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"***Special Issue: Early nutrition and outcomes in preterm and term infants***"

Guest Editors: Enrico Bertino, Sertac Arslanoglu, Guido Moro

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MEDICATION AND BREASTFEEDING

A. VARALDA[1], A. COSCIA[1], P. DI NICOLA [1], G. SABATINO[2], I. ROVELLI[1], F. GIULIANI[1], A. SOLDI[1], C. PERATHONER[1], E. BERTINO[1]

[1]*Neonatal Unit, University of Turin , Italy*
[2] *Neonatal Intensive Care Unit, University of Chieti, Italy*

It is well accepted that the best feeding method for infants is breastfeeding, due to its numerous biological and clinical effects on child and maternal health. The use of medication by the nursing mother and the physician's advice to stop nursing are the most common reasons for the cessation of breastfeeding. The physician plays an extremely delicate role and should be able to assess risks and benefits for both mother and child. The main factors that must be taken into account include pharmacokinetics, the duration of maternal therapy, the age of the infant and the general health of the infant. All physicians should have access to reliable and updated information on medication safety during breastfeeding (reference books, online medical literature). Few drugs have been demonstrated to be absolutely contraindicated during breastfeeding. Nevertheless clear, safe and reliable information is still lacking for most drugs and it would be desirable to improve the knowledge about mechanisms and consequences of infant exposure to drug present in milk.

It is well accepted that the best feeding method for infants is breastfeeding, exclusive for the first six months, then followed by a complementary diet and carried on, if possible, for the first year of life or even more (1,2). Due to its numerous biological (3,4) and clinical effects (5,6), breast milk is acknowledged to be the optimal nourishment not only for term infants but also for preterm: several data confirmed the great advantages of fresh mother's milk or pasteurized donor breast milk (that maintains some biological properties and clinical benefits when mother's milk is unavailable or in short supply) also for feeding very low and extremely low birthweight preterm infants (7,8).

Often there is a need to decide whether a mother who is breastfeeding and who needs treatment with drugs can take the necessary medication and still continue breastfeeding safely. Because of the potential harm that medications may cause the breastfed child, the use of medication by the nursing mother is therefore a common reason for the cessation of breastfeeding (9). Sometimes also physicians are reluctant to prescribe a variety of medications and they improperly counsel a mother to discontinue breastfeeding. Although cessation of breastfeeding may seem the safest alternative, most drugs likely to be prescribed to breastfeeding mothers have minimal or no effect on the infants or on the milk supply (9). The physician plays an extremely delicate role in this situation and should be able to assess risks and benefits for both mother and child.

Assessing safety of a certain drug during lactation is quite complicated. The main factors that must be taken into account include pharmacokinetics and assessment of the risk to the infant and to the lactation.

Safety of drugs during lactation
Pharmacokinetics

Concerning pharmacokinetics, it is important to know the information available for the drug, its metabolites, the ability of the mother and the infant to absorb, metabolize and excrete the medication (10-13). *Oral bioavailability* is one of the principal pharmacokinetic properties of drugs; it describes the fraction of an orally administered dose of drug that reaches the systemic circulation. A drug with low oral bioavailability, even if it's excreted in human milk, is considered compatible with breastfeeding because the percentage of the drug absorbed into the infant's systemic circulation via the gut is low. Most drugs administered only by injection (e.g. insulin, heparin) are not orally available.

Key Words: Drugs, breastfeeding

Corresponding Author: Dr. A. Varalda
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3135775
ale.varalda@alice.it

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Most *drugs pass into milk* by passive diffusion, although other mechanisms have also been identified, including carrier mediated diffusion, active transport, pinocytosis and reverse pinocitosys (11). The size of the molecule, its ionization, the pH of the substrate, the solubility in water and lipids and the protein binding influence the passage of a drug into milk. In general, low plasma protein binding, low molecular weight, high lipophilicity and cationic drugs facilitate a higher excretion of the drug into milk.

A parameter proposed to estimate drug excretion in human milk is the *milk/plasma (M/P) ratio* (10). It is the concentration of the drug in the milk measured at the same moment that the concentration in maternal plasma. Low M/P ratios (<1) indicate that there is no accumulation in the mother's milk. This is a time dependent parameter and it assumes that the relationship between the two concentrations remains constant, but in most cases it does not.

A more unified approach is based on the *Relative Infant dose (RID*)(10,13). The RID is calculated by dividing the infant's dose via the milk (mg/kg/day) by the mother's dose (mg/kg/day). Compared to M/P ratio the RID is more appropriate for estimating the exposure risk for the child via breast milk because it considers the distribution volume of the drug.

The theoretical knowledge of the pharmacokinetic is not sufficient to evaluate the safety of a drug and in daily practice, the duration for which maternal therapy is needed, the age of the infant, the general health of the infant, and whether or not there is any likelihood of an idiosyncratic (non-dose-related) adverse event are also important factors to consider.

When medication is taken over a long period, even drugs with a low relative dosage can accumulate as a result of the prolonged half-life in infancy and lead to symptoms. For this reason, the repeated administration of a medication must be more critically viewed than a single dosage. The infant should be hold in check for possible side-effects and for symptoms possibly caused by the medication (14).

Individual characteristics

The safety of certain drugs also depends on the age of the infant and health condition. Newborns, and especially premature ones have a different capacity to absorb and excrete drugs than older infants because of the immaturity of liver metabolism and renal excretion (10). Thus, in general, extra caution is needed for these infants.

The age of an infant makes a difference in the total volume of milk consumed; a drug that appears in minimal concentrations in the milk may present a significant problem when one considers that 1000 ml of milk may be consumed in a day by an infant, especially during the first six months of life. In older children the diet includes other items so that the milk does not compose the total intake.

There are very few kinds of treatment during which breastfeeding is absolutely contraindicated (14): antineoplastic drugs (ie: Cyclosporine, Cyclophosphamide, Methotrexate, and Doxorubicin), redionuclides, combination therapy with several psychotropic drugs (lithium) or antiepileptics, ergotamine, tetracycline antibotics (long term use may lead to discoloration of baby's teeth). In these cases, when administration cannot be avoided, it must be decided whether to abandon breastfeeding temporarily or permanently. In case of temporarily cessation, the mother should try to pump milk to maintain milk production and freeze it before the drug assumption to feed the baby during the time the milk is unsafe.

