, used to estimate the relative "risk" of obtaining the best performance in each visual item, were run as sensitivity analysis, including GA and ROP, to control for their potential confounding effect. The results are presented as odds ratios (OR) and 95% CI.

All tests were two-tailed, and $p < 0.05$ was considered significant for all tests.

Statistical analyses were performed using R version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Overall, 70 infants (EI n = 34, SC n = 36) were recruited and randomized for intervention between April 2014 and January 2017. According to the protocol 3 infants allocated to EI did not receive treatment because: 2 developed surgical NEC and 1 family became a single-parent family after written informed consent was signed by both parents. All babies in the SC group received allocated treatment as part of routine clinical practice.

At TEA ten infants were excluded from visual assessment because: 6 developed ROP > stage 2 (3 for each group), 2 infant developed cPVL (1 for each group) and 2 infants belonging to SC group developed surgical NEC.

Fifty-seven infants (EI = 27, SC = 30) were assessed for visual functions at TEA .

Parent and infant characteristics were similar between the two groups (Table 1).

Demographic feature	**Early Intervention (n=27)**	**Standard Care (n=30)**	**P value**
Gestational age at birth (weeks), mean (SD)	28,4 (0,9)	27,8 (1,3)	0,06 *
Birth Weight (g), mean (SD)	1032 (249)	1092 (312)	0,42 §
Male, n (%)	13 (48)	16 (53)	0,90 °
Singleton, n (%)	15 (56)	18 (60)	0,94 °
CRIB II score, mean (SD)	7,7 (1,7)	8,1 (2,3)	0,64 *
Apgar score at 1', median (range)	7 (4-9)	6 (2-8)	0,31 *
Apgar score at 5', median (range)	8 (7-10)	8 (5-9)	0,32 *
Cesarean Section, n (%)	25 (93)	26 (87)	0,67 °
Days of Mechanical Ventilation, mean (SD)	3,9 (7,5)	4,3 (6,3)	0,24 *
Days of NCPAP, mean (SD)	25,7 (13,7)	25,6 (14,0)	0,81 *
Days of High Flow Nasocannula, mean (SD)	15 (26,5)	7,2 (15,3)	0,79 *
Small for Gestational Age, n (%)	6 (22)	4 (13)	0,49 °
Sepsis, n (%)	11 (41)	11 (37)	0,96 °
Severe Bronchopulmonary Dysplasia, n (%)	8 (30)	5 (17)	0,35 °
GMH-IVH grade 1-2, n (%)	3 (11)	4 (13)	1,00 °
Retinopathy of prematurity <3, n (%)	1 (4)	6 (20)	0,06 °
Medical Necrotizing Enterocolitis, n (%)	0 (0)	1 (3)	N/A
Days of Hospitalization, mean (SD)	76 (24,0)	82,4 (35,1)	0,82 *
Gestational Age at Discharge, mean (SD)	39,2 (3,5)	39,6 (4,1)	0,90 *
Maternal Age, mean (SD)	33,9 (3,9)	33,8 (6,2)	0,99 §
SES, mean (SD)	50,7 (9,7)	44,8 (13,9)	0,12 *
Gestational Age at visual assessment, mean (SD)	40,7 (1,0)	41 (1,1)	0,23 *

§ Student's t test, * Mann–Whitney test, ° Fisher Exact Test

Table 1: Infants and maternal characteristics

Visual Function

The assessment was performed from June 2014 to April 2017 at TEA in the 2 groups (mean age EI: 40.7 ± 0.99, mean SC: 41 ± 1.05), and all infants completed the evaluation.

The infants in the EI group showed a more mature visual performance compared to the SC group. In the EI group, 59% of the infants achieved the highest score possible on all 9 items of the assessment compared to 17% of the infants in the SC group (p = 0.001, Fisher Exact Test).

Descriptive results for each item of the assessment are presented below and specified in Table 2.

Neonatal Visual Assessment	**Item Categories**	**Early Intervention (n=27)**	**Standard Care (n=30)**	**P value**
Spontaneous ocular motility	**Mainly conjugated**	**26 (96.3%)**	**21 (70%)**	0.013 °
	Occasional strabismus / occasional or lateral nystagmus	1 (3.7%)	9 (30%)	
	Intermittent strabismus /nystagmus	0 (0%)	0 (0%)	
	Continuous strabismus /nystagmus	0 (0%)	0 (0%)	
Ocular movements with target	**Mainly conjugated**	**23 (85.2%)**	**16 (53.3%)**	0.012 °
	Occasional strabismus / occasional or lateral nystagmus	4 (14.8%)	14 (46.7%)	
	Intermittent strabismus /nystagmus	0 (0%)	0 (0%)	
	Continuous strabismus / nystagmus	0 (0%)	0 (0%)	
Fixation	**Stable (> 3 sec)**	**27 (100%)**	**30 (100%)**	n.a.
	Unstable (< 3 sec)	0 (0%)	0 (0%)	
	Absent	0 (0%)	0 (0%)	
Tracking - Horizontal	**Complete**	**27 (100%)**	**30 (100%)**	n.a.
	Incomplete	0 (0%)	0 (0%)	
	Brief	0 (0%)	0 (0%)	
	Absent	0 (0%)	0 (0%)	
Tracking – Vertical	**Complete**	**27 (100%)**	**29 (96.7%)**	1°
	Incomplete	0 (0%)	1 (3.33%)	
	Brief	0 (0%)	0 (0%)	
	Absent	0 (0%)	0 (0%)	
Tracking – Arc	**Complete**	**27 (100%)**	**24 (80%)**	0.025°
	Incomplete	0 (0%)	6 (20%)	
	Brief	0 (0%)	0 (0%)	
	Absent	0 (0%)	0 (0%)	
Tracking colored stimulus	**Present**	**27% (100%)**	**30 (100%)**	n.a.
	Absent	0 (0%)	0 (0%)	
Visual Acuity	**7 – 8 cards**	**21 (77.8%)**	**10 (33.3%)**	0.001 °
	5 – 6 cards	6 (22.2%)	15 (50%)	
	3 – 4 cards	0 (0%)	5 (16.7%)	

	< 3 cards	0 (0%)	0 (0%)	
Attention at distance	**≥ 70 cm**	**20 (74.1%)**	**6 (20%)**	< 0.001 °
	51- 69 cm	6 (22.2%)	17 (56.7%)	
	30 – 50 cm	1 (3.7%)	7 (23.3%)	
	< 30 cm	0 (0%)	0 (0%)	

'°' Fisher exact test.

The last column shows the p-value for a Fisher exact test comparing the best performance versus all the others. The best performance is shown in bold.

Table 2: Visual assessment in the 2 groups.

Spontaneous ocular motility: In the EI group, 96.3% of infants showed conjugated ocular motility and the remaining 3.7% showed occasional strabismus or nystagmus. In the SC group, conjugated ocular motility was observed in 70% of the infants, and occasional strabismus or nystagmus in the remaining 30%.

Ocular movements with target: In the EI group, 85.2% of infants showed conjugated ocular motility and the remaining 14.8% showed occasional strabismus or nystagmus. In the SC group, conjugated ocular motility was observed in 53.3% of the infants, and occasional strabismus or nystagmus in the remaining 46.7%.

Fixation: Stable fixation was observed in all infants in both groups.

Tracking: Horizontal tracking was complete in all infants in the 2 groups. The ability to track vertically was complete in all infants in the EI group and in 96.7% of infants in the SC group; the remaining 3.33% presented incomplete vertical tracking. Arc tracking was complete in the whole EI group and in 80% of infants in the SC group; incomplete arc tracking was observed in the remaining 20%.

Reaction to a colored contrast target: All infants in the 2 groups were able to track a colored target.

Visual acuity: In the EI group, 77.8% of the infants discriminated cards 7-8, and the remaining 22.2% discriminated cards 5-6. In the SC group, 33.3% of the infants discriminated cards 7-8, 50% cards 5-6 and the remaining 16.7% cards 3-4.

Attention at distance: In the EI group, 74.1% of the infants could keep attention on the target for more than 70 cm, 22.2% up to 51-69 cm and the remaining 3.7% up to 30-50 cm. In the SC group, 20% of the infants could keep attention on the target for more than 70 cm, 56.7% up to 51-69 cm and the remaining 23.3% up to 30-50 cm.

The differences in GA and ROP ≤ 2 in the EI and SC groups were not statistically significant; however, both p-values were close to the significance level of 0.05. To account for the possible uncontrolled effect that resulted from the different distribution in the two groups, logistic regression models, to compare infants that obtained the best performance in each item versus all others, were computed including terms for GA and ROP. The multivariate analyses were computable for attention at a distance (OR, 14.9; 95% CI, 4.1 to 67.4; $p < 0.001$), visual acuity (OR, 7.5; 95% CI, 2.3 to 28.0; $p = 0.001$), ocular movements with target (OR, 5.9; 95% CI, 1.6 to 26.3; $p = 0.01$) and spontaneous ocular motility (OR, 13.7; 95% CI, 2.1 to 279; $p = 0.02$), and they confirmed the higher visual performance in the EI group.

Discussion

This is, to our knowledge, the first study focusing on the effects of a multisensory early intervention program on the maturation of visual function in preterm infants at term age. Our findings suggest that early intervention strategies may have a positive effect on visual function and result in a possible acceleration of visual performance maturation.

More specifically, our data show that the difference between the EI and group and the SC group was obvious in some items but negligible in others. The discrepancy between the findings in the two groups of items can be easily explained by the known maturational pattern of individual function. Some items, such as fixation, horizontal and vertical tracking and tracking a colored stimulus, were already mature in the infants in our cohort, as expected at TEA and as observed in previous studies in low-risk preterms[26]. Thus, all infants in the study achieved a maximum score, and no significant differences could be found between the groups.

In contrast, other items did not show a ceiling effect and could provide an opportunity to assess the differences in maturation in response to an intervention. In these items, whereas the SC group showed a level of maturation consistent with the previously reported range[26], the EI group showed higher scores, suggesting more mature findings. This supports the hypothesis that an EI program may accelerate the development of visual function[26].

Among the not completely mature items at TEA, some are dependent on subcortical structures, whereas others require cortical maturation; however, both showed acceleration in the EI group. More specifically, ocular motility and tracking for an arc at this age are mainly dependent on subcortical functioning. As these items are known to be influenced by experience[26,44], the accelerated maturation of these abilities is likely to be partly related to the increased visual stimulation that infants in the EI group experienced from 34 weeks postmenstrual age.

The combination of massage and increased visual stimulation may affect the maturation of more cortical aspects of visual function, such as visual acuity and attention at a distance, reported as being primarily dependent on postmenstrual age[45,46]. Infants in the EI group, in fact, showed more mature responses in these items.

These findings are consistent with a recent study reporting the effect of infant massage on the maturation of visual function and brain electrical activity in low-risk preterm babies[23]. In this study, infants received a multisensory intervention including body massage and an auditory stimulation. Visual Evoked Potential (VEP) and Electroencephalogram (EEG) were performed before and after the massage, and the functional visual assessment was performed only at 3 months corrected age. The results showed that enriching the environment using a multisensory stimulation positively affects brain development and visual system maturation. Although the two protocols differ in the number and type of tactile stimulation, and in the actor performing the massage, our RCT confirms the potential benefit of a multisensory stimulation on the development of both cortical and subcortical visual function already at TEA.

Based on previous evidence, we designed our RCT to study the effect of a multisensory approach (including both tactile and visual stimulation) to promote early visual function and child neurodevelopment, thus limiting our ability to disentangle the contribution of each intervention as both have been proven to promote visual maturation. Due to the early nature of the intervention, a baseline assessment of visual function could not be performed; however, the randomization supports the homogeneity of the groups before intervention.

One of the advantages of our study is that we included only preterms with normal or mildly abnormal sequential cUS, thereby excluding those with brain lesions who are more likely to develop visual disorders. We could therefore avoid confounding factors (severe brain lesions and severe neonatal comorbidities potentially affecting neurodevelopment) when assessing the effects of EI on preterms, while previous studies evaluating the effect of PremieStart on neurodevelopment also included preterms with major brain lesions. However, these strict exclusion criteria led to a relatively small sample, which represents a limitation of the study and makes our risk estimates unstable. Although not conclusive, we consider our results important to support a biologically well-described hypothesis that would deserve subsequent confirmation from larger studies in the future.

Another potential limitation of the study is the higher, but not significant, rate of ROP ≤ 2 observed in the SC group. However, this finding is unlikely to affect the robustness of our results, as demonstrated by the logistic regression models. Moreover, several studies reported that lower grades of ROP do not affect visual function[47,48].

A key aspect of our protocol was that parents were engaged as first actors in the EI protocol; starting from PremieStart, they were then involved in performing massage and visual interaction, thus potentially helping parents build a stronger dyadic relationship. It may also be speculated that improvement in visual function could improve infants' ability to interact with their parents, with a positive effect on parents' responsiveness.

Conclusion

Even though it is preliminary, our study, which assesses infants at TEA, suggests that the positive

effect of a multisensory approach can already be recorded at that age for specific aspects of visual function, thus supporting the introduction of early intervention in the care of very preterm infants in addition to Standard Care.

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Chapter 5

Effects of Early Intervention on feeding behavior in preterm infants: a Randomized Controlled Trial

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Abstract

Background: Although highly beneficial, human milk feeding is challenging in preterms due to adverse NICU factors for infant and mother.
Aim: to investigate the effect of an early intervention in promoting infant's breast milk feeding and acquisition of full oral feeding
Methods: This study is part of a RCT. We included preterm infants born between 25^{+0} and 29^{+6} weeks of gestational age (GA) without severe morbidities and their families. Infants were randomized to either receiving standard care (SC) or early intervention (EI). EI included PremieStart and parental training to promote infant massage and visual attention according to a detailed protocol. SC, in line with NICU protocols, included Kangaroo Mother Care. Time of acquisition of full oral feeding and human milk assumption at discharge were recorded.
Results: Seventy preterm (EI n=34, SC n=36) infants were enrolled. Thirteen were excluded according to protocol. Fifty-seven (EI n=29, SC n=28) were evaluated at discharge. The two groups were comparable for parent and infant characteristics. A significantly higher rate of infants fed with any human milk was observed in the EI group (75.9%) compared to SC group (32.1%) (p=0.001) and EI infants were four times more likely to be fed exclusively with human milk. Full oral feeding was achieved almost one week before in EI infants (mean postmenstrual age 36.8±1.6 vs 37.9±2.4 weeks in EI vs SC, p=0.04).
Conclusions: Early interventions promoting mother self-efficacy and involvement in a multisensory stimulation have a beneficial effect on breast milk feeding in preterm infants

Background

Preterm birth is the leading cause of infants' mortality across the world [1] and it is associated with several neonatal morbidities – the main ones include sepsis, bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC) and brain lesions [2]. Infants' life quality may also be negatively affected by long term neurodevelopmental delays [3,4].

In premature infants, breastmilk plays a key role with several studies reporting a significant decrease of sepsis and NEC or lower rates of retinopathy of prematurity (ROP) [5–7]. Similarly, it is proved to positively affect neurodevelopment in the long term with benefits on motor and cognitive outcomes as well as neurobehavioral organization [8,9].

Therefore, exclusive human milk is recommended by the American Academy of Pediatrics as the first choice for preterms' enteral nutrition, especially during the first six months of life [10].

However, preterm birth and admission to a Neonatal Intensive Care Unit (NICU) are the strongest predictors of not being exclusively breastfed at discharge [11,12]. Vohr et al report that 78% of mothers initiate human milk feeding in the NICU, but only 31% provide it at discharge [13].

Human milk feeding is particularly challenging for preterm infants and their mothers because of the negative factors they are exposed to, such as NICU environment, neonatal morbidities, paucity of parental contact, delayed breastfeeding etc. [14] All these factors can affect mother-infant relationship which is essential to start and continue lactation [15,16]. An established practice to improve the mother-infant relationship in NICU is the Kangaroo Mother Care (KMC) and its benefits on breastfeeding are well-known [17]. Skin-to-Skin contact promotes a greater closeness between infant and mother helping her to interpret infant cues [18]. Recent studies are exploring the effect of more active tactile contact such as preterm baby massage on neurobehavior or duration of hospital stay, but the lack of a randomized control trials (RCT) approach has raised concerns on their validity [19].

At the same time the positive effects of early intervention strategies on sensitive and responsive interaction between preterm infants and their mother has been recently confirmed [20].

However the effects of an early multisensory intervention that includes preterm baby massage and early mother-infant interaction on infant's feeding behavior have not been investigated yet.

The present study is part of a RCT aimed to assess the effectiveness of an early intervention program in promoting visual function and neurodevelopment in preterm infants. Within this context further analyses have been performed with the exploratory purpose to investigate the effect of the early intervention in promoting infant's breast milk feeding and acquisition of full oral feeding.

Methods

Subjects

The trial was approved by the Ethical Committee on the 14th of March 2014. Written parental informed consent was obtained from the parents.

All the preterm babies, consecutively born between 25^{+0} and 29^{+6} weeks gestational age (GA) from April 2014 to January 2017 at the same institution were eligible for the study. Exclusion criteria were as follow: multiple pregnancy (triplets or higher); genetic syndromes and/or major congenital malformations; NEC stage III according to Bell [21]; major brain lesions, including Germinal Matrix Intraventricular Hemorrhage (GMH-IVH) > 2° grade according to Papile [22], documented by early cranial ultrasound (cUS). Also infants who developed stage II NEC were excluded from the present exploratory study due to the potential adverse effect of any stage NEC on oral feeding acquisition related to protracted suspension of oral feeding.

Mothers were selected according to the following inclusion criteria: age over 18 years, good comprehension of Italian language, no single-parent families, no obvious cognitive impairments or psychiatric disorders, and no drug addiction.

Infants were recruited after the first week of life and if clinically stable (no need of invasive mechanical ventilation and no active sepsis).

Study design

This study is part of a larger RCT (Trial Registration Number: NCT02983513).

Infants were randomised either to receive Early Intervention (EI) or Standard Care (SC) by using sealed envelops prepared in groups of 10 through computer-generated randomization.

The EI program was delivered in addition to routine care during the NICU stay by the same investigator (CF), according to the PremieStart Protocol [23], in order to train parents to: recognize signs of infant stress and alert-available behaviour to promote mother-infant interaction; adopt principles of graded stimulation; optimize interactions and avoid overwhelming infants through facilitation strategies (for example, engage and support the visual attention of the newborn). The program was held in eight main sessions and one additional post-discharge session. In addition parents were trained and invited to daily promote preterm baby massage therapy and visual attention when babies were in an alert or active behavioural state according to Brazelton [24]. A diary was given to parents to daily register the interventions. Preterm baby massage therapy was performed twice a day by parents after receiving two training session. Each massage session consisted on 10 minutes of slow tactile stimulation of the back giving moderate pressure stroking with both hands. During massage the infant was placed prone. Each session was performed at least 2 hours after the previous one.

Parents promoted visual attention at least once a day using either a black and white toy or parents face. This interaction took place when baby was in an alert behavioural state and not before 34 weeks of GA. Infants were supine, either on parents lap or in their crib, and nested with a blanket to avoid excessive stimulation.

SC, according to the NICU protocols, included Kangaroo Mother Care (KMC), nesting and minimal handling. During the study period no specific interventions to decrease stress (e.g. Newborn Individualized Developmental Care Assessment Program - NIDCAP) were in use.

Baseline characteristics of the two groups were collected from hospital charts. Recorded data included: gender, birth weight and GA, Small for Gestational Age (SGA) [25], twin birth, mode of delivery, Apgar score at 1 and 5 minutes, Clinical Risk Index for babies (CRIB) [26], number of days on invasive mechanical ventilation or on nasal continuous positive airway pressure (NCPAP)

or High Flow nasocannula, duration of hospital stay and GA at discharge.

The following neonatal morbidities were considered: ROP [27], BPD [28], GMH-IVH [22] and sepsis (increased plasmatic levels of C reactive protein associated with a positive blood culture).

Family socio economic status (SES) was calculated and classified according to Hollingshead's criteria [29].

Feeding protocol was the same during the study period and all mothers were provided with a pump and encouraged to start pumping on day 1 and to increase it to every 3 hours.

In case of unavailable or insufficient human milk, formula feeding was started. Infants' human milk intake at discharge was calculated from the infants' computerized medical chart, completed by nurses blinded to group allocation, and expressed as percentage of the total milk intake. Infants were categorized as receiving exclusive formula, exclusive human milk and human milk plus formula and data are presented accordingly.

For further analysis infants fed any extent of human milk, irrespective of the quantity or the exclusivity, were categorized as fed any human milk [10].

Fortification of human milk was started when the enteral intake reached 90 ml/kg/day. The volume of enteral feeding was increased based on the infants' cardio-respiratory stability and gastrointestinal tolerance. Human milk was fortified with a target fortification to comply with the guidelines from the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). The target levels of the human milk macronutrients were as follows: 3 g/100 ml of proteins, 8.8 g/100 ml of carbohydrates and 4.4 g/100 ml of fat [30].

Statistical analysis

All data were analysed with R software, version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria). Categorical variables were compared by Fisher's exact test and continuous variables by Mann-Whitney U test. A P value < 0.05 was considered as significant.

Results

A total of 70 infants (EI n=34, SC n=36) were recruited between April 2014 and January 2017.

According to the protocol 3 infants allocated to EI did not receive treatment because: 2 developed stage III NEC and 1 family became a single-parent family after written informed consent was signed by both parents. All babies in the SC group received allocated treatment as part of routine clinical practice.

At discharge 10 infants (EI n=2; SC n=8) were excluded from feeding behavior evaluation as: 5 infants in the SC group developed NEC (stage II n=3, stage III n=2) and 5 mothers (EI n=2; SC n=3) decided not to express milk from day one.

Fifty-seven infants (EI n=29, SC n=28) were then eligible for evaluation on type of feeding at discharge.

Parental and infant characteristics were similar between the two groups (Table 1).