Some medications can affect lactation (14). Medication with an antidopamine effect, such as phenotiazine and haloperidol, and other neuroleptics, such as sulpirine and rispedidone, as well as the antihypertensive α-methyldopa, and medications used to stimulate intestinal peristalsis, domperidone and metoclopramide, can, as a result of increasing the secretion of prolactin, stimulate milk production. On the other hand, diuretics, estrogen and dopamine agonists from the group of ergotamine derivatives, such as bromocriptine, cabergolin, lisuride and quinagolide, all of which have an antiprolactin action, can reduce the production of milk.

Updated informations on medication safety during breastfeeding

All physicians treating breastfeeding mothers should have access to reliable and updated information on medication safety during breastfeeding.

Most of the time, the primary source of drug information is the PDR (Physician's Desk Reference). Recommendations are based on the idea that no drug should be taken by a nursing mother unless it has been proven absolutely safe in all circumstances. Few drugs can be said to be absolutely safe all the time. The PDR is not the best source of breastfeeding information, because pharmaceutical manufacturers often discourage breastfeeding solely for legal reasons, rather than for well-founded pharmacologic scientific evidence. For the same reason pharmaceutical information leaflets could not be considered always reliable (10). An excellent resource of information about the safety of drugs during breastfeeding is the reference book by Dr. Thomas Hale *Medications and Milk* (10), updated every year. It provides detailed information on drugs and includes useful information about the drug's half-life, milk/plasma ratio, side effects, AAP ratings. Other excellent resource books are the ones by Lawrence (11) and Briggs (12). Reliable information

Table I. *Drugs and breastfeeding: suggestions.*

General suggestions for the Clinician
• *Find and use the most correct sources of information*
• *Improve effective doctor-patient communication:* Make the mother aware of the decision and let her share the clinical judgment (especially if in contrast with the pharmaceutical leaflet)
• *Always sustain breastfeeding:* especially when breastfeeding must be temporarily suspended
• *Drugs commonly prescribed for infants are most often safe to take while nursing:* the baby generally gets a much lower dose from the milk than he would from taking it directly
• *Drugs considered safe during pregnancy are usually safe to take while nursing*: during pregnancy medication gets into mother's bloodstream and then into the baby's bloodstream through the placenta; during breastfeeding, drugs that enter mother's bloodstream are filtered through the breast, and less gets into her milk.
• *Drugs that are not absorbed from the GI tract are usually safe*: many of these drugs are injected (e.g: heparin, insulin, lidocaine, or other local anesthetics) Topical medications are nearly always safe because they don't enter the bloodstream
• *Avoid drugs with long-half lives, sustained-release preparations*
• *Avoid new drugs if a therapeutically equivalent alternative that has been more widely used is available*
General suggestions for the Nursing Mother
• *Limit use of over-the-counter products and seek advice on their suitability before use*
• *Schedule the doses so that the least amount possible gets into the milk*: Drug exposure to the nursing infant may be minimized by having the mother take the medication immediately after breastfeeding or just before the infant is due to have a lengthy sleep period
• *Watch for any unusual signs or symptoms:* although reactions are rare, keep the doctor informed of any change in feeding pattern, sleeping habits of the baby

can be found also from the Committee on Drugs of the American academy of Pediatrics (AAP) (9) and from the World Health Organization list of essential drugs (15). For the most updated information it would be useful to consult the website of the National Library of Medicine of the United States Institutes of Health (16) and the website of the UK Drugs in Lactation Advisory Service (17).

Unfortunately, because of the paucity of scientific evidence and different interpretation of pharmacokinetic data, there is often a significant variability of advices among all sources of information (18). Contradictory advices from different authoritative sources can cause confusion and complicate clinicians' evaluation about the safety profile of a drug taken by the breastfeeding mother.

Table I presents some suggestions to help the physician in counseling a nursing mother regarding breastfeeding when the mother has a condition for which a drug is medically indicated.

CONCLUSIONS

Almost all medications taken by a breastfeeding mother enter the milk to a certain degree. With important exceptions, most medications have no effect on milk supply or on infant health. This information is important not only to protect nursing infants from untoward effects of maternal medication but also to allow effective pharmacologic treatment of breastfeeding mothers.

A clear, secure and reliable information is still lacking for most drugs. It is desirable to improve the knowledge on drug 's transfer mechanisms in the milk, to analyze drug 's biotransformation process and to study clinical consequences of infant exposure to drug present in milk.

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CRITICAL APPRAISAL OF DIFFERENT ANTHROPOMETRIC CHARTS TO EVALUATE POST-NATAL GROWTH OF PRETERM INFANTS

E. BERTINO[1], A. COSCIA[1], S. ARSANOGLU[3,4], F. CRESI[1], G. SABATINO[2], F. GIULIANI[1], F. CHIALE[1], L. OCCHI[1], C. MARTANO[1], G. GILLI[1]

[1]*Neonatal Unit, University of Turin, Italy*
[2] *Neonatal Intensive Care Unit, University of Chieti, Italy*
[3]*Italian Association of Human Milk Banks, Milan, Italy*
[4]*Department of Neonatology, Dr. BehcetUz, Children Hospital, Izmir, Turkey*

Preterm infants' survival has greatly increased in the last few decades thanks to the improvement in obstetrical and neonatal care. The correct evaluation of postnatal growth of these babies is nowadays of primary concern, although the definition of their optimal postnatal growth pattern is still controversial. Concerns have also been raised about the strategies to monitor their growth, specifically in relation to the charts used. At present, the charts available in clinical practice are fetal growth charts, neonatal anthropometric charts and postnatal growth charts for term infants. None of these, for different reasons, is suitable to correctly evaluate preterm infant growth. Recently, an international project has recently started aiming to create prescriptive standard for the evaluation of postnatal growth of preterm infants (INTERGROWTH-21st). Alternatively, at present, while specific charts for evaluating preterm infant postnatal growth are lacking, the best compromise is likely to be as follows: from birth to term neonatal anthropometric charts; International longitudinal charts WHO 2006 or CDC 2002 from term to childhood.