Demographic feature	Early intervention (n=29)	Standard care (n=28)	Pvalue
Gestational age at birth (weeks), mean±SD	28.1 ± 1.3	27.6 ± 1.5	0.09 *
Birth Weight (g), mean±SD	1020 ± 274	1040 ± 322	0.91 *
Male, n (%)	13 (44.8%)	13 (46.4%)	1.00 °
Singleton, n (%)	18 (62.1%)	15 (50%)	0.43 °
CRIB II score, mean±SD	8± 2.3	8.6 ± 2.6	0.42 *
Apgar score at 1', median (range)	7 (4-9)	6 (2-8)	0.17 *
Apgar score at 5', median (range)	8 (6-10)	8 (5-9)	0.14 *
Cesarean Section, n (%)	26 (89.7%)	23 (82.1%)	0.47 °
Days of Mechanical Ventilation, mean±SD	3.8 ± 6.5	6.6 ± 10	0.17 *
Days of NCPAP, mean±SD	27.4 ± 15.3	27.1 ± 12.9	0.82 *
Days of High Flow Nasocannula, mean±SD	10.8 ± 20.4	11.3 ± 19.4	0.78 *
Small for Gestational Age, n (%)	8 (27.6%)	9 (32.2%)	0.77 °
Sepsis, n (%)	13 (44.8%)	11 (39.3%)	0.79 °
ROP			0.42°
stage I-II	1 (4%)	4 (14%)	
stage III	3 (10%)	3 (11%)	
Severe Bronchopulmonary Displasia, n (%)	9 (31%)	7 (25%)	0.77 °
GMH-IVH 1-2, n (%)	2 (6.9%)	3 (10.7%)	0.67 °
Maternal Age (years), mean±SD	33.4 ± 4.2	33.6 ± 5.9	0.81 *
SES, mean±SD	50.3 ± 9.6	43.6 ± 13.3	0.06 *

* Mann-Whitney U Test, ° Fisher Exact Test

Table 1. Baseline characteristics of the EI and SC groups

No differences were found between the two groups in terms of lenght of stay (75.3±21.1 vs 85.9±33.2 days in EI and SC group respectively, p=0.35) and gestational age at discharge (38.9±3.0 vs 39.9±3.8 weeks in EI and SC group respectively, p=0.36).

The feeding characteristics of the two groups are described in Table 2.

	Early intervention (n=29)	**Standard Care (n=28)**	**Pvalue**
Acquisition of full oral feeding (weeks), mean±SD	36.8 ± 1.6	37.9 ± 2.4	0.04 *
Percentage of human milk assumption, mean±SD	57.6 ± 41.6	22.9 ± 36.9	< 0.001 *
Type of feeding at discharge, n (%)			0.003 °
Exclusive Human Milk	12 (41.4)	3 (10.7)	
Human Milk + Formula	10 (34.5)	6 (21.4)	
Exclusive Formula	7 (24.1)	19 (67.9)	

* Mann-Whitney U Test, ° Fisher Exact Test

Table 2. Feeding characteristics of the EI and SC groups.

Infants enrolled in EI group achieved full oral feeding almost one week before SC infants (p=0.04) and showed a higher assumption of human milk at discharge (p<0.001).

More specifically, a higher rate of babies fed with any human milk was observed in the EI group compared to SC group (EI=75.9% versus SC=32.1%, p=0.001) and EI group babies were four times more likely to be fed exclusively with human milk.

Discussion

Our findings suggest that early intervention strategies, based on a parental training program, are successful in improving breast milk feeding in very preterm infants at discharge. Accordingly, the EI program resulted in a higher proportion of infants exclusively fed with human milk compared to SC group. This result is of primary importance given the widely acknowledged beneficial effect of breast milk for the short and long term outcomes of preterm infants [5,6,8].

The lactation rates observed in the SC group are consistent with those previously reported in infants with similar GA [13] whereas mother's milk assumption in the EI group is approximately four times higher.

The percentage of human milk intake was assessed at discharge, thus supporting the hypothesis that the EI program may contribute not only to sustain initiation but also to maintain lactation until term age.

Both components (the parental training program - PremieStart - and infant massage) of our early intervention program may be involved in the observed beneficial effect on mother's lactation. However, due to the combined nature of our intervention is not possible to disentangle each single contribution.

PremieStart [23] is based on the promotion of mother-infant relationship through facilitation strategies that help parents recognize signs of alert and stress behavior. This program, together with its original version Mother Infant Transaction Program (MITP) [31], has been proven to encourage mother's responsiveness and to reduce stress and depressive symptoms [20,23,32]. thus theoretically promoting the attainment of the maternal role, which is threatening in case of preterm birth.

The second major element of our protocol is infant massage delivered by mothers during NICU stay, which has also been reported to be effective in reducing depressed mood and anxiety in mothers of preterm infants [33].

We hypothesize that both elements of the intervention contributed to sustain mother milk provision. This is in line with studies showing how parental participation and involvement has a crucial importance on maintaining breastfeeding [34,35] and with research on how depression and stress could negatively affect breastfeeding [36].

Another significant result of the present study is the effect on timing of acquisition of full oral feeding. Infants in the EI group showed a mature oral feeding pattern approximately one week corrected age before infants in the SC group. This finding may be partially explained by the attainment of one of the objectives of the PremieStart, namely training parents to recognize signs and respond to infant cues in daily care, which is reported to enhance the development of preterms' oral skills [37,38].

Surprisingly, the observed beneficial effects of EI did not result in a shortened NICU stay. This is in contrast with a wide meta analysis reporting that massage intervention in preterm infants decreased average length of stay of 4.5 days. However, the same meta analysis reports concerns about the methodological robustness and blinding of this outcome [39]. Additionally, this meta analysis included studies performed also on more mature preterm babies suffering milder postnatal morbidities. We focused on very preterm infants (<30 weeks gestation) and although babies with major morbidities were excluded, all of them experienced postnatal complications potentially prolonging the time needed to acquire the physiologic stability and the full respiratory competency, which are mandatory for home discharge.

Previous studies report a beneficial effect of human milk in reducing the occurrence of NEC, however this effect could not be evaluated as NEC was settled as exclusion criteria of the study.

One of the advantages of the present study is that medical staff completing infants' computerized chart were blinded to group allocation.

One limitation of the study is represented by the failure to differentiate between breastmilk feeding and breastfeeding as this information was not clearly available in the nutritional dedicated section of the infants' computerized medical chart.

Another possible limitation is the lack of a baseline evaluation of mothers' psychosocial aspects, however the randomization supports the homogeneity of the two groups.

Based on previous reports [40] the slightly higher SES observed in EI group could have influenced breastmilk feeding rates; however, this difference is not statistically significant and maternal age, one of the most reported limiting factor for breastmilk feeding [11], was similar in the two groups.

Conclusions:

Even if preliminary, our RCT highlights the role of early intervention strategies in promoting breastmilk feeding. Early approaches promoting mother self efficacy and involvement in a multisensory stimulation to enhance mother-infant closeness and dyadic relationship should be implemented in the care of very preterm infants in addition to Standard Care.

Conflicts of interest:

Authors have no conflict of interest to declare.

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Chapter 6

LINE-1 Methylation Status in Preterm Infants and Effects of Early Intervention Strategies

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Abstract:

Background: NICU stressful environment may alter brain development in preterm infants. Epigenetic mechanisms are likely to have a role in mediating brain maturation and seem to be associated with long-term effects of exposure to stress in early life. Long Interspersed Nuclear Elements (LINE-1) methylation, covering the 22% of the human genome, are per se proxy of genome-wide methylation and may contribute to the pathogenesis of several neurodevelopmental disorders. We hypothesize that LINE-1 methylation levels may be a novel epigenetic biomarker to evaluate the effect of stress reduction strategies for preterm infants.
Aim: to explore the effect of an early neurodevelopmental intervention program on changes in LINE-1 methylation status at term corrected age in preterm infants.
Methods: This study is part of a RCT. We included preterm infants born between 25^{+0} and 29^{+6} weeks of gestational age (GA) without severe morbidities and their families. Infants were randomized to either receiving Standard Care (SC) or Early Intervention (EI). EI included PremieStart and parental training to promote infant massage and visual attention according to a detailed protocol. SC, in line with NICU protocols, included Kangaroo Mother Care.
LINE-1 methylation analyses were conducted using a cord blood sample, collected at birth, and a peripheral blood sample, harvested at hospital discharge (around term corrected age).
Results: 70 preterm infants (EI n =34, SC n = 36) were recruited and randomized for intervention between April 2014 and January 2017. For the purpose of this ancillary study blood samples were collected starting from August 2015. LINE-1 methylation analyses were performed in fifteen infants (EI = 9, SC = 6) with matched cord and peripheral blood samples at discharge. The two groups were comparable for parent and infant characteristics. LINE-1 methylation increased at term corrected age for both groups but was more pronounced in the EI group (p=0.0077) especially when looking at single CpG sites.
Conclusions: Even if very preliminary this study suggests that early intervention strategies during a window of both epigenetic and brain plasticity might modulate DNA methylation processes in preterm infants.

Background

Preterm infants are exposed to the NICU stressful environment, characterized by excessive sensory stimulation, paucity of parental contact and painful procedures[1,2]. Early postnatal life represents a critical stage of development for the brain that is particularly vulnerable to extrinsic insults and several preclinical and clinical studies have documented that reiterated exposure to high levels of pain and adverse environmental stimulation during the postnatal period can affect brain microstructural development.[2,3]

As a result, when stress is experienced during this critical early-life period, it may have a long-lasting effect and may be implicated in impaired neurobehavioral outcomes [4–6] that are reported as long term consequences of preterm birth in 25-50% infants.[7]

Mechanisms of epigenetic regulation such as DNA methylation are likely to have a key role in mediating brain maturation during this critical developmental window[8]. Epigenetics refers to changes of the genome's function that occur without any alteration in the DNA sequence itself[9] and DNA methylation is an epigenetic mechanism that occurs by the addition of a methyl (CH_3) group to DNA, thereby often modifying the function of the genes and affecting gene expression.

The major function of epigenetic processes is to dynamically regulate gene activity in response to environmental events.[10]

Recent findings from animal studies highlight how the long-term effects of exposure to stress in early life[11,12] are mediated by the epigenetic regulation of gene expression. Different rodent models and different experimental settings support these findings.[13,14]

In preterm infants, exposed to numerous skin-breaking procedure, pain-related stress was associated with an altered methylation of SLC6A4, the serotonin transporter gene.[15]

Also maternal deprivation during NICU stay represents a stressful condition for the preterm infants. Interestingly, animal studies designed to assess the effects of maternal separation, and variation in maternal care on offspring's behavior, suggest that exposure to stressful conditions in early life (such as maternal deprivation) may lead to neuroendocrine perturbations thereby affecting cognitive

functions and stress responses in later life.[10,16–18]

In particular it is proved how offspring exposed to high quality of maternal care, consisting in high level of licking / grooming and arched-back nursing present greater expression of glucocorticoid receptors in the hippocampus and increased sensitivity to cortisol feedback in the hypothalamic-pituitary axis, showing a more adaptive responsiveness to stress.[19,20] Subsequently, cross-fostering studies suggest that this is not a purely genetic issue, as maternal care in the first week of rat life, hence an environmental event, plays a crucial role in gene expression and responses to stress.[17]

Additionally, low quality of care in the early neonatal period is associated with a high methylation status on the nerve growth factor-induced protein A (NGFI-A) transcription factor, located in the glucocorticoid receptor promoter gene: the Nr3c1 gene.[21] The methylation process therefore appears to be sensitive to maternal care that can modulate the epigenetic effects of exposure to environmental stressful events.

Recently, animal studies have demonstrated that the process of methylation of specific genes is reversible and postnatal treatment modifications can diminish the effects on methylation induced by previous exposure to stressful events.[22]

An innovative approach to connect the epigenetic status of DNA methylation with neurological aspects is to study the level of methylation in a particular class of repetitive elements that are the Long Interspersed Nuclear Elements 1 (LINE-1), that covering the 22% (over 500,000 copies) of the human genome are per se proxy of genome-wide methylation. LINE-1 belong to the class of retrotransposons, that are capable of duplication by a copy-and-paste genetic mechanism, thus increasing their number of copies. They have been shown to be transiently activated during the processes of cellular differentiation in adults, embryogenesis but particularly in neurogenesis where this phenomenon contributes in a phisiological way to the normal brain development and to create somatic plasticity in the neurons inside the brain. Conversely, the deregulation of this mechanism has been associated to the occurrence of schizophrenia or brain disorders as in the Rett syndrome[23–

[25]. As with any biological phenomena, misregulation of retrotransposition can have detrimental effects and can possibly contribute to neuropathological diseases. LINE-1 elements are epigenetically strictly regulated being mostly transcriptionally silent thanks to the process of DNA methylation of their promoter. LINE-1 elements can be modulated and activated in response to biotic and abiotic stress conditions and during perturbation of cellular metabolism.[26]

Several studies have highlighted the positive effect of Developmental Care, as a strategy to reduce stressfull NICU environmental factors, promote maternal involvment and improve brain maturation at MRI and neurodevelopmental outcomes[27–29] but the epigenetic impact of early interventation has not been investigated yet.

We hypothesize that methylation levels (total but more precisely at CpG site level) of LINE-1 may be a novel epigenetic biomarker to assess the effect of early intervention strategies aimed to reduce stress, enhance maternal care and in the long term improve child neurodevelopment

The present study is an ancillary study of a larger RCT aimed to assess the effectiveness of an early intervention program in promoting visual function and neurodevelopment in preterm infants. Within this context further analyses have been performed with the exploratory purpose to investigate the effect of the early intervention on LINE-1 methylation status.

Objective: To assess LINE-1 methylation in preterm infants at birth and to explore the effect of an early intervention program, based on mother-infant interaction combined with a multisensory stimulation, on changes in LINE-1 methylation status at term corrected age.

Study population

We designed a randomized controlled trial (Trial Registration Number: NCT02983513).

All preterm infants, consecutively born between 25^{+0} and 29^{+6} weeks of gestational age (GA) from April 2014 to January 2017 at the NICU, Fondazione IRCCS Cà Granda Ospedale Maggiore

Policlinico, Milano, were eligible for the study.

The exclusion criteria were as follows: multiple pregnancy (triplets or higher); genetic syndromes and/or major congenital malformations; surgical Necrotizing Enterocolitis (NEC); major brain lesions, including Germinal Matrix Intraventricular Hemorrhage (GMH-IVH) > 2° grade according to Papile[30] documented by early cranial ultrasound (cUS).

Mothers were selected according to the following criteria: age over 18 years, good comprehension of Italian, no single-parent families, no obvious cognitive impairment or psychiatric disorders, and no drug addiction.

Methods

Intervention

Infants were recruited after the first week of life and if they were clinically stable (i.e., no need for invasive mechanical ventilation and no active sepsis).

After obtaining parental written informed consent, infants were randomized to receive either Early Intervention (EI) or Standard Care (SC) using sealed envelopes that were prepared in groups of 10 through computer-generated randomization. The randomization sequence was concealed until the group allocation was assigned, and the examiner remained blinded for the entire study period.

The EI program was delivered in addition to routine care during the NICU stay by the same investigator (CF), according to the PremieStart Protocol[29], to train parents to: recognize signs of infant stress and alert-available behavior to promote mother-infant interaction; adopt principles of graded stimulation and avoid overwhelming infants through facilitation strategies. The program was held in eight main sessions and one additional post-discharge session.

Moreover, parents were trained to promote massage therapy and visual attention when their infants were in an alert behavioral state.[31] A diary was given to parents to register the interventions.

Massage therapy was performed twice per day by parents after they received two training sessions. It started not before the third week from birth and was performed until TEA. Each massage session consisted of 10 minutes of slow tactile stimulation of the back, giving moderate pressure stroking

with both hands. During the massage, the infant was placed prone. Each session was performed at least 2 hours after the previous one.

Parents promoted visual attention at least once a day using either a black-and-white toy or the parent's face. This interaction occurred not before 34 weeks of GA and it was performed until TEA. Infants were in an alert behavioral state, supine, either on a parent's lap or in their crib, and nested with a blanket to avoid excessive stimulation.

SC, according to the NICU protocols, included Kangaroo Mother Care (KMC), nesting and minimal handling.

During the study period, no specific interventions (e.g., Newborn Individualized Developmental Care Assessment Program - NIDCAP) to decrease stress were used.

The baseline characteristics, collected from hospital charts, included: gender, birth weight and GA, Small for Gestational Age (SGA)[32], twin birth, mode of delivery, Apgar score at 1 and 5 minutes, Clinical Risk Index for babies (CRIB)[33], number of days on invasive mechanical ventilation or on nasal continuous positive airway pressure (NCPAP) or High Flow nasocannula, duration of hospital stay and GA at discharge.

The following neonatal morbidities were considered: ROP[34], NEC[35], Bronchopulmonary Displasia (BPD)[36], GMH-IVH[30] and sepsis (increased plasmatic levels of C reactive protein associated with a positive blood culture).

Family socioeconomic status (SES) was calculated and classified according to Hollingshead's criteria.[37]

DNA methylation analysis

The DNA methylation analyses were conducted using two blood samples (0.5 ml of blood for single collection): a cord blood sample, collected at birth, and a peripheral blood sample, harvested at hospital discharge (around term corrected age). Peripheral blood was obtained during blood sampling performed for routine blood examination, according to clinical practice.

All the blood samples were obtained by trained doctors or nurses to avoid haemolysis and

immediately stored at - 80°C.

Methylation analyses were carried out at the Genome Biology Unit, INGM, Milan directed by Dr. Beatrice Bodega. All the analyses were performed by the same biologist (LP) blinded to allocated intervention.

DNA extraction

Genomic DNA was extracted from cord and peripheral blood samples using standard phenolclorophorm extraction techniques.

The concentration and purity of the DNA were determined by absorbance at 260 and 280 nm, measured by NanoDrop TM 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA).

Bisulfite treatment

A total of 500 ng genomic DNA from each sample was bisulfite-treated using the MethylEdge® Bisulfite Conversion System (Promega, Madison, USA) following the manufacturer's protocol.

Sequencing results confirmed that >95% of cytosine residues were converted.

LINE-1 methylation analysis

To obtain the overall DNA methylation status of the LINE-1 promoter region, we used the same primers used by Coufal et al.[38] to amplify a 363 bp fragment of the LINE-1 promoter, from a constellation of L1s, which included both young Ta-1 and older subfamilies of the L1Hs/L1PA1 family such as Ta-0 due to the high degree of L1 sequence conservation [39,40].

The 363-bp amplified fragment contains 19 CpG sites.

The primer sequences used to amplify bisulfite-converted DNA were the following:

For: 5'- AAGGGGTTAGGGAGTTTTTTT

Rev: 5'- TATCTATACCCTACCCCCAAAA

The 50-µL reactions for LINE-1 promoter were run for 30 cycles as follows:

- pre-denaturation at 95°C for 2 minutes;

- denaturation at 95°C for 45 seconds;
- annealing at 56°C for 1 minute;
- extension at 72°C for 30 seconds;
- final extension at 72°C for 4 minutes.

The resulting PCR products were checked by agarose gel electrophoresis and then purified by PureLink ™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen- Thermo Fisher Scientific, USA).

Once purified, they were cloned into pGEM- T® Easy Vector System I (Promega) using a molar ratio insert: vector of 6:1. Ten clones form each sample were randomly selected for DNA sequencing.

Sanger sequencing was performed by GATC Biotech, using the following primer: pGEM Seq (Rev: 5'-GACCATGATTACGCCAAGCTA).

To analyse the methylation status of the 19 CpG sites of the LINE-1 5'UTR, we took advantage of the QUMA (*QUantification tool for Methylation Analysis*) software (CDB, Riken, Japan)[41]. We excluded from the analysis three of the 19 CpGs due to their high degree of variability among the analysed sequences.

To obtain the actual methylation status of each CpG site, we used the percentage of methylation of each CpG site calculated as the number of methylations at a specific CpG site divided by the total number of clones that were sequenced.

Statistical Analysis

Descriptive statistics are given as mean ± SD, median and range or number and percentage. Independent t-test and Mann-Whitney U test were used in the comparison of continuous variables with normal distribution and non-normal distribution respectively. For the comparison of qualitative data, Fisher's exact test was used. Shapiro-Wilk test was used to test the normal distribution of the data.

For the analyses of the total methylation, independent t-tests were used to assess differences

between levels in EI and SC infants, while paired t-tests were used to compare the methylation levels between cord blood and peripheral blood for each group. Methylation levels for each CpG and the comparison between different groups of infants were assessed by a two-way analysis of variance (ANOVA), followed by post hoc comparisons (Tukey's HSD test) checking for individual differences. All tests were two-tailed and values of $p < 0.05$ were considered to be significant.

Results:

Overall, 70 preterm infants (EI n =34, SC n = 36) were recruited and randomized for intervention between April 2014 and January 2017. According to the protocol 3 infants allocated to EI did not receive treatment because: 2 developed surgical NEC and 1 family became a single-parent family after written informed consent was signed by both parents. All babies in the SC group received allocated treatment as part of routine clinical practice.

For the purpose of this ancillary study blood samples were collected starting from August 2015. We therefore harvested blood samples from 35 infants. Of those, only 20 infants had matched blood samples (both cord blood at birth and peripheral blood at NICU discharge).

Five infants had to be excluded from the study due to technical issues occurring during DNA methylation analyses. LINE-1 methylation analysis was performed in fifteen infants (EI = 9, SC = 6) with matched cord and peripheral blood samples at discharge.

Infant and Maternal Characteristics

Descriptive statistics for infants and maternal characteristics subdivided in the EI and SC group are reported in Table 1. No statistically significant differences were observed between the two groups.