Preterm newborns constitute the large majority of the population in neonatal intensive care units (NICUs). The correct evaluation of postnatal growth of these babies is nowadays of primary concern, although the definition of their optimal postnatal growth pattern is still controversial. Concerns have also been raised about the strategies to monitor their growth, specifically in relation to the charts used.

The current nutritional recommendations aim at supporting a growth pattern that mimics physiological foetal growth (1). Such goal is seldom attained, because, beside genetic factors (2), postnatal environment and metabolic conditions are quite different from intrauterine ones (3), weight loss being the most known effect of these differences in the first days after birth; moreover even when postnatal weight growth mimics intrauterine growth, body composition is quite different from a fetus of the same postmenstrual age, mainly because of excessive fat deposition (4). Furthermore, neonatal morbidity often interferes with the adequate nutritional intake. Finally, some preterm very low birth weight (VLBW) infants suffer from intrauterine growth restriction (IUGR) and all subjects present a physiological postnatal weight loss. Thus, to counterbalance the combined effects of IUGR and neonatal weight loss, their postnatal growth velocity should be higher than the average foetal growth velocity. It's interesting to note that, in order to identify intrauterine malnutrition, some anthropometric indexes have been proposed. They compare variables, such as body length and head circumference which are less influenced in cases of malnutrition to those variables which are more compromised such as body weight.

The most well known is the Rörher ponderal index (5), corresponding to the Body Mass Index (BMI) used in children and adults [BMI= body weight (kg)/length (m)^2] and it is an indirect measure of soft tissue and inferentially of fat accumulation. The evaluation of body composition can be considered also an important parameter in neonates during the first month of life (6).

It must be considered that, in order to reach foetal

Key Words: preterm infants, birth weight, birth lenght, birth head circumference, growth charts, reference, standard

Corresponding Author:
Prof. E. Bertino
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3135775

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growth rate, VLBWI homeostatic possibilities may be overcome, with severe metabolic effects: it was observed that when a rapid weight increment occurs babies are exposed to metabolic risk such as diabetes, hypertension, and dyslipidaemia at later age (8) . For these reasons, the appropriateness of foetal growth as reference for preterm infant postnatal growth is still debated.

REFERENCE VS STANDARD

The distinction between a reference, which describes how growth actually is, and a standard, i.e., a norm that indicates how growth should be, is not negligible (9). At present, postnatal longitudinal growth charts for preterm infants are essentially descriptive reference because a "healthy" population of preterm infants is hard to define or find. Therefore, no restrictive exclusion criteria have been adopted to define the target population on which the charts have been constructed. So, these references do not describe "ideal" growth, but only postnatal growth under current nutritional and care strategies. The construction, interpretation and use of longitudinal reference charts specific for preterm infants lead to several problems (10) .

The best reference charts available at present for preterm infants are those published by the Institute for Development of Human Potential from birth to 3 years (11). However, these charts-are based on data collected 30 years ago. In addition, they do not include measurements from birth to the 40th week of postmenstrual age and subsequently measurements are taken 4 months-apart or more.

To overcome the problems related to the construction and use of a reference, an international project has recently started a study aiming at prescriptive standard for the evaluation of postnatal growth of preterm infants (INTERGROWTH-21st). The conceptual issues related to the construction of such prescriptive standard have been recently described (12). The inclusion criteria of preterm infants contributing to the new standards obviously do not attempt to select a "completely healthy population" but only a population with the lowest risk for factors known to affect prenatal or postnatal growth. This strategy should provide a population that is conceptually as close as possible to the prescriptive approach used for the construction of the WHO infant and child growth standards.

Which charts for preterm babies?

In clinical practice, neonatal charts are generally used to monitor postnatal growth of preterm newborns from birth to term. These charts, however, have some limitations due to two main reasons. First, they are based on anthropometric measures taken at birth on babies of different gestational ages; therefore, they are not suitable for a longitudinal evaluation of growth. Second, they also include preterm neonates; preterm birth being an abnormal event, they are not representative of physiological fetuses of the same gestational age who will be born at term (13). The use of these charts, based on the distribution of measurements taken on neonates with different GA, should be restricted to the anthropometric auxological assessment of babies at birth.

These charts, now properly called neonatal anthropometric charts, should not be confused with the intrauterine growth charts, which are a tool for monitoring fetal growth, being based on ultrasound measurements of anthropometric traits during pregnancy. They are prone to the lack of precision of ultrasound measurements as well as to the inaccuracy of the algorithms used to convert one-dimensional ultrasound trait into a three-dimensional neonatal trait such as birth weight. Consequently, fetal growth curves derived from ultrasound technology may be not appropriate both for the longitudinal and fo the cross-sectional growth evaluation of preterm infants, mainly as regards weight (14).

After 40 weeks of postmenstrual age, postnatal charts built mostly on term babies, such as the growth charts released by the CDC (*Center for Disease Control and Prevention*) in the year 2000, are generally used for monitoring preterm infants growth. These charts represent a "reference" in which they are explicitly descriptive and represent a population's actual growth irrespective of its health status (9).

On the other hand, a typical growth standard from birth to 5 years of age has recently been released by WHO, which is based on a multiethnic population of term breastfed infants without known health or environmental constraints to growth (15). This is expected to provide a single international standard that represents the best description of physiological growth for all children. When growth traits of preterm infants are plotted on the abovementioned charts, their postnatal age should be corrected for GA at birth up to 24–36 months (16). It must be considered that, even if corrected age is used, anthropometric measures of preterm infants often fall below the 3rd centile, all these charts being built on babies born at term (17).

CONCLUSIONS

An international longitudinal standard for the evaluation of preterm infants will be available in the next years. Alternatively, at present, while specific charts for evaluating preterm infant postnatal growth are lacking, the best compromise is likely to be as follows: from birth to term neonatal anthropometric charts; International longitudinal charts WHO 2006 (14) or CDC 2002 (16) from term to childhood.

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BIOLOGICAL, NUTRITIONAL AND CLINICAL ASPECTS OF FEEDING PRETERM INFANTS WITH HUMAN MILK

E. BERTINO[1], S. ARSANOGLU[3], C. MARTANO[1], P. DI NICOLA[1], F. GIULIANI[1], C. PEILA[1], E. CESTER[1], A. PIRRA[1], A. COSCIA[1], G. MORO[2]

[1]*Neonatal Unit, University of Turin, Italy*
[2]*Italian Association of Human Milk Banks, Milan, Italy*
[3]*Department of Neonatology, Dr. BehcetUz, Children Hospital, Izmir, Turkey*

Benefits of breastfeeding are widely recognized, during the last decades human milk has been identified as the normative standard for infant feeding and nutrition. Recent evidence focused on specific bioactive and immunomodulatory factors, such as oligosaccharides, lactose, glycosaminoglycans of human milk and the variability of their concentrations during lactation in both term and preterm milk. Human milk should be fortified with proteins, minerals and vitamins to ensure optimal nutrient intake for preterm VLBWI infants. Best fortification strategies as well as the "optimal" composition of fortifiers are still object of research. Short and long-term clinical, metabolic, immunologic and neurodevelopmental advantages of breastfeeding ndividualizes fortification - particulary adjustable fortification- has proven to be effective when compared to formula are well documented. Moreover several non-experimental studies observed that clinical feeding tolerance is improved and the attainment of full enteral feeding is quicker by a diet of human milk. In addition, benefits of breastfeeding on psychological and relational aspects have to be considered. Mother's own milk remains the first choice for all neonates, when it is not available or not sufficient despite significant lactation support, donor milk represents the second best alternative and although some nutritional elements are inactivated by the pasteurization process, it still has documented advantages compared to formula.

Benefits of breastfeeding are widely recognized, during the last decades human milk has been identified as the normative standard for infant feeding and nutrition. Exclusive breastfeeding is recommended until 6 months of age followed by a complementary feeding and continued, if possible, well beyond 1 year of life. Growing evidence supports the use of human milk, due to its numerous benefits, also for sick and preterm infants in Neonatal Intensive Care Units (NICUs) (1-3).

Biological aspects

Human milk (HM) can be considered a species specific biological "dynamic" system. Particular attention is given to specific bioactive and immunomodulatory factors that can not only ensure adequate host defense against infections, but also actively modulate the immune response and modify the intestinal bacterial flora (4-7). Among these factors oligosaccharides have a relevant role. Originally described as a prebiotic "bifidus factor" that serves as a metabolic substrate for desired bacteria and shapes an intestinal microbiota composition, today oligosaccharides are known to be more than just "food for bugs": literature shows they also directly act preventing pathogen adhesion to infant mucosal surfaces, lowering the risk for infections, and modulate epithelial and immune cell responses (8). A recent study reported higher concentrations of Human Milk Oligosaccharides (HMOs) in preterm compared with term milk. This highlights the relevant role of breastfeeding for preterm infants, who, because of the immaturity of their organs and systems, are at greater risk of of developing infectious desease infectious illnesses. Lower lactose concentrations in preterm milk compared with term milk were also described. Low lactose concentrations in milk might have positive effects in preterm nutrition, because lactose contributes to lowering the milk osmolality. Low levels

Key Words: human milk; preterm infants; fortification; donor milk

Corresponding Author: Prof. E. Bertino
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3135775
enrico.bertino@unito.it

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also might represent lower substrate levels for the well-known lactase deficiency that is common among these newborns (9). Another relevant role in the immunological protection is played by IgA. IgA concentrations in both term and preterm milk have their maximum at the beginning of lactation and start to decrease quickly during the first week. This variability reflects the important role of HM in supplying immunological factors to cope with the gastrointestinal absorption of high molecular weight proteins in the first days of life. (10) In the last years an important role was ascribed to the glycosaminoglycans (GAGs). GAGs could act as an antioxidant and anti-inflammatory agent, behaving as soluble receptors having the power to interact with pathogens and to compete for their adhesion to the intestinal wall. The higher concentration of GAGs observed in preterm milk could be useful, along with oligosaccharides, for the preterm newborn in defense processes against several pathogens and contribute to the infant's defense against free radicals(11-12).

Nutritional aspects

HM is nowadays universally recognized as the optimal feeding choice for every infant (6). However, in order to meet the unique nutritional requirements of preterm infants and preserve the singular benefit of breastfeeding, human milk should be fortified to allow adequate growth and bone mineralization (13). Kuschel and Mc Cormick, in two Cochrane reviews, agree with the fact that fortification of HM with more than one nutritional component is associated with short-term improvements in weight gain, linear and head growth. Further research should be directed toward comparisons between different fortifiers, evaluating both short-term and long-term outcomes and adverse effects, to achieve the "optimal" composition of fortifiers (14-15). A great variety of protocols are currently used in the neonatal units.It has been observed that despite standard fortification,protein intakes fail to meet the reccomended intakes for preterm infants.This study also shows that actual protein intakes are consistently ans substantially lower than assumed intakes during the fortification period (16). Low protein intake has been proven to be the primary limiting factor responsible for preterm infant growth failure. Recently, good results have been obtained with individual fortification of human milk that compensates for the high variability of expressed breast milk composition. There are two models of individualization: the "adjustable fortification" based on the infant's metabolic response and the "targeted fortification" based on the analysis of human milk and on its fortification in such a way that each infant always receives the amount of needed nutrients (17). Adjustable fortification has the advantage to safeguard the excessive protein intakes (17).

Infant outcomes

Necrotizing Enterocolitis (NEC)

NEC remains one of the most critical morbidities of preterm babies. Although no individual randomized controlled trial have been sufficiently powered to examine the effect of breast milk on the incidence of NEC in preterm babies, two meta-analysis suggest a reduced incidence of NEC in infants fed with human milk (18-19). The two meta-analysis from randomized controlled trials compare formula milk with donor breast milk: since maternal breast milk contains higher levels of immunoprotective factors than donor breast milk, feeding with maternal breast milk has also a protective effect, as observed in observational studies (20-22).

Neurodevelopmental outcome

Neurodevelopmental outcome of preterm infants is improved by the feeding with human milk.