Demographic feature	Preterm Infants Early Intervention (n=9)	Standard Care (n=6)	p-values EI vs SC
Gestational age at birth (weeks), mean±SD	28.2 ± 1.4	27.8 ± 1.5	0.37 *
Birth Weight (g), mean±SD	966 ± 315	1002 ± 254	0.82 ^
Male, n (%)	3 (33%)	2 (33%)	1.00 °
Twins, n (%)	6 (67%)	4 (67%)	1.00 °
Monocorionic twins, n(%)	4 (67%)	1 (25%)	0.58 °
Laser therapy after TTTS, n(%)	3 (75%)	1 (100%)	0.60 °
Corioamnionitis, n (%)	4 (44%)	1 (17%)	0.58°
CRIB II score, mean±SD	8.1 ± 2.3	7.8 ± 2.4	0.83 ^
Apgar score at 1', median (range)	7 (4-8)	7 (5-8)	0.71 *
Apgar score at 5', median (range)	9 (6-9)	8 (8-9)	0.70 *
Cesarean Section, n (%)	9 (100%)	5 (83%)	0.40 °
Days of Mechanical Ventilation, mean±SD	5.2 ± 9.0	2.0 ± 3.5	1.00 *
Days of NCPAP, mean±SD	20.9 ± 9.7	27.0 ± 11.4	0.51 *
Days of High Flow Nasocannula, mean±SD	20.1 ± 28.9	0.0 ± 0.0	0.07 *
Small for Gestational Age, n (%)	3 (33%)	1 (17%)	0.60 °
Sepsis, n (%)	5 (56%)	3 (50%)	1.00 °
Severe Bronchopulmonary Displasia, n (%)	4 (44%)	0 (0%)	0.10 °
IVH grade I-II, n (%)	0 (0%)	0 (0%)	1.00 °
ROP			0.66°
I-II	0 (0%)	1 (17%)	
III-IV	1 (11%)	0 (0%)	
Days of Hospitalization, mean±SD	79.7 ± 29.0	69.8 ± 18.5	0.81 *
Gestational Age at Discharge (weeks), mean±SD	39.7 ± 3.2	38.2 ± 1.9	0.45 *
Maternal Age (years), mean±SD	33.1 ± 5.3	34.8 ± 5.0	0.54 *
SES, mean±SD	47.6 ± 9.0	52.7 ± 19.7	0.29 *
Days of Dexamethasone, mean±SD	3.2 ± 6.8	0.0 ± 0.0	0.23 *
Smoke during pregnancy, n (%)	0 (0%)	0 (0%)	1.00 °
Alcohol assumption during pregnancy, n (%)	0 (0%)	0 (0%)	1.00 °

Table 1 – Infant and Maternal Characteristics for EI and SC groups.

^ t-test, Mann-Whitney U Test, ° Fisher Exact Test*

Preterm infants showed an increase in LINE-1 methylation on blood collected at discharge when compared to the matched cord blood sample; these results were observed both in respect to total methylation status (Figure 1) and to single CpG sites analysis (Figure 2).

The CpG sites showing a statistically significant increase in methylation from birth and NICU discharge were: CpG 3 (p=0.029), CpG 7 (p=0.0097), CpG 8 (p=0.026), CpG 10 (p=0.004), CpG 15 (p=0.037) and CpG 18 (p=0.009).

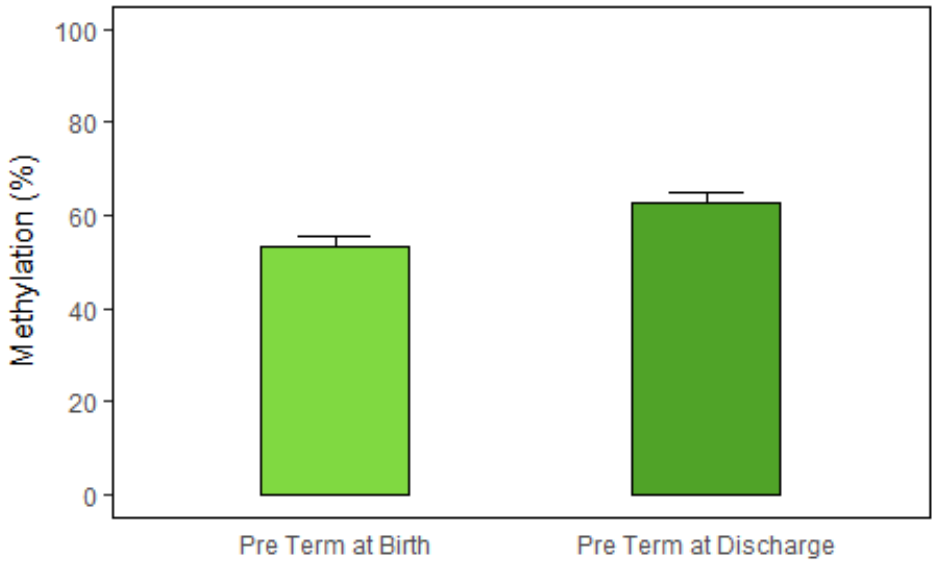

*Figure 1 – Total LINE-1 methylation status of the whole group of preterm infants at birth (cord blood) and at NICU discharge (peripheral blood)- * = p<0.05*

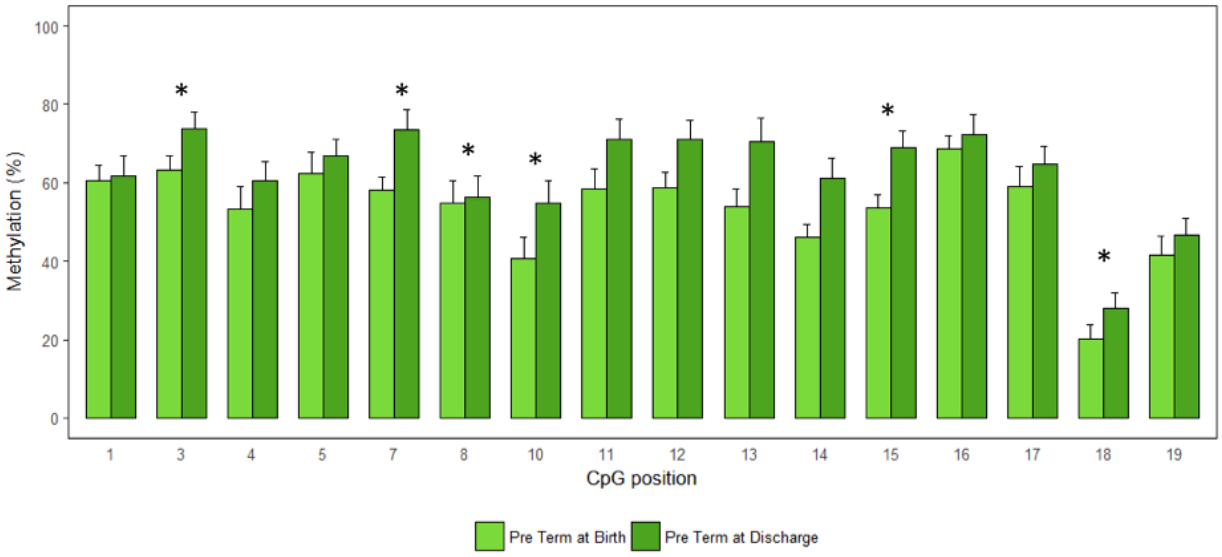

*Figure 2 – LINE-1 CpG methylation status of the whole group of preterm infants at birth (cord blood) and at NICU discharge (peripheral blood) - *= p<0.05*

Early Intervention versus Standard Care

Both EI and SC group showed an increase in LINE-1 methylation at term corrected age when compared to their methylation status at birth. However, a statistically significant increase in total methylation status (Figure 3) was observed only in the EI group (p=0.0077). Moreover, at CpG sites EI group (p<0.001) showed a trend towards a better recovery compared SC group (p=0.0037).

The two groups also differed in terms of CpG sites showing a recovery in methylation; specifically, in the EI group a statistically significant increase in methylation was observed at CpG 11 (p=0.0219), CpG 14 (p=0.016) and CpG 15 (p=0.009) while CpG 10 (p=0.032) and CpG13 (p=0.009) were more methylated in SC group. Figure 4 and 5 represent LINE-1 CpG sites methylation status for both EI and SC group respectively.

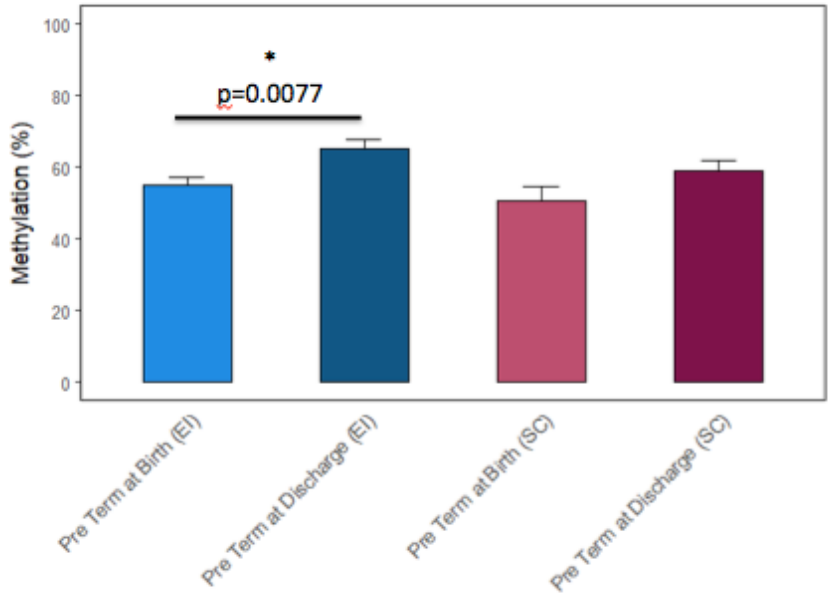

*Figure 3 – Total LINE-1 methylation status of EI and SC group both at birth (cord blood) and at NICU discharge (peripheral blood). * = p<0.05*

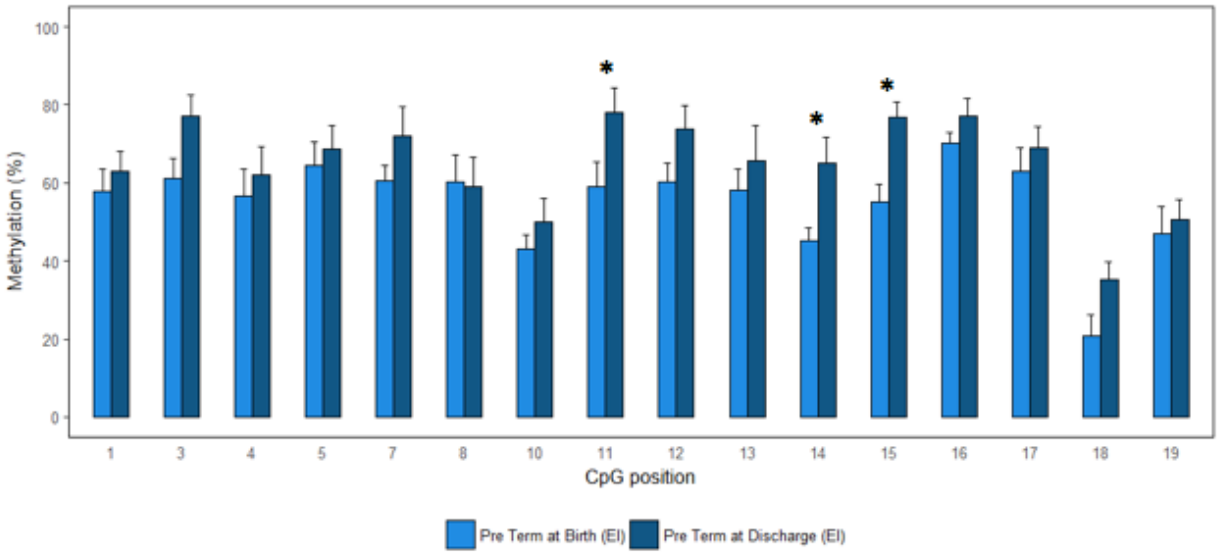

*Figure 4 – LINE-1 CpG methylation status of EI group at birth (cord blood) and at NICU discharge (peripheral blood). *= p<0.05*

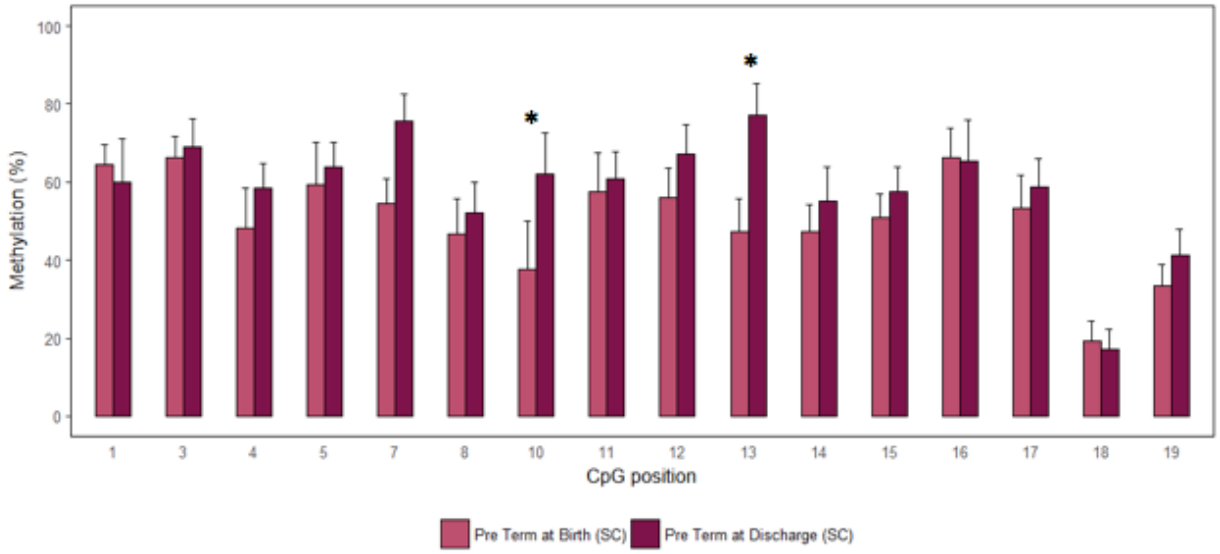

*Figure 5 – LINE-1 CpG methylation status of SC group at birth (cord blood) and at NICU discharge (peripheral blood). *= p<0.05*

Discussion:

This is, to our knowledge, one of the first studies focusing on the effects of preterm birth on LINE-1 methylation status and the very first to explore the effects of maternal care and multisensory stimulation on LINE-1 methylation.

These preliminary findings show that LINE-1 methylation in preterm infants increases from birth to term corrected age both as total level and at CpG sites,

There is no published evidence supporting these findings that need to be confirmed in a larger group. However, we speculate that several factors, related to the prematurely interrupted pregnancy and subsequent exposure to extrauterine life, may play a role in modulating LINE-1 methylation.

Firstly the gestational age at birth. Recent studies have described the effect of specific epigenetic processes in an individual's lifetime.[42] In particular, global levels of DNA methylation increase over the first few years of life while in late adulthood they start to decrease.[43]

We also need to consider the cause leading to preterm birth. It is known that preterm delivery may occur after: spontaneous labor with intact membranes, preterm premature rupture of the membranes and labor induction or caesarean delivery for maternal or fetal indications[44]; a key obstetric precursor of preterm birth is therefore represented by maternal or fetal infections leading to an inflammatory response.

Typically LINE-1 is heavily methylated, but in states of cellular stress, repetitive elements can be hypomethylated.[45] Therefore maternal inflammation could foster LINE-1 hypomethylation status observed in the cord blood of our preterm cohort.

To interpret our findings, in terms of their biological significance, we need to perform further analyses covering the whole spectrum of prematurity (from 36 weeks gestation backward) to try to disentangle the effects of detrimental factors (either fetal or maternal) leading to preterm birth from a potential physiological time-dependent increase in LINE-1 methylation according to a greater maturation. Moreover, we need to determine LINE-1 methylation status in healthy full-term infants born from uneventful pregnancies as representative of the physiological level LINE 1 methylation at birth.

The “recovery”, in terms of LINE-1 methylation, at term corrected age was observed both in the EI and in the SC group although the magnitude of changes was different in the two groups and

appeared to be more pronounced in the EI group, especially when looking at single CpG sites. These observations suggest a possible modulating effect of maternal care through reduction of stressful events which are known to affect DNA methylation in preterm infants[15]

The Early Intervention Program is based on the PremieStarts[29] which aims to promote mother-infant relationship through facilitation strategies that help parents recognize signs of alert and stress behavior. This program, together with its original version Mother Infant Transaction Program (MITP)[46], has been proven to encourage mother's responsiveness and to reduce stress and depressive symptoms [47,48] theoretically promoting the attainment of the maternal role, which is threatening in case of preterm birth.

An impaired mother-infant interaction (as induced by maternal separation) during the early postnatal period was shown to disrupt the neuroendocrine regulations, as described in several preclinical studies.[19,49] These biochemical modifications have been shown to be related to changes in gene expression and associated epigenetic alterations.[10] supporting the theory that maternal care has the potential to modulate epigenetic changes.

It is known that single CpG sites can affect specific genes expression: the EI group showed increased methylation at different CpG sites compared to SC group suggesting a specific effect of maternal care; however, the meaning and the relevance of our findings at CpG level need to be further explored.

This study has some limitations. First, the analyses were performed on different samples, cord blood at birth and peripheral blood at NICU discharge. However, umbilical cord blood has already been used in epigenetic studies and it has been suggested that cord blood cells resemble those from peripheral blood.[50]

Secondly, our study population was relatively small although highly homogeneous and selected in term of baseline characteristics as part of a RCT.

The main limitation of this study is represented by the tissue we used to assess LINE-1 methylation.

Most pre clinical studies supporting the role of LINE-1 methylation in inducing neurologic disorders have been performed on cerebral tissue[51] and in particular on cells from the hippocampus.[52] Moreover, Coufal et al demonstrated LINE-1 retrotransposition in the hippocampus and this finding was observed both in rat and human neural cells.[38] We postulated that epigenetic changes in blood cells could be representative, to a lesser extent, of changes in neuronal cells; however, this hypothesis need to be confirmed in animal studies by comparing LINE-1 methylation status in blood cells and neurons simultaneously.

This study, even if very preliminary, explores the LINE-1 methylation status in a crucial phase of brain development of preterm neonates. LINE-1 transposable elements in human and mouse genomes are capable of active transposition and insertion during neuronal differentiation.[38] Moreover alterations in these repetitive sequences have been recently described in patient with Rett syndrome, autisms and schizophrenia indicating that misregulation of LINE-1 methylation may have a possible contribution in these neurobehavioral disorders.[53,54] At the present time we cannot comment on the reason why LINE-1 methylation is lower at birth than at term corrected age. However, this difference could mirror a dysmethylation of LINE-1 and we speculate that, in turn, it could play a role in minor neurobehavioral disorders that preterm babies manifest later in childhood. Early postnatal life represents a sensitive phase for infant neuroplasticity, which through a continuous series of dynamic interactions between genetic influences, environmental conditions, and experiences, leads to changes in brain architecture. Recent studies suggested that in preterm infants NICU-related stress (quantified on the basis of skin-breaking procedure during hospitalization), might be associated with alterations of serotonergic tone as a consequence of SLC6A4 methylation, which in turn, might associate with temperamental difficulties assessed at 3 months of age.[15,55]

Moreover low levels of methylation usually correlate with an increased transcription; therefore, the methylation levels we observed in preterm infants at birth arise hypotheses about the possibility that the methylation status can mirror a deregulated transcription and retrotransposition of the LINE-1,

that can be eventually associated to the neurodevelopmental impairments frequently observed in preterm infants[56].

Neurodevelopmental disabilities often take a toll in early childhood and our current ability to predict poor motor, cognitive and neurobehavioral outcomes in the neonatal period are limited. Based on these considerations, identifying biomarkers which aid in the early prediction of later neurodevelopmental delay would be a significant step towards targeted, effective interventions implemented at a time-point where their effects are likely to be greatest.

DNA methylation represents one such potential biomarker that is gaining research momentum and LINE-1 methylation status appear to be a promising early marker of impaired neurodevelopment even though long-term follow-up studies are necessary to assess possible correlations with long-term outcomes.

Although far to be conclusive, this study gives new insights into the epigenetic mechanisms related to a premature birth and suggests that early intervention strategies during a window of both epigenetic and brain plasticity might modulate DNA methylation processes in preterm infants.

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Chapter 7

Effects of Early Intervention on the development of the Preterm Brain

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Abstract

Background: Preterm infants are exposed to the NICU-related stressful environment during a period of rapid brain maturation. In this context early interventions may play a role in positively modulating brain development.
Aim: To determine the effectiveness of an early intervention program on brain development in very preterm infants.
Methods: This study is part of a RCT. We included preterm infants born between 25^{+0} and 29^{+6} weeks of gestational age (GA) without severe morbidities and their families. Infants were randomized to either receiving standard care (SC) or early intervention (EI). EI included PremieStart and parental training to promote infant massage and visual attention according to a detailed protocol. SC, in line with NICU protocols, included Kangaroo Mother Care. MRI was performed at TEA. Automated segmentation was conducted on each neonatal Axial T2 2 mm scan, in conjunction with the T1 scan. Volumetric measures of the structures were extracted from each segmentation.
Results: Seventy preterm (EI n=34, SC n=36) infants were enrolled. Seven were excluded according to protocol. MRI scans of 51 infants (EI n=26, SC n=25) were evaluated for brain volumes analyses. Parent and infant characteristics were similar between the two groups. No differences were observed between the two groups in terms of regional brain volumes for the 48 areas analyzed.
Conclusions: EI does not seem to enhance brain development in "low-risk" very preterm infants. Further MRI analyses should focus only on microstructural development and maturation of targeted structures that may benefit from stress-reduction strategies and multisensory stimulation.

Background

Worldwide almost 2 million infants born before 32nd week of gestation.[1] Among them, 5-10% suffer from major neurologic disorders like cerebral palsy and 25-50% from minor neurocognitive impairments such as attention, visual processing, academic progress, and executive function.[2]

Increasing evidence suggests that features of brain structure and functions are different between preterm infant at term and their term counterparts, even in the absence of overt brain lesions.[3,4]

The third trimester of gestation, is a critical period for human brain growth and development [5]; in this time frame the brain undergoes several changes in molecular, neurochemical and structural parameters showing a rapid neuronal proliferation and cell differentiation including oligodendroglial maturation, differentiation of subplate neurons, formation of synapses, cerebellar neuronal proliferation and migration, and major axonal development in the cerebrum.[6]

Preterm infants at term equivalent age (TEA) display total and regional brain tissue alterations compared to healthy full-term infants and these differences are more pronounced in the presence of white matter injury.[7]

Advanced Magnetic Resonance Imaging (MRI) techniques allow quantitative analysis of the developing brain and provide new insights into the microstructural characteristics of the immature brain.[8] Volumetric MR techniques permit in vivo quantification of brain compartment volumes. Overall, a reduced total brain volume has been demonstrated in preterm infants at term compared to their full-term counterparts[9] and more specifically several studies have highlighted correlations between volumetric growth impairments of specific brain areas and the risk of less-than-optimal socio-emotional development of very preterm infants.[10] In particular, the anterior temporal lobe[11,12] seems to play a critical role in socio-emotional functioning and emotion regulation[13,14] as it contains structures like amygdala, extended amygdala and anterior hippocampus, which are well-known for their involvement in socio-emotional development: these areas have been found to be reduced in volume in very preterm infants.[15] Consistently, different studies suggest that abnormalities in volumes and white and gray matter microstructure detected by MRI at term equivalent age are most

likely related to the increased risk of neurodevelopmental, cognitive, attentional or visuo-perceptual difficulties that preterm children can present at preschool and school age[16].

Many preclinical trials have clearly demonstrated that the pre natal stress (PS) effects on the offspring's brain with a reduction of brain volumes. Specific brain regions have been shown to be affected by PS both macroscopically and microscopically such as hippocampus, amygdala, corpus callosum, anterior commissure, cerebral cortex, cerebellum and hypothalamus.[17]

Sophisticated MRI techniques include Diffusion Tensor Imaging (DTI) that has emerged as the method of choice for detecting and quantifying white and gray matter microstructure in health and illness.[18] DTI allows to measure brain tissues microstructure through the calculation of Fractional Anisotropy (FA) and Mean Diffusivity (MD). These parameters have been shown to be sensitive to the physiological and pathological changes in the tissue microstructure.

Advanced MRI techniques have been recently used to further investigate the pathogenetic factors underlying the microstructural and volumetric brain abnormalities and subsequent neurodevelopmental disorders related to the premature birth. In particular, the effects of the premature exposure to extrauterine life have been investigated [19–21] and several studies have highlighted the detrimental impact of environmental stressors, including painful but necessary medical procedures and a paucity of parental contact, on brain development.[22,23]

Recently Smith and colleagues[21] showed that greater exposure to stressful procedures (i.e. heel lance/venipuncture, intubation/extubation, diaper change) in the NICU was associated with reduced brain size in the frontal and parietal regions as estimated by the bifrontal and biparietal diameters in preterm neonates assessed at TEA.

Similarly, Grunau and colleagues found that greater exposure to neonatal procedural pain (adjusted for multiple neonatal clinical factors) was associated with reduced maturation of white matter and subcortical gray matter in a cohort of very preterm infants scanned early in life and again at TEA.[24]

Thus, these studies converge to reveal the importance of early stressful and painful procedural events on brain impairment.

In this context, stress reduction interventions and the parents' role in the NICU have been recently emphasized because of their central role in the promotion of early neurodevelopment[25].

Different types of early interventions have been proposed to reduce the stressor environment such as the Newborn Individualized Developmental Care and Assessment Program (NIDCAP)[26], that focuses primarily on bedside-nurse input, and the Mother Infant Transaction Program[27] and its modified version, PremieStart[28] that targets parents. These interventions have both shown to improve neurodevelopmental outcomes of preterm infants.[28,29]

Little is known about the effect of early intervention strategies on brain development as only two studies investigated this relationship with advanced MRI techniques. Als et. al documented, in preterm infants exposed to NIDCAP, a significantly better neurobehavioral functioning and a more mature brain microstructure measured with DTI techniques.[30] The PremieStart, as well, has been reported to be effective in reducing stressful experiences and increase white matter connectivity at DTI.[31]

However, the effects of an early multisensory intervention that includes early mother-infant interaction and multisensory stimulation on brain growth have not been investigated yet.

The present study is part of a RCT aimed to assess the effectiveness of an early intervention program in promoting visual function and neurodevelopment in preterm infants. Within this context further analyses have been performed with the exploratory purpose to investigate the effect of the early intervention in promoting brain development.

Objectives: To determine the effectiveness of an early intervention program on brain development in very preterm infants.

Methods

Study Population

We designed a randomized controlled trial (Trial Registration Number: NCT02983513). All

preterm infants, consecutively born between 25^{+0} and 29^{+6} weeks of gestational age (GA) from April 2014 to January 2017 at the NICU, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milano, were eligible for the study. The exclusion criteria were as follows: multiple pregnancy (triplets or higher); genetic syndromes and/or major congenital malformations; surgical Necrotizing Enterocolitis (NEC); major brain lesions, including Germinal Matrix Intraventricular Hemorrhage (GMH-IVH) > 2° grade according to Papile[32], documented by early cranial ultrasound (cUS). Also infants who developed cPVL detected at MRI were excluded from the present exploratory study due to the reported adverse effect on brain development of major brain lesions.

Mothers were selected according to the following criteria: age over 18 years, good comprehension of Italian, no single-parent families, no obvious cognitive impairment or psychiatric disorders, and no drug addiction.

Intervention

Infants were recruited after the first week of life and if they were clinically stable (i.e., no need for invasive mechanical ventilation and no active sepsis).

After obtaining parental written informed consent, infants were randomized to receive either Early Intervention (EI) or Standard Care (SC) using sealed envelopes that were prepared in groups of 10 through computer-generated randomization. The randomization sequence was concealed until the group allocation was assigned, and the examiners that evaluated MRI scans remained blinded for the entire study period.

The EI program was delivered in addition to routine care during the NICU stay by the same investigator (CF), according to the PremieStart Protocol[28], to train parents to: recognize signs of infant stress and alert-available behavior to promote mother-infant interaction; adopt principles of graded stimulation and avoid overwhelming infants through facilitation strategies. The program was held in eight main sessions and one additional post-discharge session.

Moreover, parents were trained to promote massage therapy and visual attention when their infants were in an alert behavioral state.[33] A diary was given to parents to register the interventions.

Massage therapy was performed twice per day by parents after they received two training sessions. It started not before the third week from birth and was performed until TEA. Each massage session consisted of 10 minutes of slow tactile stimulation of the back, giving moderate pressure stroking with both hands. During the massage, the infant was placed prone. Each session was performed at least 2 hours after the previous one.

Parents promoted visual attention at least once a day using either a black-and-white toy or the parent's face. This interaction occurred not before 34 weeks of GA and it was performed until TEA. Infants were in an alert behavioral state, supine, either on a parent's lap or in their crib, and nested with a blanket to avoid excessive stimulation.

SC, according to the NICU protocols, included Kangaroo Mother Care (KMC), nesting and minimal handling.

During the study period, no specific interventions (i.e. NIDCAP)[26] to decrease stress were used.

The baseline characteristics, collected from hospital charts, included: gender, birth weight and GA, Small for Gestational Age (SGA)[34], twin birth, mode of delivery, Apgar score at 1 and 5 minutes, Clinical Risk Index for babies (CRIB)[35], number of days on invasive mechanical ventilation or on nasal continuous positive airway pressure (NCPAP) or High Flow nasocannula, duration of hospital stay and GA at discharge.

The following neonatal morbidities were considered: ROP[36], NEC[37], Bronchopulmonary Displasia (BPD)[38], GMH-IVH[32] and sepsis (increased plasmatic levels of C reactive protein associated with a positive blood culture).

Family socioeconomic status (SES) was calculated and classified according to Hollingshead's criteria.[39]

Brain MRI

MRI was performed at TEA (40±3 weeks, as part of the NICU clinical protocol, on a 3T scanner (Acheiva, Philips Healthcare, Best, The Netherlands) using a pediatric-dedicated coil (Sense Ped, Philips Healthcare, Best, The Netherlands). Clinical MRI protocol was performed including: 3D-T1

weighted sequence, 2D T2-weighted turbo spin-echo sequence for coronal and axial planes. Infants were scanned while sleeping and were monitored by pulse oximetry and electro-cardiography (Invivo Process monitoring; Invivo, Orlando, FL) throughout the MRI scans. Neonatal noise attenuators (MiniMuffs, Natus Medical Inc., San Carlos, CA) were used. MRI scans were excluded from the analysis if more than one MRI sequences was affected by motion artifacts or if scans were performed after 40±3 weeks.

Brain MRI scans were visually assessed in order to detect the presence of brain minor abnormalities classified as Germinal Matrix - Intraventricular hemorrhage (GMH-IVH) and punctuate White Matter lesions.

Brain segmentation and volumetric analysis

Automated segmentation was conducted on each neonatal Axial T2 2 mm scan, in conjunction with the T1 scan. The two images were registered, in order to segment brain tissue and extract volume measures using a neonatal specific segmentation approach[40] based on the Expectation–Maximisation (EM) technique.[41] Volumetric measures of the structures of each neonate were extracted from each segmentation. All measures are defined in terms of ratio in respect with the total brain volume, excluding ventricles.

Statistical Analysis

Baseline characteristics were described as the mean and standard deviation (SD), the median and range, or the number and percentage, as appropriate. Demographic characteristics were compared across infants in the EI and SC groups using Fisher's exact test for categorical variables and Student's t-test or Mann-Whitney U-test for continuous variables. A p value < 0.05 was considered as significant.

For the analysis of the variables (volume), independent t-tests with FDR correction for multiple comparisons were used to compare EI and SC infants in each different areas. All tests were two-tailed and values of $p < 0.05$ were considered to be significant.

All data were analyzed with R software, version 3.4.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Overall, 70 infants (EI n = 34, SC n = 36) were recruited and randomized for intervention between April 2014 and January 2017. According to the protocol 3 infants allocated to EI did not receive treatment because: 2 developed surgical NEC and 1 family became a single-parent family after written informed consent was signed by both parents. All babies in the SC group received allocated treatment as part of routine clinical practice.

MRI at TEA was acquired for all the infants in the study as part of the NICU clinical protocol.

At TEA 4 infants (EI n=1; SC n=3) were excluded from brain volumes analyses as: 2 infant developed cPVL (1 for each group) and 2 infants belonging to SC group developed surgical NEC.

Moreover 12 infants were excluded from the analyses of brain growth as: 6 infants (EI=1; SC=5) performed MRI at after 40±3 weeks and scans for 6 infants (EI=3; SC=3) had several motion artefact. The characteristics of the excluded infants were similar to the analyzed group.

Visual inspection of MRI scans revealed the presence of mild abnormalities in ten infants: seven infants presented punctate white matter lesions (EI=3; SC= 4) and three infants (EI=1; SC=2) showed hemosiderin deposits in the occipital horns of the lateral ventricles as a sign of low grade IVH.

MRI scans of 51 infants (EI n=26, SC n=25) were evaluated for brain volumes analyses.

Parent and infant characteristics were similar in the two groups (Table 1).

Demographic feature	Early intervention (n=26)	Standard care (n=25)	Pvalue
Gestational age at birth (weeks), mean±SD	28.0 ± 1.4	27.7 ± 1.3	0.14 *
Birth Weight (g), mean±SD	975 ± 257	1048 ± 292	0.35 ^
Male, n(%)	12 (46%)	14 (56%)	0.58 °
Twins, n(%)	10 (38%)	13 (52%)	0.40 °
Monocorionic twins, n(%)	9 (90%)	5 (38%)	0.35 °
Laser therapy after TTTS, n(%)	3 (33%)	2 (40%)	1.00 °
CRIB II score, mean±SD	8.3 ± 2.4	8.5 ± 2.5	0.67 *
Apgar score at 1', median (range)	7 (4-9)	6 (2-8)	0.32 *
Apgar score at 5', median (range)	8 (7-9)	8 (5-9)	0.58 *
Cesarean Section, n(%)	24 (92%)	21 (84%)	0.42 °
Days of Mechanical Ventilation, mean±SD	4.9 ± 7.9	6.9 ± 10.5	0.33 *
Days of NCPAP, mean±SD	28.9 ± 15.0	25.0 ± 11.9	0.31 ^
Days of High Flow Nasocannula, mean±SD	14.3 ± 25.3	9.6 ± 16.3	0.86 *
Small for Gestational Age, n(%)	5 (19%)	4 (16%)	1.00 °
Sepsis, n(%)	13 (50%)	9 (36%)	0.40 °
Severe Bronchopulmonary Displasia, n(%)	9 (35%)	6 (24%)	0.54 °
GMH-IVH grade 1-2, n(%)	2 (8%)	3 (12%)	0.67 °
NEC, n(%)	0 (0%)	0 (0%)	1.00 °
ROP (1-2), n(%)	1 (4%)	3 (12%)	0.60 °
ROP (3-4), n(%)	3 (12%)	3 (12%)	
Days of Dexamethasone, mean±SD	1.7 ± 4.4	1.3 ± 4.0	0.73 *
Days of Hospitalization, mean±SD	79.6 ± 24.5	80.6 ± 31.5	0.84 *
Gestational Age at Discharge (weeks), mean±SD	39.4 ± 3.4	39.3 ± 3.5	0.86 *
Maternal Age (weeks), mean±SD	33.5 ± 4.1	34.9 ± 5.5	0.31 ^
SES, mean±SD	50.2 ± 9.2	45.1 ± 13.6	0.12 ^
Gestational Age at MRI (weeks), mean±SD	41.3 ± 1.3	41.5 ± 1.3	0.59*

Table 1: *Infants and maternal characteristics - ^ t-test,* Mann-Whitney U Test, ° Fisher Exact Test*

Volumetric analysis

Scans from 51 infants (EI n=26, SC n=25) were suitable for post-acquisition analysis. Figure 1 shows an example of brain MRI segmentation.

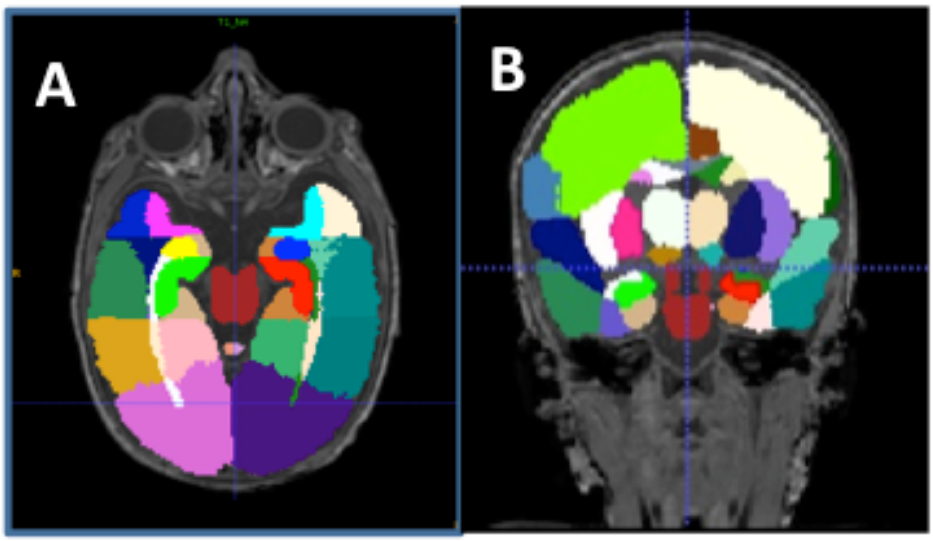

Figure 1 - Brain MRI segmentation: A. axial and B. coronal view T1-weighted images.

Comparison of regional brain volumes of the 48 segmented areas revealed no statistically significant differences between the EI and SC groups (Table 2; measures are expressed as ratio in respect with the total brain volume, excluding ventricle).

Areas	EI (Mean ± SD)	SC (Mean ± SD)	P values
Amygdala_left	0.0012 ± 0.0001	0.0012 ± 0.0001	0.982
Amygdala_right	0.0012 ± 0.0001	0.0012 ± 0.0001	0.982
Anterior_temporal_lobe__lateral_part_left	0.0047 ± 0.0005	0.0049 ± 0.0004	0.706
Anterior_temporal_lobe__lateral_part_right	0.0046 ± 0.0005	0.0049 ± 0.0006	0.571
Anterior_temporal_lobe__medial_part_left	0.0045 ± 0.0006	0.0045 ± 0.0004	0.998
Anterior_temporal_lobe__medial_part_right	0.0041 ± 0.0006	0.0043 ± 0.0005	0.812
Brainstem__spans_the_midline	0.0161 ± 0.0010	0.0162 ± 0.0011	0.982
Caudate_nucleus_left	0.0047 ± 0.0003	0.0046 ± 0.0003	0.706
Caudate_nucleus_right	0.0049 ± 0.0003	0.0047 ± 0.0003	0.706
Cerebellum_left	0.0349 ± 0.0023	0.0346 ± 0.0029	0.982
Cerebellum_right	0.0349 ± 0.0023	0.0347 ± 0.0028	0.982
Cingulate_gyrus__anterior_part_left	0.0088 ± 0.0008	0.0088 ± 0.0008	0.998
Cingulate_gyrus__anterior_part_right	0.0082 ± 0.0008	0.0080 ± 0.0008	0.982
Cingulate_gyrus__posterior_part_left	0.0080 ± 0.0005	0.0082 ± 0.0006	0.982
Cingulate_gyrus__posterior_part_right	0.0076 ± 0.0006	0.0076 ± 0.0007	0.998
Corpus_Callosum	0.0067 ± 0.0006	0.0067 ± 0.0006	0.993
Frontal_lobe_left	0.1569 ± 0.0042	0.1556 ± 0.0039	0.982
Frontal_lobe_right	0.1518 ± 0.0035	0.1515 ± 0.0037	0.982
Gyri_parahippocampalis_et_ambiens_anterior_part_left	0.0043 ± 0.0003	0.0043 ± 0.0003	0.982
Gyri_parahippocampalis_et_ambiens_anterior_part_right	0.0044 ± 0.0003	0.0044 ± 0.0003	0.982

Gyri_parahippocampalis_et_ambiens_posterior_part_left	0.0037 ± 0.0004	0.0036 ± 0.0003	0.982
Gyri_parahippocampalis_et_ambiens_posterior_part_right	0.0034 ± 0.0004	0.0035 ± 0.0004	0.982
Hippocampus_left	0.0018 ± 0.0002	0.0020 ± 0.0002	0.537
Hippocampus_right	0.0016 ± 0.0002	0.0017 ± 0.0002	0.706
Insula_left	0.0129 ± 0.0008	0.0131 ± 0.0009	0.982
Insula_right	0.0125 ± 0.0008	0.0124 ± 0.0008	0.982
Lateral_occipitotemporal_gyrus__gyrus_fusiformis_anterior_part_left	0.0043 ± 0.0003	0.0043 ± 0.0003	0.982
Lateral_occipitotemporal_gyrus__gyrus_fusiformis_anterior_part_right	0.0041 ± 0.0004	0.0042 ± 0.0004	0.706
Lateral_occipitotemporal_gyrus__gyrus_fusiformis_posterior_part_left	0.0052 ± 0.0006	0.0051 ± 0.0006	0.982
Lateral_occipitotemporal_gyrus__gyrus_fusiformis_posterior_part_right	0.0053 ± 0.0005	0.0051 ± 0.0006	0.982
Lentiform_Nucleus_left	0.0079 ± 0.0004	0.0078 ± 0.0006	0.982
Lentiform_Nucleus_right	0.0080 ± 0.0005	0.0080 ± 0.0006	0.982
Medial_and_inferior_temporal_gyri_anterior_part_left	0.0145 ± 0.0007	0.0145 ± 0.0009	0.982
Medial_and_inferior_temporal_gyri_anterior_part_right	0.0136 ± 0.0010	0.0142 ± 0.0008	0.537
Medial_and_inferior_temporal_gyri_posterior_part_left	0.0207 ± 0.0012	0.0208 ± 0.0012	0.982
Medial_and_inferior_temporal_gyri_posterior_part_right	0.0220 ± 0.0013	0.0213 ± 0.0018	0.706
Occipital_lobe_left	0.0568 ± 0.0040	0.0570 ± 0.0033	0.988
Occipital_lobe_right	0.0583 ± 0.0050	0.0591 ± 0.0034	0.982
Parietal_lobe_left	0.1033 ± 0.0037	0.1030 ± 0.0031	0.982
Parietal_lobe_right	0.1017 ± 0.0030	0.1024 ± 0.0036	0.982
Subthalamic_nucleus_left	0.0006 ± 0.0000	0.0006 ± 0.0000	0.982
Subthalamic_nucleus_right	0.0006 ± 0.0000	0.0006 ± 0.0000	0.998
Superior_temporal_gyrus__middle_part_left	0.0143 ± 0.0009	0.0146 ± 0.0012	0.982
Superior_temporal_gyrus__middle_part_right	0.0140 ± 0.0009	0.0140 ± 0.0011	0.982
Superior_temporal_gyrus__posterior_part_left	0.0073 ± 0.0008	0.0072 ± 0.0006	0.998
Superior_temporal_gyrus__posterior_part_right	0.0061 ± 0.0006	0.0063 ± 0.0007	0.982
Thalamus_left	0.0116 ± 0.0005	0.0114 ± 0.0008	0.982
Thalamus_right	0.0116 ± 0.0005	0.0114 ± 0.0009	0.982

Table 2 – Regional brain volume ratio (adjusted for absolute brain volume) in EI and SC group: values are shown as mean (SD) and p values for each single area

Discussion

This is one of the few studies investigating the effect of an Early Intervention Program on brain development.