Long-term studies at 8 years of age through adolescence suggest that intelligence test results, white matter and total brain volumes are greater in subjects who had received human milk in the NICU. Extremely preterm infants who receive the greatest proportion of human milk in the NICU had significantly greater scores for mental, motor, and behavior ratings at ages 18 months and 30 months.(1) It's noteworthy that in O'Connor's study the best neurodevelopmental and motor outcomes in very low birth weight infants fed with fortified breast milk occurs during the first year of corrected age despite the reduced growth in this period of life (23). This observation draws attention to qualitative growth aspects that had already been observed by Lucas in his trial: no differences in neurological development were observed despite a higher growth in infants fed with preterm formula (24). Both studies suggest that human milk may have an independent effect on the neurodevelopment of preterm infants.

Infections

In a systematic review de Silva focused on three randomized controlled trials and six observational studies regarding the relationship between nutrition, infection and human milk in preterm infants. Even if methodological problems in the included studies are pointed out by the Authors, they conclude that human milk compared with formula has a protective effect to the onset of infections (25). A large prospective study conducted with extremely low birth weight infants or infants with less than 28 weeks gestational age at birth was published highlighting the risk of late onset sepsis is significantly reduced by that early enteral feeding with human milk (26).

Feeding tolerance

There are a few of experimental studies about feeding

tolerance, especially regarding mother's own milk. In his meta-analysis of data from randomized controlled trials, Boyde indicates that feeding with formula milk, compared with donor breast milk (DM), leads to higher rates of feed intolerance and necrotising enterocolitis in preterm infants (18). On the other hand the meta-analysis of Henderson concluded that there are no data from randomized controlled trials to determine whether feeding with formula versus breastfeeding preterm infants affects growth, development, or other clinically important outcomes (27). There are several non-experimental studies indicating that clinical feeding tolerance is improved, and the attainment of full enteral feeding is made quicker by a diet of human milk. A recent international survey on enteral feeding practices demonstrates marked variability in neonatal feeding practices in four geographical regions. This variability is partly explained by differences in access to donor human milk. Moreover the study observed that most of the units with access to donor human milk commenced enteral feeding on the first day of life and advanced more rapidly. (28)

Long-term cardiovascular risk

Long-term studies of preterm infants also suggest that human milk feeding is associated with lower rates of metabolic syndrome and with lower blood pressures and low-density lipoprotein concentrations in adolescence as well as a lower risk of insulin resistance (1-2).

Psychological and relational aspects

In case of premature birth, mother's sense of guilt and defeat of not having been able to carry the pregnancy until term and the concern of not being able to take care of such a small and fragile creature is also associated with stress and health concerns. For this purpose, free access to the ward for parents is essential. In this case, they are encouraged in helping in the care of their own baby, touching and feeding the infants by themselves. Breastfeeding creates a great emotional involvement and a sense of gratification in mothers. In this case benefits of breastfeeding outweigh the purely nutritional aspects and they can be considered part of the neonatal and the NIDCAP care (29-30).

Donor Human Milk

American Academy of Pediatrics in its last policy statement on breastfeeding recommends that pasteurized DM, appropriately fortified, should be used for preterm infants if mother's own milk is unavailable or its use is contraindicated (1). Pasteurization is necessary to inactivate most of viral and bacterial agents but partially affects nutritional and immunological properties of breast milk, however it is well recognized that some of the mother's milk beneficial and protective effects are preserved (2). Specific Guidelines have been prepared as a tool to optimize the functioning of existing Human Milk Banks by uniforming the organization, management and procedures in these Banks and to determine the minimal essential requirements to establish a new Human Milk Bank (2).

Donor milk banks are not only meant to collect, process and store donated milk, but they also represent an instrument for breastfeeding promotion and support. In Italy, data from the Italian Neonatal Network show that in NICUs, exclusive breastfeeding at discharge is achieved for nearly 30% of neonates when banked milk is available during hospitalization and only for 16% of neonates when it is not (31). According to Quigley (19), additional randomized controlled trials are needed to compare feeding with formula versus nutrient-fortified donor breast milk. Analysis of costs and an evaluation of acceptability are also required, in particular researches exploring different cultural, religious and social attitudes to DM (32-33).

CONCLUSIONS

The term "programming" has been proposed to emphasize that early nutrition should be considered not simply in terms of meeting immediate nutritional needs, but also for its potentially long-lasting or life-long biological effects (9). The target is to achieve preterm VLBW infants growth potential and to ensure them a good health outcome and a normal neurological development. Breastfeeding and the use of human milk are the best tools to reach these goals.

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EDITORIAL

AUXOLOGICAL EVALUATION OF NEWBORNS

E. BERTINO[1], A. COSCIA[1], L. OCCHI[1], P. DI NICOLA[1], C. FABRIS[1], A. VARALDA[1], F. GIULIANI[1], G. SABATINO[2], C. VASSIA[1] and G. GILLI[1]

[1]*Neonatal Unit, University of Turin, Italy*
[2] *Neonatal Intensive Care Unit, University of Chieti, Italy*

Auxological evaluation of the newborn should be based on accurate anthropometry at birth and a reliable estimate of gestational age (GA). However, a comprehensive evaluation of the neonate should consider not only anthropometric traits at birth, but also fetal ultrasound biometry and Doppler velocimetry. Many charts have been proposed, but they are hardly comparable with each other, due to numerous methodological problems. The Italian Societies of Neonatology, of Pediatric Endocrinology and Diabetology and the Italian Society of Medical Statistics and Clinical Epidemiology promoted a multicenter survey with the aim to produce an Italian neonatal anthropometric reference (Italian Neonatal Study [INeS] charts) fulfilling the set of the criteria that a reliable neonatal chart should possess. In order to construct an international standard, an international project (INTERGROWTH-21st) has started a study aiming to create a prescriptive standard. Until an international standard is developed, the use of national updated reference charts is recommended.

The ability to recognize abnormal growth at birth and/or an intrauterine malnutrition is of great importance for the care and prognosis of neonates. For this purpose, the so called "neonatal anthropometric charts" are used, in which the percentile values of weight, length and head circumference at different gestational age (GA) are plotted. These variables must be evaluated using standardised instruments and following the techniques required for accurate measurements (1).