Although of a great interest, our exploratory study didn't show any effect of EI on regional brain

growth.

Several studies demonstrated that Early Intervention strategies have a positive effect on neurodevelopment,[28,29] on the other hand only two clinical studies used advanced MRI techniques to evaluate the effectiveness of these programs on brain development.

Consistently with our finding, Milgrom et al. didn't show any difference in brain volumes between intervention (PremieStart) and control groups.[31] Milgrom et al also investigated differences in the microstructural maturation assessed with diffusion MRI technique and found lower MD and higher FA values in white matter in the intervention group suggesting a more mature white matter microstructure [31]

Als et al. performed only microstructural analysis.[30] Similarly to Milgrom et al, they showed the effectiveness of the NIDCAP program on brain structure using DTI techniques. The beneficial effect was demonstrated by the higher relative anisotropy in left internal capsule, right internal capsule and frontal white matter, considered the locus of attention regulation and executive function, in the treated infants.[30]

Our study differs from the previous clinical ones as regard to the type of intervention and the study population. Als et al. used the NIDCAP program, which is based on a more extensive modification of the NICU care to adapt it to the individual needs of each preterm infant.[26] This intervention have some similarities with the PremieStart (used in Milgrom's study), as both of them aim to reduce stress, but in contrast to the NIDCAP in the PremieStart program the mother herself is first trained to facilitate intervention.[28] We developed a new intervention program based on parental involvement, through the PremieStart, combined with a multisensory stimulation (both tactile – through infant massage - and visual stimulation) that has never been tested before.

In contrast with the previous studies, we included only preterm infants with normal or mildly abnormal sequential cUS as confirmed by the low rate of brain abnormalities detected at MRI. We excluded babies with extensive brain lesions, at high risk for motor impairment, as we aimed to assess the effectiveness of EI on "low-risk" very preterm infants who are more likely to develop

minor neurodevelopmental deficits and may benefit the most from early intervention. Indeed, extensive brain lesions (such as IVH grade 4 with parenchymal involvement) may impact brain growth and maturation in particular when the white matter is directly affected, and it would be challenging to assess the role of early intervention strategies in enhancing brain development.[7]

Our study has several limitations. First, the number of infants with MRI scans suitable for post-acquisition analysis was quite small (n= 51), although comparable with previously published studies. One of the criteria for exclusion was the low quality of scans affected by motion artefacts. We decided to perform all the MRI scans in natural sleep after feeding and swaddling the infant: this method has the advantage of avoiding sedation but, on the other hand, it's particularly challenging with preterm infants.[42]

Secondly, we have only evaluated, so far, the potential effect of EI on brain growth as assessed by volumetric measurements. We planned to perform further MRI analyses focused on microstructural development and maturation of targeted structures that may benefit from stress-reduction strategies and multisensory stimulation, as cerebral structures involved in visual function.

Beneficial effects of multisensory stimulation have been previously demonstrated in terms of cerebral activity. In the study by Guzzetta et al the preterm massage has been related to increased maturation of cerebral electrical activity measured with EEG at the 4 weeks of age.[43]

Moreover many pre clinical studies showed that the exposure to an enriched environment elicited neuroanatomical and behavioral changes, such as enhanced dendritic arborization, gliogenesis, neurogenesis, and improved learning appreciable at the behavioral, electrophysiological, and molecular level.[44]

Based on our previous findings that demonstrated an accelerated maturation of visual function in EI infants (data under submission), further research will be focused on assessing development of white matter in the optic radiations[45] at a microstructural level using DTI technique. Rationales for this further evaluation rely on the assumption that FA in the optic radiations at term equivalent age is associated with visual function[45]

Secondly, further MRI measurements should be targeted to cerebral areas involved in stress response, in particular the temporal and frontal lobes[23,24] which regulate attention and executive functions, all cognitive domains that have been proven to benefit from early intervention in several clinical and preclinical studies.

Furthermore our previous findings also showed that infants in the EI group were four times more likely to be fed exclusively with human milk (data under submission). Given the key beneficial effect of human milk feeding, both on short and long term outcomes, further analyses are needed to disentangle the potential effect of human milk on brain growth.[46,47]

Our study, although not conclusive, provide a platform for wider analyses to identify a MRI biomarker for cerebral modification shaped by the environment in preterm infants.

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Chapter 8 – Conclusions

In recent years, the benefits of Environmental Enrichment on synaptic plasticity, visual development and cognitive processes have been investigated in both preclinical and clinical studies. Two aspects of Enriched Environment are reported to be key in planning Early Intervention (EI) strategies in preterm infants: parental involvement and multisensory stimulation.

The present work provides further insights in the field of EI. Combining the two components, our EI strategy showed an overall beneficial effect for preterm infants.

We demonstrated that the EI enhances the neurodevelopmental functions assessed at term equivalent age, in terms of both visual abilities (one of the first testable cognitive functions) and the acquisition of full oral feeding pattern (an important emerging ability in preterms).

Then, our study also highlighted the role of early approaches directed towards mother-infant closeness and dyadic relationship in promoting breast milk feeding, which is a fundamental nutritional support for preterm infants.

Finally, we explored the epigenetic effects by assessing LINE-1 methylation status, a novel biomarker that is gaining research momentum for its relevance in human genome and its susceptibility to environmental factors. Our findings suggest that EI strategies, performed during a window of both epigenetic and brain plasticity, might modulate DNA methylation processes in preterm infants with potential implications on long-term outcomes.

Although these results are very promising, at the present time, we failed in identifying a neuroimaging correlate, at advanced brain MRI, of the demonstrated improved neurodevelopmental functions

This study, despite far to be conclusive, concur with recent evidence that the quality of early experiences influences neurodevelopment in preterm infants.

Importantly, key clinical implications emerge from these results: EI strategies, focused on parental role combined with a multisensory approach, should be implemented in the care of very preterm

infants in addition to Standard Care during NICU stay.

Future research directions should focus on long term follow-up to confirm the positive effect of EI on child neurodevelopment and its correlation with epigenetic changes. Further efforts should be addressed to the study of brain microsturctural features related to neurodevelopmental functions, using different advanced MRI techinques.

Multicenter studies should be planned to strengthen the generalizability of these findings and to better understand the mechanisms of EI and their preventive role in preterm infants neurodevelopment.

Appendix 1

Neurodevelopmental outcome of Extremely Low Birth Weight infants at 24 months corrected age: a comparison between Griffiths and Bayley Scales

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Neurodevelopmental outcome of extremely low birth weight infants at 24 months corrected age: a comparison between Griffiths and Bayley Scales

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Abstract

Background: The availability of accurate assessment tools for the early detection of infants at risk for adverse neurodevelopmental outcomes is a major issue. The purpose of this study is to compare the outcomes of the Bayley Scales (Bayley-II vs Bayley-III) in a cohort of extremely low birth weight infants at 24 months corrected age, to define which edition shows the highest agreement with the Griffiths Mental Development Scales Revised.

Methods: We performed a single-centre cohort study. We prospectively enrolled infants with a birth weight of 401–1000 g and/or gestational age < 28 weeks. Exclusion criteria were the presence of neurosensory disabilities and/or genetic abnormalities. Infants underwent neurodevelopmental evaluation at 24 months corrected age using the Griffiths and either the Bayley-II (birth years 2003–2006) or the Bayley-III (birth years 2007–2010).

Results: A total of 194 infants were enrolled. Concordance was excellent between the Griffiths and the Bayley-III composite scores for both cognitive language and motor abilities (weighted $K = 0.80$ and 0.81, respectively) but poorer for the Bayley-II (weighted $K = 0.63$ and 0.50, respectively). The Youden's Index revealed higher values for the Bayley-III than for the Bayley-II (75.9 vs 69.6 %). Compared with the Griffiths, the Bayley-III found 3 % fewer infants as being severely impaired in cognitive-language abilities and 7.8 % fewer infants as being mildly impaired in motor skills while the Bayley-II showed, compared with the Griffiths, higher rates of severely impaired children both for cognitive-language and motor abilities (14.1 and 15.3 % more infants respectively).

Discussion: Our study suggests that the Bayley-III, although having a higher agreement with the Griffiths compared to the Bayley-II, slightly tends to underestimate neurodevelopmental impairment compared with the Griffiths, whereas the Bayley-II tends to overestimate it.

Conclusions: On the basis of these findings, we recommend the use of multiple measures to assess neurodevelopmental outcomes of extremely low birth weight infants at 24 months.

Keywords: Bayley-II, Bayley-III, Griffiths, Developmental assessment, Extremely low birth weight infants

Background

Survival of extremely low birth weight (ELBW) infants has dramatically increased in recent decades because of advances in perinatal and neonatal care [1, 2]. However, rates of disability, especially at the lowest gestational ages, remain high [3]. As a consequence, the availability of accurate developmental assessments for the early detection of infants at high risk of adverse neurodevelopmental outcomes has become a major issue. Indeed, early confirmation of developmental impairment is important so that early referral for intervention can be made to maximise children's abilities and to assist in their transition to school.

The Bayley Scales are widely applied to identify infants with or at risk for developmental impairment, both in clinical and research settings [4, 5]. The first two editions of the scales [6, 7] yielded only a Mental Development Index (MDI) and a Psychomotor Development

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Index (PDI). The revised structure of the Bayley-III [8], which includes distinct composite scores (Cognitive, Language and Motor), allows a more precise assessment of specific developmental domains. Nevertheless, clinicians have consistently found that Bayley-III composite scores are up to 10 points higher than those of Bayley-II [9, 10]. Thus, concerns have arisen that the Bayley-III may underestimate developmental impairment in clinical groups [11], reducing the number of children eligible for early intervention programmes.

Up to now, few studies have addressed the agreement between the Bayley Scales outcomes and other valid and reliable standardized developmental instruments on the same study group.

The Griffiths Mental Development Scales [12] are a widely used developmental assessment procedure, showing continuing validity over time and across cultures [13–15]. They were first published in 1970 and underwent a re-standardization in 1996 for the 0–2 years version [12, 16].

The Griffiths General Quotient at 2 and 3 years of age has been found to strongly correlate with intellectual ability at 5 years on the Stanford Binet [17] and moderately with the Wechsler Preschool and Primary Scale for Intelligence-Revised (WPPSI-R) [18]. McMichael [19] assessed low-birthweight infants at 1 and 3 years on the Griffiths and at 24 months on the Bayley-III, and found that the Bayley-III composite scores were almost a standard deviation higher than those on the Griffiths at both 12 and 36 months.

The aim of this study was to evaluate the developmental outcomes of a cohort of extremely low birth weight infants assessed at 24 months corrected age using both the Bayley Scales II and III and the Griffiths, so as to define which edition of the Bayley Scales better agrees with the Griffiths. The null hypothesis to be tested was that the agreement between the Griffiths and the Bayley-III would not be higher than the agreement between the Griffiths and the Bayley-II.

Methods

Study design and participants

We performed a single-centre longitudinal cohort study. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and written informed consent was obtained from all parents.

Inclusion criteria were having a birth weight between 401 and 1000 g at birth (ELBW) and/or being born between 22 and 27^{+6} weeks gestation (extremely low gestational age newborns: ELGAN). Exclusion criteria were the presence of neurosensory disabilities (blindness, deafness) and/or genetic abnormalities.

The flow chart of the study is shown in Fig. 1. Of all the 376 consecutive infants admitted to NICU Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico between 2003 and 2010, 276 (73 %) were discharged home alive. Of these, 222 (80 %) returned for the 24 months corrected age follow-up visit and 194 (70 %) infants entered the study.

All infants participating in the study were registered in the Vermont Oxford Network [20] and were scheduled to be prospectively followed up to 24 months corrected age.

The infants were divided into two groups according to the study period: Group 1 ($N = 92$) infants born between 2003 and 2006, and Group 2 ($N = 102$) infants born between 2007 and 2010.

Basic subjects' characteristics (sex, birth weight, being adequate or small for gestational age, mode of delivery, multiple birth, duration of hospital stay, number of days on mechanical ventilation) were recorded. Gestational age was based on the last menstrual period and early ultrasound examination; infants with birth weight ≥ 10th percentile or < 10th percentile for gestational age, according to the Fenton Growth Chart [21], were classified respectively as adequate or small for gestational age (AGA/SGA). The occurrence of sepsis, necrotizing enterocolitis (NEC) of stage 2 or higher (according to the classification of Bell et al. [22]), intraventricular haemorrhage (IVH) grade 3 or higher, periventricular leukomalacia

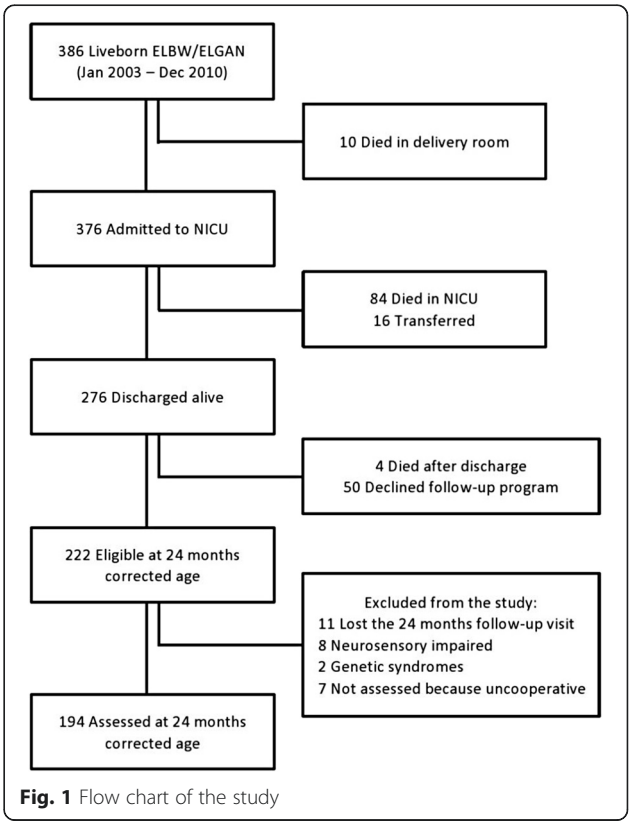

Fig. 1 Flow chart of the study

(PVL) of grade 2 or higher, retinopathy of prematurity (ROP) of stage 3 or higher and bronchopulmonary dysplasia (BPD) were also collected prospectively. Sepsis was defined by the presence of positive blood and/or cerebrospinal fluid culture. IVH and PVL were detected by brain magnetic resonance imaging examination at 40 weeks postmenstrual age. BPD was defined as treatment with supplemental oxygen at 36 weeks gestation. Corrected age was calculated up to 24 months of life, from the chronological age adjusting for gestational age. Mothers' nationality and education were also recorded. Mothers' educational level was used as a measure of socioeconomic status and classified using a 3-point scale, where 1 indicates primary or intermediate school education (≤8 years), 2 indicates secondary school education (9–13 years) and 3 indicates a university degree (>13 years).

Instruments

Bayley scales

The Bayley Scales of Infant Development, 2nd Edition [7] yields two single age-standardized composite scores (range 50–150): a Mental Development Index (MDI), which measures cognition through sensory perception, knowledge, memory, problem solving and early language abilities, and a Psychomotor Development Index (PDI), which assesses fine and gross motor skills.

The third revision of the scales (Bayley Scales of Infant and Toddler Development, 3rd Edition) [8] produces three composite scores: the Cognitive scale (range 55–145), which assesses sensorimotor development, exploration and manipulation, object relatedness, concept formation, memory and simple problem solving; the Language scale (range 45–155), which consists of Receptive Communication (verbal comprehension, vocabulary) and Expressive Communication (babbling, gesturing and utterances) subtests; and the Motor scale (range 45–155), which consists of Fine Motor (grasping, perceptual-motor integration, motor planning and speed) and Gross Motor (sitting, standing, locomotion and balance) subtests.

Both editions of the Bayley Scales have index mean scores of 100 (SD ± 15). In the present study, an index composite score of < 70 (>2 SD below the mean) is defined to indicate severe impairment, while an index composite score of 70–84 (>1 SD below the mean) is defined to indicate mild impairment. Index composite scores ≥ 85 are defined here to indicate normal development.

Because neither the Bayley-II nor the Bayley-III has been normed in Italy, the USA norms of the scales were used in this study [7, 8]. The Bayley-II administration manual was translated into Italian through the back-translation method. Before starting the study, the Italian version of the Bayley-II administration manual was tested with a group of infants to clarify any doubts on item comprehension. For the Bayley-III, the Italian validated translation of the administration manual was used [23].

Griffiths mental development scales revised

The Griffiths Mental Development Scales Revised (Griffiths) assess the development of infants from birth to 24 months [16]. They comprise five subscales (range 50–150): Locomotor, Personal-Social, Hearing and Speech, Eye and Hand Coordination and Performance. The subscales yield standardized scores for each domain (mean 100, SD 16) and a composite General Quotient (mean 100, SD 12).

For each subscale, a standardized score < 68 (>2 SD below the mean) indicates severe impairment, and a standardized score 68–83 (>1 SD below the mean) indicates mild impairment. Finally, a standardized score ≥ 84 indicates normal development.

As for the General Quotient, severe impairment is defined in the present study to be indicated by a standardized score < 76 (>2 SD below the mean), while mild impairment is categorised here with a standardized score 76–87 (>1 SD below the mean). A standardized score ≥ 88 is defined to indicate normal development.

Because normative data of the Griffiths Mental Development Scales Revised are not available in our country, we referred to the 1996 UK norms. The Manual of the Griffiths Mental Development Scales Revised was translated into Italian through the back-translation method. Before starting the study, the Italian version of the Griffiths Mental Development Scales Revised Manual was tested with a group of infants to clarify any doubts on item comprehension. Since 2007, the Italian-validated translation of the administration manual has been used [24].

Procedure

Infants underwent evaluation of the neurodevelopmental outcome at 24 months corrected age. Each infant was assessed by two trained and licensed examiners (one administering the Griffiths and the other the Bayley Scales in different sessions on the same day), both blind to the child's performance on the other test. Infants born between 2003 and 2006 (Group 1) were assessed using Griffiths and Bayley-II, while infants born between 2007 and 2010 (Group 2) were assessed with Griffiths and Bayley-III. Infants were randomly first administered either the Griffiths or the Bayley Scales to avoid a possible test order effect. A short break of 30 min was planned between the two tests to allow the infant to rest and adjust for fatigue. Except for the edition of the Bayley Scales administered, the two groups underwent the same follow-up assessment procedures.

According to Vohr [10], children who could not be assessed because they were too severely impaired ($n = 4$

Quadriplegic Cerebral Palsy) were assigned scores as follows: 49 in the Bayley-II MDI and PDI, 54 in the Bayley-III Cognitive scale, 44 in the Bayley-III Language and Motor scales and 49 in the Griffiths GQ and subquotients.

Statistical analyses

The homogeneity between the two groups of infants has been verified using a confidence interval of 95 % for the differences between the investigated variables expressed as mean or percentage. To evaluate if any infant (sex, gestational age, birth weight below the 10th percentile, being a twin, having siblings, oxygen dependency at 36 weeks postmenstrual age, magnetic resonance imaging, ROP, need for mechanical ventilation) and/or maternal variable (education, age and nationality) were associated with belonging or not to one of the two study groups, a multivariate logistic regression model was performed.

A first comparison between the results obtained at 24 months corrected age by the Bayley and the Griffiths scales was done by comparing the mean values and the 95 % confidence intervals. The obtained scores were then classified as mildly impaired (Bayley Composite Scores or Griffiths Quotients > 1 SD below the mean) or severely impaired (Bayley Composite Scores or Griffiths Quotients > 2 SD below the mean), in accordance with other authors [4, 10, 25]. Concordance between the results given by the different scales was measured using weighted K Cohen and considered poor, fair, good or excellent with Cohen's kappa 0–0.4, 0.4–0.6, 0.6–0.8, > 0.8, respectively [26]. Taking the results obtained at 24 months corrected age with the Griffiths as the gold standard, steps were taken to calculate the sensitivity, specificity and Youden's index for the two Bayley editions. The Youden's Index (sensitivity + specificity-1), with values between 0 and 1, measures the maximum potential effectiveness of a screening test.

As noted before, Bayley-II MDI includes both cognitive and language abilities, while both the Bayley-III and the Griffiths Scales yield separate scores (Cognitive and Language vs Hearing and Speech and Performance respectively). The same issue was raised for fine and gross motor abilities, measured together by the Bayley-II PDI and Bayley-III Motor Scale and separately by the Griffiths Scales (Locomotor and Eye and Hand Coordination Scales). Therefore, to compare the Bayley and Griffiths results, subscales that measured the same dimensions, as inferred by the manuals, were grouped together [Fig. 2] as follows, to have homogeneous and comparable domains:

- Griffiths Hearing and Speech-Performance Quotients (mean) vs Bayley-II MDI and vs Bayley-III Cognitive-Language Composite Scores (mean)
- Griffiths Locomotor-Eye and Hand Coordination Quotients (mean) vs Bayley-II PDI and vs Bayley-III Motor Composite Score

Results

Maternal and infants' basic characteristics are shown in Table 1.

The mean age at testing was 23.0 months (SD 1.7 months; range 22 months and 16 days-24 months and 15 days) of corrected age. Although 19.6 % of mothers in both groups were not Italian, all infants attended a kindergarten or a preschool education programme and so were exposed to Italian as a primary language in their community environment.

As shown in Table 1, there were no significant differences between the two groups for each of the variables considered, with the exception of a much higher percentage of multiple pregnancies in the second group.

The logistic regression model showed that the two study groups were homogenous with regard to maternal and infants' characteristics (likelihood ratio 21:36, df = 16, $p = 0.1650$ and rsquare rescaled = 0.1560).

Table 2 shows the means (95 % CI) of the Griffiths Hearing and Speech-Performance vs Bayley-II MDI or vs Bayley-III Cognitive-Language and the Griffiths Locomotor-Eye and Hand Coordination (mean) vs Bayley-II PDI or vs Bayley-III Motor composite scores.

The Bayley-II MDI composite score was 6.6 points lower than the Griffiths Hearing and Speech-Performance combined score, whereas the Bayley-III Cognitive-Language combined score was almost equal to it.

For the Griffiths Locomotor-Eye and Hand Coordination combined score, the discrepancy with the Bayley-II PDI composite score was even larger (7.9 points lower), whereas the Bayley-III Motor composite score was only 1.2 points higher. Table 3 reports the concordance between Griffiths and Bayley II/Bayley III.

Griffiths and Bayley-III composite scores for both cognitive-language and motor abilities showed an excellent concordance. On the contrary, concordance between Griffiths and Bayley-II was lower, especially with regard to motor skills. Table 4 outlines the ranges of developmental impairment. Compared with the Griffiths, the Bayley-II showed consistently higher rates of severe impairment both in cognitive and language abilities (14.1 % more infants) and in motor skills (15.3 % more infants). There was a higher agreement between the Bayley-III and the Griffiths rates with regard to mild and severe impairment in all domains, except for motor mild impairment, which appeared to occur in a slightly lower percentage of infants when the Bayley-III was used (7.8 % fewer infants). The comparison between single subscales revealed that the Bayley-III Cognitive Index

Bayley-II vs Bayley-III vs Griffiths divided in Cognitive-Language and Motor abilities			
	Bayley-II	**Bayley-III**	**Griffiths**
Cognitive-Language abilities	***Mental Development Index*** Includes items that assess habituation of attention, problem solving, reasoning and classification. This scale also assesses both receptive and expressive language, such as reaction to sounds, responding to spoken requests, pointing to pictures, vocalizations, babbling and vocabulary development.	***Cognitive Scale*** Includes items that assess sensorimotor development, exploration and manipulation and object relatedness, for example placing the pieces correctly in a form board, putting blocks inside a cup or finding hidden objects.	***Performance Scale*** Assesses a set of performance tests, drawing on the developing ability to reason in practical situations or manipulate materials intelligently, for example placing insets in the correct hole of a form board, completing a set of boxes containing bricks or finding hidden objects. Visual spatial skills including speed and precision of working are assessed.
		Language Scale *Receptive communication* Assesses preverbal behaviours and vocabulary development, such as being able to identify objects and pictures that are referenced. *Expressive communication* Measures preverbal communication, such as babbling and gesturing, and vocabulary development, such as naming objects and pictures.	***Hearing and Speech Scale*** Evaluates hearing, in the sense of active listening, together with the child's progress in acquiring first a vocabulary of sounds, vocalisation and bubble or pre-speech, that are finally superseded by adult language.
Motor abilities	***Psychomotor Development Index*** Assesses control of the gross and fine muscle groups. This includes fine motor manipulations involved in prehension, adaptive use of writing implements and imitation of hand movements. This scale also tests movements associated with rolling, crawling and creeping, sitting, standing and walking.	***Motor Scale*** *Fine motor* Measures fine motor skills, associated with prehension, object manipulation and perceptual-motor integration. *Gross motor* Measures gross motor abilities related to static positioning, dynamic movement and motor planning.	***Locomotor Scale*** Measures in some detail all the series of developing skills that result in the achieving of the upright posture by the child and lead on to learning to walk, run, climb and so on.
			Hand and Eye Coordination Scale Assesses the child's level at manipulation, including a study of the development of the hand itself as well as certain manipulative activities. It focuses on fine motor skills, manual dexterity and visual monitoring skills.

Fig. 2 Bayley-II vs Bayley-III vs Griffiths divided into Cognitive language and motor abilities. Manual definitions of Bayley and Griffiths Subscales, grouped in comparable domains: Cognitive language and motor abilities

detected 7.9 % fewer infants as being mildly impaired and 4.9 % fewer infants as being severely impaired compared with the Griffiths Performance subscale. The Bayley-III Language Index showed mild impairment in a higher percentage of cases (4.9 % more infants) and severe impairment in a lower percentage of cases (4.9 % fewer infants) compared with the Griffiths Hearing and Speech subscale.

Finally, considering motor skills, the Bayley-III Motor Index highly agreed with the Griffiths Eye and Hand Coordination subscale but identified 9.8 % fewer infants as being severely impaired compared with the Griffiths Locomotor subscale.

As noted in Table 5, in comparison to the Griffiths Scales, the sensitivity of the Bayley-II was greater than that of the Bayley-III, especially for cognitive-language abilities. On the contrary, Bayley-III appeared to have an increased specificity compared with its previous edition. However, the Youden's Index (combining sensitivity and specificity) reveals much higher values for the Bayley-III

Table 1 Maternal and infant characteristics

Characteristics	Group 1 (*n* = 92)	Group 2 (*n* = 102)	C.I. 95 % of differences
Maternal			
Age, years (mean)	34.2	34.4	−1.22–1.65
University degree, %	23.9	33.3	−4.2–23.1
Non-Italian nationality %	19.6	19.6	−12.2–12.2
Infant			
Birth weight, g, (mean)	796.0	813.3	−18.2–49.4
GA, weeks, (mean)	27.7	27.2	−0.1–1.1
Males, %	43.5	44.1	−14.4–15.6
SGA, %	50.0	38.2	−3.1–2.74
Multiple birth, %	18.5	38.2	6.3–33.16
Cesarean delivery, %	92.4	92.2	−8.3–8.7
Sepsis, %	37.0	27.5	−4.7–23.7
NEC stage 2–3, %	2.2	4.9	−3.5–8.9
IVH grade 3–4, %	2.2	5.9	−2.8–10.2
PVL, %	1.1	2.0	−3.6–5.4
BPD, %	43.4	35.3	−6.6–22.9
ROP grade 3–4, %	16.3	14.7	−9.6–12.8
Days in hospital, (mean)	95.2	104.2	−3.7–21.6
Days on ventilation, (mean)	14.3	12.4	−2.8–6.5

than for the Bayley-II both for cognitive language and motor abilities.

Discussion

Our study shows that the Bayley-II and the Bayley-III yield significantly different outcomes, with the latter displaying higher composite scores both in the cognitive-language and motor abilities. Concerning the comparison with the Griffiths Scales, the Bayley-III mean composite scores revealed a higher agreement than the previous edition.

The increased scores obtained using the Bayley-III, compared with the previous edition, might be because of the improved outcomes of ELBW/ELGAN infants over time [27]. However, it must be taken into account that, in our cohort, there were no significant differences between the rates of impairment detected using the Griffiths throughout the whole study period. A possible explanation of our finding could rely on the changes in the structure of the scales. Indeed, in the Bayley-III, Cognitive and Language scores are separated so as to minimize the effects of language impairment on cognitive assessment. Thus, it can be speculated that the MDI scores were lower because cognitive assessment was negatively affected by the presence of impairments in language abilities. In addition, the Bayley-II uses item sets with established start and stop points, which may create an artificial ceiling. On the contrary, in the Bayley-III, although a start point based on age is also present, the examiner continues to administer the test items until the child receives scores of 0 for five consecutive items. Consequently, a bright child is allowed to achieve a higher level. Furthermore the Griffiths basal and ceiling rules are similar to those of the Bayley-III, as the manual recommends that the child successfully answers six consecutive items for each subscale, while administration should be discontinued when the child misses six consecutive items. It is therefore clear that both the test design and the administration rules of Bayley-III are more consistent with the Griffiths, which may explain the higher agreement between the scales' outcomes. However, concern persists that the Bayley-III may tend to underestimate both mild and severe neurodevelopmental impairment.

Indeed, whereas the degree of concordance between the Griffiths and the Bayley-III is high at an overall (non-severity-specific) level, a more detailed analysis on single subscales shows that the Bayley-III detects 5 % fewer infants as being severely impaired in language

Table 2 Griffiths vs Bayley-II – Bayley-III

	Mean (C.I. 95 %)	Mean (C.I. 95 %)
Group 1	Griffiths	Bayley-II
Cognitive-Language abilities[a]	86.0 (82.0–89.9)	79.4 (74.7–84.0)
Motor abilities[b]	91.7 (87.9–95.5)	83.8 (79.6–87.9)
Group 2	Griffiths	Bayley-III
Cognitive-Language abilities[c]	90.3 (87.2–93.5)	90.2 (87.6–92.8)
Motor abilities[d]	91.8 (88.4–95.2)	93.0 (89.6–96.4)

[a]Griffiths Hearing and Speech-Performance Quotients (mean) vs Bayley-II MDI
[b]Griffiths Locomotor-Eye and Hand Coordination Quotients (mean) vs Bayley-II PDI
[c]Griffiths Hearing and Speech-Performance Quotients (mean) vs Bayley-III Cognitive-Language Composite Scores (mean)
[d]Griffiths Locomotor-Eye and Hand Coordination Quotients (mean) vs Bayley-III Motor Composite Score

Table 3 Concordance between Griffiths and Bayley-II (Group 1) or Bayley-III (Group 2)

	Concordance (%)	Weighted K	C.I. 95 % of K
Group 1			
Cognitive-Language abilities[a]	70.7	0.63	0.51–0.75
Motor abilities[b]	67.4	0.50	0.35–0.65
Group 2			
Cognitive-Language abilities[c]	89.2	0.80	0.69–0.92
Motor abilities[d]	90.2	0.81	0.69–0.93

[a]Griffiths Hearing and Speech-Performance Quotients (mean) vs Bayley-II MDI
[b]Griffiths Locomotor-Eye and Hand Coordination Quotients (mean) vs Bayley-II PDI
[c]Griffiths Hearing and Speech-Performance Quotients (mean) vs Bayley-III Cognitive-Language Composite Scores (mean)
[d]Griffiths Locomotor-Eye and Hand Coordination Quotients (mean) vs Bayley-III Motor Composite Score

Table 4 Rates of developmental impairment

	n (%)	*n* (%)	
Group 1	Bayley-II	Griffiths	
Cognitive-Language abilities[a] within normal limits	40 (43.5)	54 (58.7)	
Cognitive-Language abilities[a] mild impairment	21 (22.8)	20 (21.7)	
Cognitive-Language abilities[a] severe impairment	31 (33.7)	18 (19.6)	
Motor abilities[b] within normal limits	53 (57.6)	66 (71.7)	
Motor abilities[b] mild impairment	13 (14.1)	14 (15.2)	
Motor abilities[b] severe impairment	26 (28.3)	12 (13.0)	
Group 2	Bayley-III	Griffiths	
Cognitive-Language abilities[c] within normal limits	78 (76.5)	74 (72.6)	
Cognitive-Language abilities[c] mild impairment	16 (15.7)	17 (16.7)	
Cognitive-Language abilities[c] severe impairment	8 (7.8)	11 (10.8)	
Motor abilities[d] within normal limits	84 (82.4)	77 (75.5)	
Motor abilities[d] mild impairment	7 (6.9)	15 (14.7)	
Motor abilities[d] severe impairment	11 (10.8)	10 (9.8)	
	n (%)	*n* (%)	*n* (%)
	Bayley-III	Griffiths	
Group 2-for single subscales			
Cognitive abilities[e] within normal limits	87 (85.3)	74 (72.5)	
Cognitive abilities[e] mild impairment	8 (7.8)	16 (15.7)	
Cognitive abilities[e] severe impairment	7 (6.9)	12 (11.8)	
Language abilities[f] within normal limits	75 (73.5)	75 (73.5)	
Language abilities[f] mild impairment	17 (16.7)	12 (11.8)	
Language abilities[f] severe impairment	10 (9.8)	15 (14.7)	
Motor abilities[g] within normal limits	84 (82.4)	73 (71.6)	84 (82.4)
Motor abilities[g] mild impairment	7 (6.9)	8 (7.8)	9 (8.8)
Motor abilities[g] severe impairment	11 (10.8)	21 (20.6)	9 (8.8)

[a]Bayley-II MDI vs Griffiths Hearing and Speech-Performance Quotients (mean)
[b]Bayley-II PDI vs Griffiths Locomotor-Eye and Hand Coordination Quotients (mean)
[c]Bayley-III Cognitive-Language Composite Scores (mean) vs Griffiths Hearing and Speech-Performance Quotients (mean)
[d]Bayley-III Motor Composite Score vs Griffiths Locomotor-Eye and Hand Coordination Quotients (mean)
[e]Bayley-III Cognitive Composite Score vs Griffiths Performance Quotient
[f]Bayley-III Language Composite Score vs Griffiths Hearing and Speech Quotient
[g]Bayley-III Motor Composite Score vs Griffiths Locomotor Quotient vs Eye and Hand Coordination Quotient

abilities and 13 % fewer infants as being mildly and severely impaired in cognitive abilities.

Our findings suggest that scores classified as "severe impairment" and "mild impairment" according to the Griffiths tend to shift up towards "mild impairment" and "normal" levels, respectively, when using the Bayley-III.

It is possible that the Bayley-III identifies fewer infants with language impairment because it separates the receptive and expressive subscales, so a child can reach a higher score by passing all the receptive items even if the production is compromised. On the contrary, as the Griffiths Hearing and Speech subscale mixes production and comprehension items, the achievement of a high score requires a greater integration of verbal skills. We also hypothesize that the Griffiths Performance subscale requires a greater integration of cognitive functions, providing a score that is more consistent with the actual level of the infant's cognitive functioning. Conversely, the Bayley-III Cognitive Index consists of a greater number of items with simpler and more graded tasks, so it is easier for a child to gain a higher score. The Bayley-III combination of fine and gross motor abilities makes it difficult to identify specific impairments in one of the two areas. Indeed, the comparison with the Griffiths Locomotor and Eye and Hand Coordination subscales shows that the Bayley-III Motor Index fails in identifying 10 % of severe gross motor impairments.

Table 5 Sensitivity, specificity and Youden's Index of Bayley-II and Bayley-III vs Griffiths

	Sensitivity (%)	Specificity (%)	Youden's index (%)
Group 1			
Cognitive-Language abilities[a]	97.4	72.2	69.6
Motor abilities[b]	80.8	72.7	53.5
Group 2			
Cognitive-Language abilities[c]	78.6	97.3	75.9
Motor abilities[d]	68.0	98.7	66.7

[a]Bayley-II MDI vs Griffiths Hearing and Speech-Performance Quotients (mean)
[b]Bayley-II PDI vs Griffiths Locomotor-Eye and Hand Coordination Quotients (mean)
[c]Bayley-III Cognitive-Language Composite Scores (mean) vs Griffiths Hearing and Speech-Performance Quotients (mean)
[d]Bayley-III Motor Composite Score vs Griffiths Locomotor-Eye and Hand Coordination Quotients (mean)

Our findings on the Bayley-II and the Bayley-III outcomes are consistent with previous studies reporting > 7 points of difference between the Bayley-II MDI and the Bayley-III Cognitive score [28].

In cohorts of infants born earlier than 25 weeks' gestation, Hintz et al. [29], using the Bayley-II at 18–22 months' corrected age, reported rates of mild to severe cognitive impairment ranging from 40 to 47 %, while mild to severe motor impairment ranged from 31 to 32 %. In our cohort, the rates of mild and severe developmental impairment, according to the Bayley-II, were slightly lower than those commonly reported in the literature. This is probably because of the higher assessment age of our study group (24 months corrected age) that may have reduced the impact of health and medical issues on child neurodevelopmental outcome. On the contrary, the rates of mild and severe impairment found in the present study according to the Bayley-III slightly

exceeded those reported by Anderson et al. [30], who found mild to severe cognitive impairment in 10 and 3 %, respectively, and mild to severe language impairment in 16 % of their preterm cohort.

As for the Griffiths outcomes, Claas et al. [25], studying a cohort of preterm infants with birth weight ≤ 750 g at 2 years, reported that none of the infants assessed with the Griffiths had a GQ of < 76 (<2 SD), whereas 9.6 % infants assessed with the Bayley-II had a MDI < 70. Similarly, in our cohort, rates of severely impaired infants according to the Griffiths (ranging from 10 to 20 %) were found to be lower than those revealed by the Bayley-II (ranging from 28 to 34 %), but greater than those of the Bayley-III (ranging from 8 to 11 %).

Our rates of agreement between the Griffiths and the Bayley-III average scores are higher than those reported by Milne et al. [31] Y. The authors, comparing a cohort of 100 preschoolers referred for assessment of developmental impairment at 32 months using the Bayley-III and reassessed at 52 months using the Griffiths Scales, found that the Bayley-III average composite scores identify 7 % fewer children as being mildly impaired and 28 % fewer children as being severely impaired compared with the Griffiths General Quotient. Thus, underestimation of the Bayley-III, in comparison to the Griffiths Scales, seems more evident at later ages even though it must be taken into account that 59 % of children studied by Milne et al. were affected by autism.

The main strength of our study is that it provides a comparison with one of the most recognized instruments for neurodevelopmental assessment, the Griffiths, which gives a standardized independent criterion on which performances at the Bayley Scales can be referred. The main limitation of the current study is that the two editions of the Bayley Scales were not administered to the same study group. In addition, because none of the neurodevelopmental assessments used in the present study have been normed in Italy, we had to use the USA norms for the Bayley-II and the Bayley-III and the UK norms for the Griffiths.

Conclusions

The findings of our study indicate that the Bayley-III has a higher agreement with the Griffiths Scales compared with the Bayley-II. Conversely, the Bayley-II yields higher rates of severe impairment than the Griffiths both in cognitive-language and motor abilities.

However, it is clinically relevant to note that the Bayley-III slightly tends to shift up scores classified as "severe impairment" and "mild impairment" according to the Griffiths towards "mild impairment" and "normal range", thus making it sometimes difficult to ascertain the real extent of neurodevelopmental impairment.

These findings have important implications for clinical services, follow-up programmes and clinical trials that rely on the Bayley-III for the assessment of developmental impairment. As the Bayley scores are often used to determine eligibility for early intervention services, the use of the Bayley-III may result in the lack of qualification for early intervention programmes of infants that would have been previously eligible. On the basis of the present findings, the use of multiple measures could be recommended to assess neurodevelopmental outcome of ELBW infants at the age of 2 years. Additional studies are needed to replicate the current findings in larger populations and at different ages of assessment.

Abbreviations

ELBW: Extremely low birth weight; ELGAN: Extremely low gestational age newborns; AGA/SGA: Adequate/small for gestational age; NEC: Necrotizing enterocolitis; IVH: Intraventricular hemorrhage; PVL: Periventricular leukomalacia; ROP: Retinopathy of prematurity; BPD: Bronchopulmonary dysplasia; MDI: Mental development index; PDI: Psychomotor development index.

Competing interests

The authors declare that they have no competing interests to disclose.

Authors' contributions

OP, CS and CF conceptualised and designed the study, interpreted the clinical data for follow-up, drafted the initial manuscript and critically reviewed the manuscript. MG and IC designed the data collection instruments and critically reviewed the manuscript. SG, LG and GP carried out the initial analyses and reviewed and revised the manuscript. MF and FM interpreted the clinical data for follow-up and critically reviewed the manuscript. All authors read and approved the final manuscript.

Authors' information

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Appendix 2

A longitudinal ICF-CY-based evaluation of functioning and disability of children with Very Low Birth Weight

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A longitudinal ICF-CY-based evaluation of functioning and disability of children born with very low birth weight

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This paper aims to describe the longitudinal changes in disability, defined by the International Classification of Functioning, Disability, and Health – Children and Youth version (ICF-CY) biopsychosocial model, and developmental outcomes in a cohort of 56 very low birth weight children over 14–20 months. We used a neurofunctional assessment, the Griffiths Mental Development Scales-Revised: 2–8 years (Griffiths 2–8) to evaluate psychomotor development and the ICF-CY questionnaire for ages 0–3 and 3–6 to address children's disability. Extension indexes on the basis of ICF-CY categories were computed, and longitudinal change was tested. Complete follow-up was available for 55 children (mean age 36.7 months, SD 6.7). Considering the sample as a whole, neurofunctional assessment, Griffiths score and disability were basically stable. When the subsample of children with the higher baseline functioning was taken into account, some degree of worsening, in terms of an increase in the number of impairments and limitations, was found. Our results show that disability profiles, neurofunctional assessment and global development were basically stable, except for the subgroup of children who were in the intermediate/high-functioning cluster at baseline. The increased disability among these children might be because of the possibility to observe a wider set of age-specific problems, such as emotional, regulation and social abilities that are not detectable at an early stage of development and that might lead to reduced participation in social activities. *International Journal of Rehabilitation Research* **39:296–301**

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Introduction

In high-income countries, 7% of children are born preterm, that is, before 37 weeks of gestation, and up to 1% are born with very low birth weight (VLBW), that is, birth weight below 1500 g [Certificati di Assistenza al Parto (CEDAP), 2015]. The reasons for this are older maternal age, increase of multiple births, increased use of assisted reproductive technology and advances in maternal–foetal medicine (Kalra and Molinaro, 2008). Prematurity and VLBW are associated with impaired neurodevelopmental outcomes including cognitive delay, cerebral palsy, and visual and hearing problems (El-Dib *et al.*, 2010). Considering the improved healthcare, preterm children with VLBW are more likely to survive: this results in an increased number of children and adults with learning and developmental disabilities, behavioural or psychiatric diseases, attention deficit disorder and hyperactivity, and cognitive, communicative, regulatory, social and emotional disturbances (Hack *et al.*, 2002; Aarnoudse-Moens *et al.*, 2009).

Approximately 73% of preterm children receiving active perinatal care have mild or no disability and neurodevelopmental outcome improves consistently with increasing gestational age (Serenius *et al.*, 2013): as shown in the EPICure study, neurodevelopmental impairments were present in 45% of children born at 22–23 weeks and in 20% of those born at 26 weeks (Moore *et al.*, 2012). Another important factor is birth weight: as reported by Claas *et al.* (2011), the survival of infants with a birth weight up to 750 g was associated with the presence of neurodevelopmental impairment at 2 years of corrected age.