In order to identify intrauterine malnutrition, some anthropometric indexes have been proposed. They compare variables, such as body length and head circumference which are less influenced in cases of malnutrition to those variables which are more compromised such as body weight.

The best known is the Rörher ponderal index [PI = body weight (grams) x 100/height (centimeters)^3] whose normal value in a full - term newborn lies between 2.2 and 3 (3° and 97° percentile) (2) This neonatal body proportionality index corresponds to the Body Mass Index (BMI) used in children and adults [BMI= body weight (kg)/height (m)^2] and it is an indirect measure of soft tissue and inferentially of fat accumulation. Body composition was also considered an important parameter in neonates during the first week of life (3).

The validity of neonatal auxological evaluation is also based on reliable estimates of gestational age. There is unanimous agreement that the best estimation is obtained by a combination of anamnestic-based on reported last menstrual period and early ultrasound assessment (4).

In clinical practice, a neonate is classified as small-for-gestational-age (SGA) (anthropometric trait below the 10th centile for gestational age), appropriate-for-GA (AGA) (anthropometric trait between 10th and 90th centile for gestational age), or large-for-GA (LGA) (anthropometric trait above 90th centile for gestational age) on the basis of threshold values derived from the distribution of a given anthropometric trait (e.g. birth weight [BW]) in a population of neonates regarded either as standard or more often as a reference. A standard is based on highly restrictive criteria aimed at excluding all neonates exposed to any risk factor for fetal growth, thus describing "how growth should be." In the absence of these exclusion criteria, a chart is considered a reference, which describes "how growth actually is." At present, the large majority of neonatal charts in use are references (5).

Key Words: neonatal anthropometric charts, small for gestational age (SGA), intrauterine growth restriction (IUGR), birth weight, birth length, head circumference

Corresponding Author: Prof. E. Bertino
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3135775
enrico.bertino@unito.it

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The use of neonatal anthropometric charts, based on the distribution of measurements taken on neonates with different GA, should be restricted to the auxological assessment of babies at birth. These charts, called neonatal anthropometric charts, should not be confused with the intrauterine growth charts, which are a tool for monitoring fetal growth, based on ultrasound measurements of anthropometric traits during pregnancy: preterm births are abnormal events and preterm neonates cannot be equated to fetuses of the same gestational age who will be born at term (6) .

Which neonatal anthropometric charts are appropriate?

During the past decades charts have been proposed, but they are hardly comparable with each other, due to methodological problems such as different inclusion and exclusion criteria, measurement techniques and statistical analysis. In a commentary published in 2007, we defined the characteristics that a reliable neonatal anthropometric chart should possess to be of both clinical and epidemiological use (7).

With the aim of assessing to what extent the neonatal charts published in the last decade present such characteristics, we examined the relevant literature published in the last decade (5) .

General agreement seems to have been achieved over the debated question whether a neonatal chart should be a reference or a standard, as the large majority (80%) of charts included in this analysis are a reference (which describes ‘‘how growth actually is’’ in a given population), with the only exclusion being stillbirths and major congenital anomalies, rather than a standard (which indicates ‘‘how growth should be’’), with the exclusion of all neonates exposed to any known risk factor for intrauterine growth. About two thirds of the charts are specific for gender and single pregnancy, whereas only a few of them are specific for parity (primiparae/multiparae), which is a maternal constraint known to affect fetal growth. In 10 cases, the minimum gestational age considered in the charts is 30 weeks or higher, so these charts are unsuitable for the assessment of many preterm neonates. Reliable neonatal charts require reliable estimates of gestational age. Unfortunately, in 80% of the studies under examination, gestational age is derived from the reported last menstrual period and not from the ultrasound assessment. In only four papers are both estimates considered (8-11). Reliable neonatal charts require reliable anthropometric measurements. Regrettably, only 30% of the surveys meet these requirements.

The Italian Society of Neonatology, the Italian Society of Pediatric Endocrinology and Diabetology, and the Italian Society of Medical Statistics and Clinical Epidemiology promoted a multicentric survey with the aim to produce an Italian neonatal anthropometric reference (Italian Neonatal Study [INeS] charts) (12) fulfilling the set of the criteria that a reliable neonatal anthropometric chart should possess to be of both clinical and epidemiological use (7).

To overcome the problems related to the construction and use of a reference, an international project has recently started a study aiming at prescriptive standard (INTERGROWTH-21st).

Small for Gestational Age (SGA) and Intrauterine Growth Restriction (IUGR)

The terms Small for Gestational Age (SGA) and Intrauterine Growth Restriction (IUGR) are often used as synonyms, however they reflect two different concepts. SGA refers to a statistical definition, based on an auxological cross-sectional evaluation (prenatal or neonatal), and denotes a foetus or a neonate whose anthropometric variables (usually weight) are lower than a given threshold value computed on a set of infants having the same gestational age. SGA includes infants who have not achieved their own growth potential because of maternal, uterine, placental and fetal factors and also small but otherwise healthy infants (7). IUGR refers to a clinical and functional condition and denotes fetuses unable to achieve their own growth potential: a fetus with IUGR would have been larger, with no adverse environmental or genetic factors affecting growth. Such a condition can be assessed by ultrasonography during pregnancy by a longitudinal evaluation of fetal growth rate. A neonate identified as SGA by neonatal anthropometric charts is not necessarily a case of IUGR and, conversely, a neonate identified as having IUGR during the fetal period by intrauterine growth charts may not be SGA , also considering that the genetic component plays an important role in fetal growth (13) .

Furthermore, Doppler velocimetry can detect altered flow states in the fetal–placental and uterine–placental circulation. It may help to distinguish, among SGA fetuses, subjects with IUGR from constitutionally SGA. In the first case Doppler flussimetry can often be altered (14).

CONCLUSION

The INeS charts are an updated national reference having the properties that a chart should be of both epidemiologic and clinical use (7) . They allow for sex and birth order and apply to all single-born neonates with GA between 23 and 42 weeks of gestation. Last and most important, the INeS charts are summarized through the LMS parametrization that enables the user to express anthropometric traits as SDS, even in the case of skewed

distribution. The differences observed with other European charts are partly due to methodological discrepancies, but a role of differences among populations, such as diet, environment, and prevalence of risk factors, cannot be excluded. Therefore, caution should be used against the extension of any national chart to other countries. Until an international standard is developed, we would recommend the use of updated national reference charts constructed according to an accurate methodology.

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DONOR HUMAN MILK VERSUS MOTHER'S OWN MILK IN PRETERM VLBWIS: A CASE CONTROL STUDY

F. GIULIANI[1], G. PRANDI[1], A. COSCIA[1], F. CRESI[1], P. DI NICOLA[1], M. RAIA[1], G. SABATINO[2], L. OCCHI[1], E. BERTINO[1]

[1]*Neonatal Unit, University of Turin, Italy*
[2]*Neonatal Intensive Care Unit, University of Chieti, Italy*

As for term infants, over the past decades there has been increasing evidence of the benefits of human milk in the feeding of Very Low Birth Weight Infants (VLBWI), influencing not only short-term health outcomes but also long-term neurodevelopmental, metabolic outcomes, and growth. Mother's own milk is the first choice for all neonates including preterm infants, when it is unavailable or in short supply, pasteurized donor breast milk offers a safe alternative and is considered the next best choice. The main aim of this case-control retrospective analysis was to evaluate short term advantages of mother's own milk as a sole diet compared to donor milk as a sole diet, in terms of growth, antiinfectious properties, feeding tolerance, NEC and ROP prevention in a population of VLBWI born in a tertiary center. We did not find significant differences in clinical outcome from mother's own milk compared with pasteurized donor milk. Only a slight and statistically not significant difference in growth could be observed, in favour of maternal milk. We conclude that the maximum effort should always be put in supporting and promoting breastfeeding and donor milk used not only as an alternative to mother's milk but also as a breastfeeding promotion and support strategy.

As for term infants, over the past decades there has been increasing evidence of the benefits of human milk (HM) even in the feeding of Very Low Birth Weight Infants (VLBWI), influencing not only short-term health outcomes but also long-term neurodevelopmental, metabolic outcomes, and growth (1-4). Many factors are known to play a role in the biological activity of HM, e.g. Ig, lactoferrin, lysozime, nucleotides, oligosaccharides, growth factors, enzymes, antioxydant factors and cellular components, which provide adequate host defence against infections, modulate immune response and favourably modify intestinal bacterial colonization. Growing scientific evidence indicates the benefits of human milk for appropriate growth and development of a newborn infant: the particular nutrient composition, hormonal and enzymatic components, anti-infective and growth factors of human milk make it a unique and inimitable nutrient (5-8).

Mother's own milk is the first choice for all neonates including preterm infants, when it is unavailable or in short supply, pasteurized donor breast milk offers a safe alternative and is considered the next best choice (9). Pasteurization only partially affects nutritional and immunological properties of breast milk, in fact it is well known that pasteurized milk maintains some of the mother's milk protective effects (10). The Holder method is the most widely employed by human milk banks. Even though it inactivates immunological and anti-infectious factors, reduces levels of active IgAs, IgGs, lisozime, lactoferrin and abolishes IgMs and complement factors this method of pasteurization does not affect some other key nutritional compounds such as lactose, PUFA, fatty acids, gangliosides (11-12), oligosaccharides (13) and vitamins A, D and E (14). Therefore, pasteurization only partially affects nutritional and immunological properties of breast milk, in fact it is well known that from a clinical point of view pasteurized milk maintains some demonstrated protective effects. Donor human milk advantages for VLBWI have been deeply studied over the past years and are still object of research. The main benefit deriving from

Key Words: donor milk, very low birth weight infant, mother's milk

Corresponding Author: Dr. F. Giuliani
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3135775
quo.g@libero.it

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the use of donor human milk (vs. formula) in preterm infant feeding is the reduction of the NEC incidence as indicated in three recent meta-analyses (15-17).

A recent systematic review that has considered three randomized controlled trials (RCT) and six observational studies (18) indicated the protective effect of human milk against infections in preterm infants. Following this review, a large prospective study (19) showed that fresh human milk or donor milk feeding reduced the risk of late onset sepsis in extremely low birth weight or extremely premature infants. These findings have been confirmed only for fresh mother's own milk in a successive study (20). Thus, further studies evaluating specifically the antiinfective effects of donor human milk are necessary. It has been shown that donor human milk feeding significantly reduces the incidence of bronchopulmonary dysplasia in neonates with a gestational age below 30 weeks (20). This observation suggests that human milk exerts an antioxidant activity which is likely to be maintained also after pasteurization. Also, there is increasing evidence of a protective effect of donor milk vs formula in developing cardiovascular risk profile in adolescence (21-23).

WHO and UNICEF jointly developed the Global Strategy for Infant and Young Child Feeding to revitalize world attention to the impact that feeding practices have on the nutritional status, growth and development, health, and thus the very survival of infants and young children. In that document, donor human milk is mentioned as an advisable practice for breastfeeding promotion: "…Only under exceptional circumstances can a mother's milk be considered unsuitable for her infant. For those few health situations where infants cannot, or should not, be breastfed, the choice of the best alternative – expressed breast milk from an infant's own mother, breast milk from a healthy wet-nurse or a human-milk bank, …– depends on individual circumstances.(24)

The main aim of this case-control retrospective analysis was to evaluate short term advantages of mother's own milk as a sole diet compared to donor milk as a sole diet, in terms of growth and short term clinical outcomes in a population of VLBWI born in a tertiary center.

MATERIALS AND METHODS

Subjects

The case-control study was performed selecting infants from all VLBWI with a gestational age >23 weeks admitted to the NICU, Department of Pediatrics, Turin University, Italy, from 1st January 2000 to 30th June 2006.