Longitudinal studies with a follow-up evaluation up to 2 years generally use standardized datasets, such as the Bayley Scales of Infant Development-II or Bayley III (Mercier *et al.*, 2010), whereas, with longer follow-up, measures need to vary consistently with the age of children: the effects of this are the lack of longitudinal evaluations of children born with VLBW and the lack of

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longitudinal data on disability. Regular follow-up for preterm children usually ends at 2 years of corrected age, and only a few studies investigate the development profile or stability of the diagnosis thereafter. Current instruments provide limited possibility to predict disability outcomes of very preterm/VLBW infants and the 2-year period for neurodevelopmental follow-up is not sufficiently reliable (Roberts *et al.*, 2010).

Children's assessment was traditionally focused on gross and fine motor skills, cognitive and communicative skills, and vision and hearing performance (Msall, 2006). Some efforts have been made to limit the division between neurological and behavioural approaches, but much still needs to be done to link neurodevelopment outcome to social and environmental factors (EF), that is, to comprehensively address disability (World Health Organization, 2007). Most of the current neurodevelopmental assessments are impairment-based models of disability, which basically ignore the relevant contribution of contextual factors. Conversely, the importance of these factors is recognized by the biopsychosocial model endorsed by the International Classification of Functioning, Disability, and Health – Children and Youth version (ICF-CY) (World Health Organization, 2007). Two different studies showed the value of ICF-CY-based datasets to cross-sectionally compare functioning and disability data in children of different ages (Ibragimova *et al.*, 2009; Meucci *et al.*, 2014). These studies showed that ICF-CY-based methods enable capture of similarities and specificities, for example, the fact that the overall prevalence of problems peaks at the age of 4–6 years (Meucci *et al.*, 2014).

We previously showed that the ICF-CY-based approach can be implemented successfully in routine follow-up programmes for VLBW children through ICF-CY questionnaires (Giovannetti *et al.*, 2013), and that this approach allows for the collection of information on the EFs that impact on children's functioning, irrespective of the birth weight and gestational age. We divided a group of 56 children into four groups on the basis of neurofunctional assessment and mental development (very low, low, intermediate and high functioning) and showed that traditional assessment tools tend to poorly evaluate the interaction between the individual's functioning and environment factors (Giovannetti *et al.*, 2013). However, to our knowledge, no study exists that has longitudinally assessed the course of disability using an ICF-CY-based approach. This study aims to provide a longitudinal description of change in disability, and its link to change in neurofunctional and mental development, in VLBW infants.

Materials and methods

This observational longitudinal study was based on the same cohort of babies enrolled in the previous study between November 2011 and March 2012 at the Neonatal ICU of the Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico of Milan (Giovannetti *et al.*, 2013). The cohort included 56 VLBW children (58.9% females, average gestational age 28.3 weeks, average birth weight 1052 g) undergoing regular follow-up assessment and consisting of multidisciplinary evaluation and assistance, when needed, for all the different health problems that might be associated with prematurity. Specialists in different fields such as cardiology, paediatric surgery or ophthalmology take part in the follow-up programme, which is scheduled at 3, 6, 9, 12 and 24 months of adjusted age and when the child is 3, 5 and 7 years old. For the purpose of this study, the cohort was re-evaluated 14–20 months later (mean 17, SD 1.7): parents provided written consent for the inclusion in the follow-up evaluation.

Measures and procedures

The protocol was similar to that used for the baseline evaluation and included a neurofunctional assessment (NFA) (Vohr *et al.*, 2000; Picciolini *et al.*, 2006); the Griffiths Mental Development Scales-Revised: 0–8 years (Griffiths 0–2 and Griffiths 2–8) (Griffiths, 1970); and the ICF-CY questionnaires for age less than 3 and 4–6 (French WHO Collaborating Centre for the Family of International Classification, 2015). It was administered during a single follow-up visit and required around 60 min to be performed. In addition to the results of the different outcome measures, we also recorded the kind of interventions that were carried out during the period, including medical/rehabilitative (i.e. physiotherapy, psychomotor therapy and speech therapy) and psychosocial ones (i.e. educative intervention such as mother–child group and attendance to kindergarten) that are aimed to increase children's participation. The ICF-CY questionnaires were completed at the end of the two assessment by the parents and a member of the follow-up team who took part in the evaluations.

NFA assesses neurosensory, behavioural and motor functions (Vohr *et al.*, 2000; Picciolini *et al.*, 2006). A neurofunctional score was assigned according to the Tardieu classification: 0-normal function; 1-mild impairment, but no limitations; 2-moderate impairment (the function is possible, but limited); 3-severe impairment of function (possible only with the use of facilitators or assisted devices); and 4-function not possible. The global NFA score was defined by the highest score, thus reflecting the most severe impairment (Tardieu, 1984).

The Griffiths 0–2 is a five-scale assessment of babies' mental development. The five scales (locomotor, personal-social, hearing and language, eye and hand coordination and performance) reflect age-appropriated activities and describe children's psychomotor skills. The Griffiths 2–8 adds a practical reasoning subscale that measures children's ability to solve practical problems, understand basic mathematical concepts and moral

issues. Raw scores are converted into a weighted score that enables the calculation of a general quotient (mean = 100, SD = 12) (Griffiths, 1970).

The ICF-CY questionnaires for age less than 3 and 3–6 were used to describe disability profiles (French WHO Collaborating Centre for the Family of International Classification, 2015). The two questionnaires comprise 89 and 101 categories derived from the four ICF-CY domains: body functions (BF), body structures (BS), activities and participation (A and P) and EF. Information on the presence and extent of problems was used to assign appropriate qualifiers, ranging between 0-no problem and 4-complete problem. Sources of information included clinicians' direct observation, assessments, medical documentations and information from parents.

Statistical analysis

For each ICF-CY domain, a count-based method was used to obtain an 'extension' index reflecting the number of categories in which qualifiers 1–4 (i.e. mild to complete problem) were assigned. Given that different ICF domains, as well as the two ICF-CY questionnaires, are composed of a different number of items, a linear transformation (count/max × 100) was performed: transformed values range from 0 to 100, with lower values representing integrity of BF and BS, no limitations in A and P, absence of facilitators and barriers, respectively, in EF.

With respect to change in NFA, we computed the variation between the baseline and the follow-up evaluation and defined cases in which children were stable (if the two scores are equal), worsened (if the follow-up score is higher than baseline NFA) or improved (if the follow-up score is lower than baseline NFA). Using these three NFA change categories, we carried out a χ^2-test analysis to test whether there are differences in the distribution of NFA change between children included at baseline in the low/very-low cluster (26 children) and in the intermediate/high cluster (30 children) (Giovannetti *et al.*, 2013).

The longitudinal change was assessed at the whole group level as well as at cluster-based subgroups level. Dependent variables were the six ICF-CY extension indexes and the Griffiths general quotient. Longitudinal differences were assessed using Wilcoxon's W nonparametric test: significance was set at P less than 0.0024 after Bonferroni's correction. Parallel to this, effect sizes were calculated as the change in the means between baseline and follow-up divided by baseline SD: effect sizes of 0.2, 0.5 and 0.8 reflect small, moderate and large changes (Kazis *et al.*, 1989).

To test the relationship between change in disability and change in neurofunctional and mental development, we calculated the delta between the two evaluations for Griffiths and ICF-CY-based extension indexes, and used Spearman's correlation to test the association: significance was set at P value less than 0.0083 after Bonferroni's correction.

Results

Of the 56 children assessed at baseline (33 females, mean gestational age 28.3, SD 2.9; average birth weight 1052.1 g, SD 280.3; and mean corrected age 17.9 months, SD 4.9), 55 completed the follow-up: one child, included in the very-low functioning cluster, died as a consequence of his health condition (Trisomy 18). The mean postnatal age was 36.7 months (SD 6.7). All children with NFA scores greater than 0 during follow-up visits underwent an early intervention: 48 underwent physiotherapy, six underwent psychomotor therapy, five underwent speech therapy, three attended an educative intervention (mother–child group) and 44 attended kindergarten.

With respect to NFA change, 16 children worsened (29.1%) and 12 of these were in the intermediate/high cluster at baseline; 12 improved (21.8%) and nine of these were the low/very low cluster at baseline; and 27 were stable (49.1%) and 14 of these were the in low/very low cluster at baseline ($\chi^2 = 11.68$; $P = 0.003$).

Table 1 reports the results of the longitudinal evaluation. The Griffiths scale was basically stable. Considering the entire sample, a large reduction in the EF-facilitators index and an increase in BF and A and P-capacity

Table 1 Analysis of longitudinal change for Griffiths and ICF-CY extension indexes

	2011–2012 evaluation	2013–2014 evaluation	*P*-value	ES
Entire sample (*n*)	56	55		
Griffiths	86.0 (17.3)	85.6 (14.3)	0.382	0.02
BF	13.9 (14.2)	20.2 (15.2)	0.002*	0.44
BS	10.0 (14.0)	8.7 (12.7)	0.285	0.09
A and P-performance	19.6 (19.0)	26.0 (19.5)	0.032	0.34
A and P-capacity	16.8 (20.3)	25.3 (19.6)	0.002*	0.42
EF-facilitators	23.5 (13.5)	13.2 (13.9)	< 0.001*	0.76
EF-barriers	3.5 (5.5)	1.9 (4.5)	0.016	0.29
Low and very low functioning (*n*)	26	25		
Griffiths	74.2 (18.4)	77.7 (17.0)	0.472	0.19
BF	22.5 (16.5)	29.2 (16.0)	0.038	0.41
BS	16.6 (17.9)	14.1 (15.4)	0.332	0.14
A and P-performance	30.3 (21.1)	32.8 (21.8)	0.485	0.12
A and P-capacity	27.9 (23.8)	31.7 (22.3)	0.326	0.16
EF-facilitators	30.2 (14.0)	16.8 (16.1)	0.002*	0.96
EF-barriers	4.4 (5.8)	1.0 (2.7)	0.004	0.59
Intermediate and high functioning (*n*)	30	30		
Griffiths	96.3 (6.6)	92.3 (6.6)	0.041	0.61
BF	6.4 (5.1)	12.6 (9.5)	0.002*	1.22
BS	4.4 (6.3)	4.2 (7.8)	0.410	0.03
A and P-performance	10.4 (10.7)	20.4 (15.5)	0.011	0.93
A and P-capacity	7.1 (9.2)	19.9 (15.4)	< 0.001*	1.39
EF-facilitators	17.7 (10.0)	10.1 (11.2)	0.005	0.76
EF-barriers	2.7 (5.2)	2.7 (5.5)	–	–

Notes: Reported values are means (SD).
A and P, activities and participation; BF, body functions; BS, body structures; EF, environmental factors; ES, effect size; ICF-CY, International Classification of Functioning, Disability, and Health – Children and Youth version.
*Wilcoxon's W significant at $P < 0.0024$.

indexes were observable. Considering the two clusters, the variation in the EF-facilitators index was detected only in the lower functioning group, whereas the variations in BF and A and P-capacity indexes were detected only in the higher group.

Finally, the correlations between change in Griffiths and in ICF-CY extension indexes were all inverse and nonsignificant.

Discussion

Our study found three main results: first, NFA and the general mental development quotient were basically stable over 17 months when the entire cohort was taken into account; second, BF and A and P capacity indexes worsened, particularly in the subgroup of children with baseline higher functioning; and third, facilitator indexes' use decreased, in particular, among the subgroup of children with lower functioning at baseline.

With respect to the issue of stability over time, our results are consistent with others, showing no or minor differences over 6–30 months (Picciolini *et al.*, 2006; Romeo *et al.*, 2012). In our sample, approximately half of the children were stable over time: those who showed a decrease in NFA were in the high/intermediate cluster at baseline, and this also corresponded to a similar trend in the Griffiths scale. The Griffiths scale was basically stable with slight, but not significant, differences in the entire sample and in the two subgroups. In fact, those children who were classified as normal at baseline, according to the test standards, were still in the 'normal' group at follow-up. The same stability was also observed for those children who were classified as 'mildly impaired'. In our opinion, this trend is because of the older age of children and to the difficulties in detecting age-specific emerging problems at an early stage of development (Greene *et al.*, 2012): examples of this include regulation problems, especially in sleep–wake rhythms, sphincter control and feeding problems. Similarly, language delay is common in preterm children and it is possible that language delay is connected to the increase in BF impairments among children who were in the intermediate/high functioning group at baseline. Children with lower functioning at baseline already showed several neurodevelopmental impairments and were basically stable at follow-up: this finding is consistent with the study of Marlow *et al.* (2005), in which 86% of children with severe disability still had moderate-to-severe disability at preschool age, whereas developmental disabilities shown at the age of 30 months were poorly predictive of later developmental problems.

Both Griffiths and NFA at baseline were much more distant between children in the lower and higher cluster than at follow-up: it seems that the two groups are becoming more similar under a clinical profile, and it would be interesting to explore, in future research, whether such a phenomenon endures over time. Similarly, the worsening in BF and A and P-capacity indexes is somehow consistent with the trend observed for NFA. In our opinion, this may be because of the commonalities between the contents of NFA, such as mobility, postural adaptability, variability of motor patterns, neuromotor and behavioural skills, and impairments in BF and BS, which are usually present in younger babies. As children grow older, the NFA highlights other aspects that could not be evaluated at younger ages, such as minor dysfunctions in social or emotional areas that are fully included in the A and P domains.

Other features that can only be evaluated in older children are cognitive, emotional and social abilities, which are often delayed in preterm infants who show several minor dysfunctions involving the motor (e.g. clumsiness), mental or behavioural areas (e.g. hyperactivity) (Alexander and Slay, 2002). In previous studies, cognitive development was abnormal in 5% of cases and borderline in 20% among babies born before 32 weeks of gestation (Bos and Roze, 2011), and 23% of adolescents born preterm (vs. 9% of healthy controls) had psychiatric problems, particularly attention deficit disorders, anxiety disorders and autism (Johnson *et al.*, 2010). NFA at 12 months predicted cognitive performance (Giannì *et al.*, 2007) and neurodevelopmental delay (Picciolini *et al.*, 2016) at 36 months. The children included in our study were preschool children, but most of them attended kindergarten, and minor dysfunctions can also emerge in such a context. On the one hand, children experience richer psychosocial contexts and situations: this is likely to produce positive effects in terms of participation, such as in dealing with relational situations and peer interactions. On the other, these children 'experience' more complex social situations that may determine relevant difficulties in carrying out daily activities that would be precluded if they would not attend kindergarten. The use of instruments derived from the ICF-CY, such as the questionnaires or other structured assessments (e.g. the ICF-PEI schedule) (Raggi *et al.*, 2014), facilitates the recognition of problems not directly connected to clinical parameters and the planning of rehabilitation process through the improvement of information sharing between families and services (Järvikoski *et al.*, 2013).

With respect to the decrease in the number of facilitators, some hypotheses can be made. First, after the first 2 years, a reduction in the frequency of health interventions (e.g. number of visits) is normal, but might be perceived by parents as a reduction in the amount or the quality of provided services. Second, as they grow up, children and their families are exposed to challenging contexts, including attendance to kindergartens and interaction in nonstructured contexts, that is, the 'normal' open-to-public environments of a city where the facilitators that are present in private environment are not

present. The decrease in facilitators, that is, the increase of personal independence, and the inclusion in social situations are linked to the promotion of autonomy and participation, and are relevant indicators of overall health, well-being and future life outcomes (King *et al.*, 2003; Coster and Khetani, 2008): an ICF-CY-based approach enables to address such a perspective.

To our knowledge, this is the first time that ICF-CY-based procedures have been used to address disability change in children born with VLBW. Our results are important as they stress the advantages of the use of ICF-CY in such situations. Previous studies showed that neurosensory outcomes are relatively stable across ages (Picciolini *et al.*, 2006; Romeo *et al.*, 2012) and minor dysfunctions in social or emotional areas cannot be addressed with NFA or other commonly used neurological tools. ICF-CY-based procedures, in contrast, enable reporting of information, such as those connected to social situations, that would otherwise be ignored.

The limitations of this study include the small sample size, which significantly hampers our ability to generalize the results, and the fact that the two evaluations were carried out relying in part on slightly different instruments. However, it has to be taken into account that the general quotient is corrected for age and that the ICF-CY extension indexes are also based on age-specific items. Moreover, we did not include school-age children, in whom learning disabilities are usually diagnosed and constitute a relevant domain of disability. Future studies focusing on longitudinal follow-up cohorts of children born preterm up to school age are needed to address the impact of disability on functioning of children born preterm.

Conclusion

In conclusion, we presented a 17-month follow-up examination of a cohort of VLBW children. Taken as a whole, the results show that functioning and disability profiles, NFA and the general mental development quotient were basically stable, except for the subgroup of children who were included in the intermediate/high functioning cluster at baseline. The onset of disabilities in this group might be because of the shift between what is observable in very young children, that is, neurodevelopmental impairments, and the wider set of age-specific problems that can be observed in older children. The use of ICF-CY procedures enables to capture information connected to social situations that would not be addressed with the NFA alone.

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Conflicts of interest

There are no conflicts of interest.

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Appendix 3

Support to mother of premature babies using NIDCAP method: a non-Randomized Controlled Trial

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Support to mothers of premature babies using NIDCAP method: a non-randomized controlled trial

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ABSTRACT

Background: The Newborn Individualized Developmental Care and Assessment Program (NIDCAP) is based on preterm infant's observation during hospitalization and considers infant's behavior as the key to evaluate the level of neurobehavioral maturation.
Objectives: To evaluate the effectiveness of NIDCAP program on mother's support and infant development.
Study Design: Non-randomized controlled study, including 43 infants of 32 weeks gestation receiving either a Standard Care (SC) or NIDCAP assessment. The Nurse Parent Support Tool (NPST) was given to mothers before discharge to evaluate the support given by NICU staff. Infants' motor, visual and auditory development was investigated by a neurofunctional assessment (NFA) at term and at 3 months. The effect of NIDCAP assessment on length of hospital stay and feeding status at discharge were also evaluated.
Results: Mothers in the NIDCAP group awarded higher scores in the majority of the NPST items than mothers in the SC group. NFA at term resulted to be normal in a significant higher percentage of infants that underwent NIDCAP, while no difference could be detected at 3 months.
Conclusions: NIDCAP is an effective program to promote mothers' involvement in infants' care, that, in turn, could endorse infants' neurofunctional development in the short term.

1. Introduction

Preterm birth accounts for 12% and 5–8% of total births in the United States and in Europe, respectively [1,2]. Survival of infants born extremely preterm or with extremely low birth weight has markedly increased as a result of advances in obstetric and neonatal care. As a consequence, concern has arisen on the occurrence of potential adverse cognitive outcomes in these infants in the short and long term. Indeed, due to the physiological immaturity, preterm infants have difficulty adapting to extrauterine life [3].

When birth occurs early, a premature detachment of the infant from the mother occurs. In addition, preterm infant is cared for survival in the neonatal intensive care unit (NICU) [4,5] and, hence, completes growth and development in a non physiological environment. The association between the preterm infant's physiological immaturity and the NICU environment represents a highly stressful factor that can negatively affect the adaptive capacity of the preterm infant. As a result, the preterm infant may develop neurobehavioral disorders or emotional difficulties in the mother–child dyadic interaction and in the process of parent–infant attachment [6].

The quality of the early relationship between mother and child is regarded as facilitative and protective during the process of care. In the long-term it has also been reported to promote the emergence of infant's skills [7]. To facilitate the attachment process between parents and infant in a hospital environment, parents should be supported in playing an "active" role in the care of their infant through the creation of a "therapeutic alliance". This alliance is based on an empathic professional collaboration between parents and the NICU staff [8,9].

The Newborn Individualized Developmental Care and Assessment Program (NIDCAP) is an individualized care program based on the observation of the preterm infant during the entire period of hospitalization and considers infant's behavior as the key to evaluate the attained level of neurobehavioral maturation [10,11]. Preterm infant is observed before, during and after the interaction with the parents/caregiver. The

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quality of this interaction and the default behavioral signals are then recorded. According to the infant's specific needs and to the achieved and emerging capacities, parents are advised by the NICU staff on how to interact with their infant so that the individualization of the plan of care [12] can be further implemented [13].

The aim of the study was to evaluate the effectiveness of NIDCAP during hospital stay in preterm infants. The hypothesis to be tested was that infants undergoing NIDCAP assessment would show a better neurofunctional evaluation at term as compared to infants not undergoing NIDCAP assessment. Furthermore, we aimed to evaluate the effect of NIDCAP on mothers' perception of the support given by the NICU staff and, hence, in the involvement of their infants' process of care.

2. Materials and methods

We performed a single-center, non-randomized controlled study. The study was approved by the departmental ethics committee of Authors' Institution and written informed consent was obtained from all parents. Infants were enrolled from June 2011 to July 2013. The study was performed is an open space level III NICU where parents have access 24 h a day.

Inclusion criteria were being born between 32 + 0 and 32 + 6 weeks of gestational age to mothers having a good comprehension of written and spoken Italian. We have decided to include only infants of 32 weeks of gestational age because in most cases they show stable clinical conditions, allowing for early involvement of the mother in newborn's care as reported by Montirosso et al. [14] Exclusion criteria were: multiple birth (>2 neonates), the presence of neurosensory disabilities (blindness, deafness) and/or genetic abnormalities, the need for major surgery and brain ultrasound showing intraventricular hemorrhage >2 [15] or periventricular leukomalacia >2 [16].

All the consecutively infants that fulfilled the inclusion criteria were enrolled. Infants were frequency matched for gender and received either a standard care (SC) or underwent NIDCAP assessment. To avoid contamination between groups the NIDCAP group was enrolled only after hospital discharge of all the neonates in the SC group.

NIDCAP assessment was performed by two NIDCAP trained professionals, from birth to discharge every 10 ± 2 days. A caretaking interaction, like diaper change or feeding, was observed and the infants' current ability to organize and modulate the subsystems was assessed, as described by Als at al [12,13]. Caregiving recommendations to reduce stress and to support the individual infants' competence and development were then formulated and a written report was handed to the parents and nurses. Accordingly, parents and NICU staff were trained by the NIDCAP trained nurses to use these recommendations when taking care of the infant. NIDCAP implementation in the NICU went along with the current study and started when the first neonate in the NIDCAP group was enrolled. NIDCAP trained professionals had 5 years of experience in the NIDCAP program prior to the NICU implementations. The SC group comprised the developmental care usually practiced in the NICU including primary care nursing, skin-to-skin holding, postural support and breastfeeding [17].

The neurofunctional assessment (NFA) [18,19,20] is a comprehensive neurodevelopmental assessment based on the International Classification of Functioning Children and Youth (ICF-CY) framework. The NFA has been proposed as a useful clinical tool in evaluating the preterm infants' neurodevelopmental profile and is based on the evaluation of evoked and spontaneous motricity, postural adaptability, variability of motor patterns, and neuromotor and behavioral skills. The items are evaluated according to the emerging functions and characteristic of each age considered.

A neurofunctional score was assigned to each item evaluated and categorized as follows: 0, normal function; 1, mild impairment of function (no limitations); 2, moderate impairment of function (possible but limited); 3, severe impairment of function (possible only with the use of facilitators or assisted devices); and 4, function not possible. NFA score was defined as the maximum value obtained at assessed items, reflecting the most severe functional impairment. In the current study the scores were then pooled into 3 categories: normal (score 0 and 1), moderate impairment (score 2) and severe impairment (score 3 and 4).

At term inanimate visual and auditory orientation was further assessed using the Neonatal Intensive Care Unit Network Neurobehavioral Scale (NNNS — items 35–39) [21,22] since we aimed to evaluate more specifically the visual and auditory functions. The inanimate visual and auditory orientation appears more easily reproducible than the animate one. The NNNS was performed by the same trained physician, who had not been involved in the infants' intensive care and was blinded to the intervention. However, we have not used the entire NNNS since we have chosen the NFA for evaluating neurofunctional development. The following scores were given: score = 0 (visual orientation and tracking about 60° on horizontal axis and 30° in vertical axis or performing an arch complete of 180°, corresponding to 7/8/9 scores on the NNNS); score = 1 (visual orientation and tracking on the horizontal axis for at least 30° or visual tracking with eyes and head for at least 30°, corresponding to 4/5/6 scores on the NNNS); score = 2 (visual fixation or occasional visual tracking impossible, corresponding to 1/2/3 scores on the NNNS). With regard to the auditory orientation, we awarded a score = 0 in case of evidence of alert orientation with eyes and head towards the sound source at least once over 4 stimuli (corresponding to 7/8/9 scores on the NNNS); score = 1 in case of alerting and reactions of orientation by shifting the eyes with the head turning to source once or twice (corresponding to scores 4/5/6 in the NNNS) and score = 2 in case of modification in the behavioral state and alertness related to the sound stimulus (corresponding to scores 1/2/3 in the NNNS).

To evaluate the early involvement of mothers in the care of their child we used the Nurse Parent Support Tool (NPST) [23,14] that has been previously used in an Italian population. The NPST is a questionnaire including 21 multiple choice questions, assessed through a Likert scale with 5 options — (almost never: 1, sometimes: 2, not most of the time: 3, very often: 4, always: 5). The questionnaire was administered to parents 1–2 days before discharge. The NPST evaluates four aspects:

1. communications of information related to the child's illness (9 items)
2. the support given by staff members to parents mainly directed to enhance compliance and parental role (4 items);
3. emotional support to help parents cope with the child's illness (3 items) and
4. quality of care and support (5 items).

The answer to each question was categorized as follows: option 1 and 2, “adverse opinion”; option 3, “neutral judgment”; option 4 and 5, “positive opinion”.

We decided to involve only mothers in answering the questionnaire since they generally spend longer time in NICU than fathers. Indeed, Italian laws allow only a few days of paternity leave while mother can benefit of at least 3 months of maternity leave after delivery.

Maternal age, nationality and education were also recorded. Maternal educational level was used as a measure of socioeconomic status and classified using a 3 point scale, where 1 indicates primary or intermediate school education (≤8 years), 2 secondary school education (9–13 years) and 3 university degree (>13 years). Length of NICU stay, the number of days needed to achieve exclusively bottle or breast feeding (full oral feeding) and the type of milk at discharge (human/formula or both) were also collected to evaluate the effect of NIDCAP assessment on those variables. The following neonatal data were recorded: gender, gestational age (GA, based on the last menstrual period and early ultrasound examination), birth weight, being small for gestational age (SGA, defined as infants with birth weight < 10th percentile for gestational age, according to the Fenton Growth Chart [24], mode of delivery, Apgar score (1′ and 5′), twins, administration of antenatal steroids, surfactant treatment, the occurrence of sepsis (defined by the presence of positive blood and/or cerebrospinal fluid culture), number of days on

continuous positive airway pressure (NCPAP), and postmenstrual age at discharge.

3. Statistical analyses

Assuming a proportion of 50% of impairment at NFA among usual (control) newborns and 10% among the NIDCAP (treated) group, power = 80%, and alpha = 0.05 (two-tailed), we calculated that a sample size of 20 newborns per group would have been sufficient to detect a statistically significant difference.

Descriptive data are expressed as mean (SD) or number of observations (percentage).

Comparison among groups was performed by the chi-square test for discrete variables, by the T-test or the Mann–Whitney U-test, when appropriate, for continuous variables. Statistical significance was set at a = .05 level. All statistical analyses were performed by using SPSS (version 12, SPSS, Chicago, IL).

4. Results

The flow chart of the study is reported in Fig. 1. The study involved a total of 43 infants (SC group: = 22; NIDCAP group = 21). Infants' and maternal basic characteristics are shown in Table 1. There were no statistically significant differences in the infants' and maternal basic characteristics between the two groups. Length of NICU stay (days) and postmenstrual age (weeks) were similar in the SC group and in the NIDCAP one (33.4 ± 8.4 vs 32.6 ± 9 and 36.5 ± 1.4 vs 36.4 ± 1.3, respectively).

The percentage of infants fed any human milk at discharge was significantly higher in the infants in the NIDCAP group than in the infants in the SC group (76% vs 41%, $p < 0.0001$). Among the infants in the NIDCAP group, 19% (N = 4) was fed with human milk exclusively whereas no infant in the SC group was fed with human milk exclusively. Timing of achievement of full oral feeding (days) was similar in infants of the SC group and in infants in the NIDCAP group (26.9 ± 6 vs 25.3 ± 7.6).

With regard to the NPST questionnaire, 2 mothers (1 in the NIDCAP group and 1 in the SC group) refused to fulfill the questionnaire. Mothers in the NIDCAP group were awarded significantly higher scores in the majority of the items as compared to the mothers in the SC group (Table 2). Relatively to the aspect "communications of information related to the child's illness ", results shows that mothers of the NIDCAP

Table 1
Infants' and maternal basic characteristics.

	NIDCAP (N = 21)	Control (N = 22)
Gestational age (weeks)	32	32
Birth weight (g)	1542 ± 229	1568 ± 229
Apgar score at 1 min.	7.23 ± 1.7	7.45 ± 1.7
Apgar score at 5 min.	8.9 ± 0,8	8.9 ± 0.8
Duration of NCPAP (days)	3.9 ± 3.2	2.8 ± 3.2
Male % (n)	47.6 (10)	50 (11)
Cesarean section % (n)	90.4 (19)	90.9 (20)
Twin % (n)	42.8 (9)	63.6 (14)
Surfactant treatment % (n)	47.6 (10)	36.3 (8)
Sepsis % (n)	–	10 (2)
Antenatal steroids	76.2 (16)	77.7 (17)
Mothers (n)	17	16
Maternal age (years)	35.6 ± 7.1	35.4 ± 5.4
Mothers' educational level % (n)		
Low	10 (2)	18.2 (4)
Intermediate	50 (10)	36.4 (8)
High	40 (8)	45.4 (10)

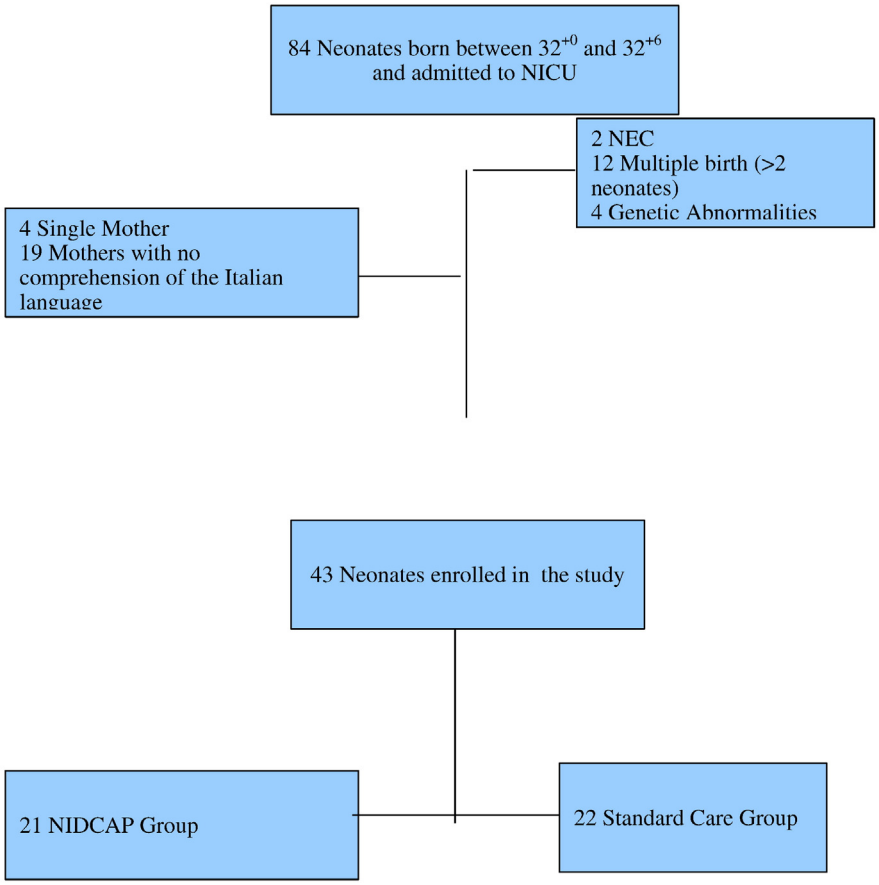

Fig. 1. Flow chart of the study.

Table 2
Nurse parental support tool.

Nurse Parent Support Tool Item % (n)	NIDCAP (20)			Control (21)			
The nursing staff at this hospital has:	Adverse opinion	Neutral judgment	Positive opinion	Adverse opinion	Neutral judgment	Positive opinion	P value
1. Helped me talking about my feelings, worries or concerns	–	30% (6)	70% (14)	85.7% (18)	4.8% (1)	9.55 (2)	0.001
2. Helped me to understand what was done to my child	–	30% (6)	70% (14)	28.6% (6)	28.6% (6)	42.8% (9)	0.029
3. Taught me how to take care of my child	–	–	100% (20)	9.5% (2)	52.4% (11)	38.1% (8)	0.001
4. Made me feel important as a parent	–	10% (2)	90% (18)	9.5% (2)	57.1% (12)	33.3% (7)	0.001
5. Let me decide whether to be present during medical procedures	33% (6)	27.8% (5)	38.9% (7)	55% (11)	35% (7)	10% (2)	N.S.
6. Answered to my questions or found someone who could	–	16.7% (3)	83.3% (15)	19% (4)	28.6% (6)	52.4% (11)	0.06
7. Told me about the changes in my infant's condition	5% (1)	5% (1)	90% (18)	9.5% (2)	28.6% (6)	61.9% (13)	0.09
8. Let me participate to the discussions regarding my baby	20% (4)	30% (6)	50% (10)	33.3% (7)	28.6% (8)	38.1% (8)	N.S.
9. Helped me understand my infant's behaviors/reactions	–	5% (1)	95% (19)	14.3% (3)	61.9% (13)	23.8% (9)	0.01
10. Helped me to understand how to comfort my child	–	10% (2)	90% (18)	14.3% (3)	47.6% (10)	38.1% (8)	0.02
11. Let me know I was doing a good job in taking care of my baby		5% (1)	95% (19)	23.8% (5)	66.7% (14)	9.5% (2)	0.00
12. Answered to my worries or concerns		5% (1)	95% (19)	19% (4)	57.2% (12)	23.8% (5)	0.00
13. Showed concern about my well-being		40% (8)	60% (12)	33.3% (7)	66.7% (14)	–	0.00
14. Helped me to know names and roles of the NICU staff	11.1% (2)	16.7% (3)	72.2% (13)	19% (4)	57.2% (12)	23.8% (5)	0.009
15. Provided good care to my infant	–	–	100% (20)	–	14.3% (3)	85.7% (18)	N.S.
16. Encouraged me to ask questions about my child	5% (1)	15% (3)	80% (16)	14.3% (3)	47.6% (10)	38.1% (8)	0.02
17. Was sensitive to my child's individual needs	–	5% (1)	95% (19)	9.5% (2)	9.5% (2)	81% (17)	N.S.
18. Allowed me to be involved in my infant's care	–	–	100% (20)	–	60% (12)	40% (8)	0.00
19. Showed they liked my child	–	–	100% (20)	9.5% (2)	19% (4)	71.5% (15)	0.03
20. Responded to my infant's needs in timely fashion	–	–	100% (20)	9.5% (2)	4.8% (1)	85.7% (18)	N.S.
21. Was optimistic about my baby	–	–	100% (20)	–	23.8% (5)	76.2% (16)	0.02

group reported an overall good sharing of information with NICU staff, except for the items concerning the active participation of parents during medical procedures and the opportunity to be involved in the decision regarding the treatment that has to be carried out and the care of their baby.

For the aspect "the support given by staff members to parents mainly directed to enhance compliance and parental role results from the questionnaire show that mothers in the NIDCAP group feel that doctor and nurses helped them to learn how to take care of their baby. The analysis of the answers of the aspect related to "emotional support to help parents cope with the child's illness" shows that mothers in the NIDCAP group, compared to mother in the SC group, feel more able to cope with their child's illness and long hospitalization thanks to the support given by the NICU staff. No significant difference among groups could be detected in the majority of the scores related to the items concerning the aspect of "quality of care and support". However, mothers in the NIDCAP group express a positive opinion concerning the items "Showed they liked my child" and "Was optimistic about my baby" in a significant higher percentage of cases than mothers in the SC group.

Table 3
Neurofunctional assessment at term equivalent age and at 3 months of corrected age.

NFA at 40 weeks			
	NIDCAP (N = 21)	Control (N = 22)	P value
Normal % (n)	90.5 (19)	61.9 (13)	.030
Moderate impairment % (n)	9.5 (2)	38.1 (9)	
Severe impairment % (n)	–	–	
Auditory orientation at 40 weeks			
Normal % (n)	66.7 (14)	66.7 (14)	.584
Moderate impairment % (n)	33.3 (7)	33.3 (8)	
Severe impairment % (n)	–	–	
Visual orientation at 40 weeks			
Normal % (n)	81.0 (17)	52.4 (12)	.122
Moderate impairment % (n)	19.0 (4)	42.8 (9)	
Severe impairment % (n)	–	4.8 (1)	
NFA 3 m			
Normal % (n)	66.6 (14)	47.6 (11)	.449
Moderate impairment % (n)	33.4 (7)	52.4 (11)	
Severe impairment % (n)	–	–	

NFA at term equivalent age resulted to be normal in a significantly higher percentage of infants that underwent NIDCAP assessment as compared to infants that had received a SC. In addition, the visual orientation at 40 weeks was normal in 81% compared to 52.4% of SC group and NFA at 3 months had normal scores in 66.6% of children compared to 47.6% of the control group while no difference among groups could be detected in the visual and auditory orientation at term. (Table 3).

5. Discussion

These preliminary findings indicate that mothers of infants that have undergone NIDCAP assessment perceived to be more supported by NICU staff in the learning process of their babies' needs than mothers of infants that have undergone standard care. Specifically, mothers in the NIDCAP group felt more confident, able to talk about their concerns, to understand and take care of their child. Furthermore, mothers in the NIDCAP group showed a good sharing of information with NICU staff. It can be speculated that these results might be due to the fact that mothers in the NIDCAP group were more involved in their infants' process of care as compared to mothers of infants that had received the SC group. NICU staff-led education actually promotes maternal role, while reducing maternal stress related to NICU physical environment [14]. These results are consistent with previous studies that have found a higher level of satisfaction in parents receiving the NIDCAP model of care as compared to mothers receiving the traditional care for their preterm infants [25,26]. Indeed, the NIDCAP program is based on family support during hospitalization through a process of empowerment [10]. Specifically, the NIDCAP program supports parents in recognizing the needs of a preterm baby, in order to help them finding the most effective strategies to respond to their babies needs. Accordingly, the literature emphasizes the need for mothers to hold active role over the decisions that affect their child [27,28]. In addition, the perception of being more supported by NICU staff could also decrease the state of anxiety related to the preterm birth, leading to the reinforcement of maternal role [29,30].

Our results are in agreement with the study of Wielenga et al. [31]. The authors reported that nurses are perceived as those who, in addition to providing direct care to their infant, provide emotional support, facilitating mother–infant relationship. In addition, early mothers' involvement, using NIDCAP program, has been demonstrated to make them aware of their importance. The involvement of the mother in

her infant's care promotes, in turn, the establishment of a good mother–infant relationship and can be regarded as one of the factors that can decrease the traumatic experience of preterm birth and positively affect preterm infant's development [32]. Kelberg et al. [27] further underline the importance of mothers' early involvement in infants' care. The authors demonstrated that mothers, who have received assistance with the NIDCAP method, felt closer to their baby, since their early involvement allowed them to interact with their baby through eye contact. As a result, both a dyadic connection between the mother and her infant and the development of the visual system was promoted. Furthermore, mother's early involvement has been reported to reduce parental stress [28].

The NIDCAP method appears to be effective also in promoting a better neurofunctional evalutation at term equivalent age although its positive effect on the neurofunctional development was no more detectable at 3 months of corrected age. In a recent study on application of NFA in neonatal intensive care unit, the authors found a good predictability of NFA when applied in VLBW infants at term equivalent age [19]. Indeed, NFA is a comprehensive neurodevelopmental assessment based on the ICF framework and it simultaneously evaluates autonomic, behavioral, neurosensory and motor items, taking into account the adaptability to the dynamic stimuli and the emerging functions. However, it has been previously reported that the sensitivity of NFA at 3 months of corrected age is relatively low [20]. The NIDCAP method has been described as a useful tool in promoting preterm infants' neurobehavioral development and in providing parents' support [7,26,33].

Specifically, Als et al. [33] reported a better motor performance and self-regulation evaluated with Assessment of Preterm Infants' Behavior (APIB)/Prechtl scores in infants treated with NIDCAP as compared to infants assisted with SC group at 42 weeks. Positive results on infant's cognitive and psychomotor development are reported as an effect of NIDCAP implementation, as underlined by the systematic review of Wallin et al. [34]. Improvements are mainly related to higher scores in the APIB, Prechtl and Bayley Scales. However, these improvements seem to reduce when looking at long term follow up. Accordingly, the authors underline the need of a sufficiently comprehensive study with extended follow-up and a clear focus on outcome variables.

The lack of persistency of the beneficial effect of the NIDCAP method is in accordance with the systematic review of Ohlsson et al. [35], who showed no difference in outcomes at medium and long term between infants that underwent NIDCAP and infants that did not. Indeed, several environmental factors can interfere with infants' neurodevelopment process after discharge.

In the present study no significant difference in the duration of hospital stay among the two groups was found. This result is in line with the study by Wielenga et al. [31]. On the contrary, other authors have reported a shorter hospital stay in infants that had undergone NIDCAP assessment [36]. We can hypothesize that the lack of any difference in hospital length may be due the inclusion of infants born at 32 weeks, that rapidly achieve independent full oral feeding, which is regarded as the limiting step for being discharged [37]. Accordingly, in contrast with the data reported by Wallin et al. [34] and with the meta-analyses proposed by Jacobs et al. [38], where the authors report an earlier achievement of full oral feeding with NIDCAP, we did not find any difference among groups in terms of days of acquisition of oral skills. However, percentage of infants fed any human milk at discharge was higher in the NIDCAP group than in the SC group. This might be due to the fact that mothers in the NIDCAP group, since they shared good information with NICU staff, could have had received more information about breastfeeding. On the other hand, the NIDCAP group might have been more alert and, hence, could have breastfed more easily as compared with the infants who had received SC.

While this study is of clinical interest, it presents several limitations. First, the sample size is relatively small so that these results need to be further validated in future research. In addition randomization of the two groups could have increased the rigor of the study design. Second, a potential bias of the current study could result from the type of population studied. Given that selection biases may result from geographical causes, it cannot be possible to generalize findings obtained from a cohort of infants from a single center to the population of preterm infants. A further limitation of the study could rely on the fact that only mothers have been involved in the study. However, it is well acknowledged that mothers show a high level of stress related to their infant's hospital stay [27,32,39]. In addition, it can be speculated that the NPTS answers could actually reflect mother's perception of the support received by the nursing staff rather than the support received by the medical staff. Lastly, it has not been possible to control for the nursing staff as well due to the fact that not all the staff had finished their developmental care training due to their high turn over.

On the basis of the present findings, NIDCAP implementation in NICU, although being time consuming and requiring specifically trained care givers, appears to be a useful additional tool for the enhancement of the NICU staff support perceived by mothers and the promotion of mothers' involvement in infants' care. This, in turn, could endorse developmentally supportive family-centered care and infants' neurofunctional development in the short term, contributing to the reduction of the burden of prematurity.

Conflict of interest

Authors have no conflict of interest to declare.

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