Patients who were moved to another unit within the first week of life or infants with severe congenital anomalies [http://eurocat.ulster.ac.uk] were excluded. During hospitalization, nutritional practices were standardized for all babies as follows. Both enteral and parenteral nutrition were begun simultaneously on day 1. Fluids were started at 60-80 ml/kg/day on day 1 and increased to 130-160 ml/kg/day by day 7. Carbohydrates were begun at 6-8 g/kg/day on day 1 and increased progressively to a maximum of 15 g/kg/day. Protein intake was started at 1.5 g/kg/day on day 1 and increased to 3.5 g/kg/day by day 7. Lipids were introduced at 0.5 g/kg/day on day 1 and increased to 3 g/kg/day by day 7. Enteral nutrition was started with trophic feeding with 10-24 ml/kg/day of human milk and increased daily by 10-20 ml/kg/day. Mother's own milk (MM) was used or, if the supply of their own maternal milk was inadequate, human pasteurized donor milk (DM). Human milk fortifier was added to HM or DM when the intake reached 100 ml/kg/day and continued until the infant attained a weight of 1.8 kg or was discharged. If no MM was available, feeding was carried on with fortified DM until 32 weeks of gestational age and then progressively replaced by preterm formula until the infant attained a weight of 1.8 kg or was discharged. All babies received a daily feeding supplementation with probiotics, with Lactobacillus GG ($3*10^9$ Lactobacilli/day) or Lactobacillus casei ($8*10^9$ Lactobacilli/day).

Cases were defined as babies whose feeding was made up of more than 80% human donor milk during the first 20 days of life. 46 cases were selected and matched with 46 controls by birthweight (±110g) and gestational age (GA). Controls received enteral feeding made up of more than 80% of mother's milk to be included.

Data collection

Demographic variables were recorded for all infants; information was obtained on all subjects from the NICU database and the medical record, using a specific data collection form. Maternal age, hypertensive disease of pregnancy, mode of delivery, prolonged rupture of membranes (defined as >24 hours) and single or twin pregnancies were recorded. For cases and controls, gestational age (in complete weeks), birth weight, sex, being born small for gestational age (defined as birth weight below the 10th centile of the reference for North-West Italy)(25) and Apgar score were studied. Infants with severe congenital heart disease were identified by cardiac ultrasound and excluded from analysis. All of the case infants with hemodynamically significant PDA were treated with intravenous ibuprofen with a first dose of 10mg/kg, followed at intervals of 24 hours by 2 doses of 5mg/kg. Only when pharmacological therapy failed was surgical ligature performed. Number of days with an umbilical vein catheter was also recorded. Proven NEC (Bell stage >2)(26) before death or hospital discharge was confirmed by abdominal X-ray. Respiratory distress syndrome (PaO2<50mmHg or cyanosis without O2 supplementation and consistent chest radiographs), bronchopulmonary dysplasia (defined as need a supplement of oxygen at 28 days or at 36 weeks), late onset sepsis (clinical syndrome characterized by systemic signs of infection with increased C-reactive protein or positive blood culture), intraventricular haemorrhage (IVH) graded according to Papile classification by cerebral ultrasonography within 28th day of life (27) and periventricular leucomalacia (PVL) were also recorded. Examination of the eyegrounds was used to determine and classify retinopathy of prematurity (ROP) on the basis of international classification (28). Feeding tolerance was assessed by the following characteristics: number of infants with

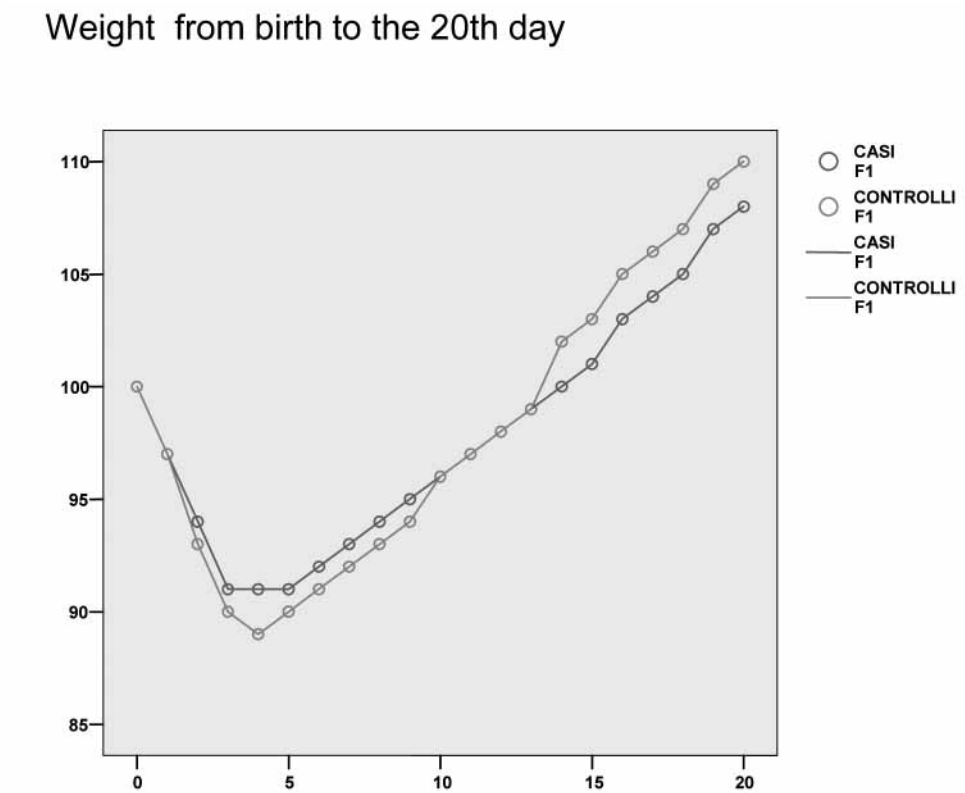

Fig. 1. *Percentage difference of the weight the day with respect to birthweight from birth to the 20th day (cases=red, controls=green)*

gastric residual volume > 10cc or with gastric residual volume > 3cc detected twice a day and aspect of residuals (assessed as clear, bilious, blood-stained or haemorrhagic). Gastric residual volume was determined by aspiration of gastric contents from the indwelling orogastric tube every 3 hours in all infants before the following feeding. Moreover, age in days at exclusive enteral feeding and age in days at the beginning of human milk fortification were recorded for each baby. Weight was daily assessed in the two groups.

Data analysis

Odds ratio with 95% confidence intervals was used for categorical variables. Independent sample t-test, or Mann-Whitney test if the distribution was not normal, were used for continuous variables to report mean difference and standard deviation. All of the statistical computations were performed using SPSS for Windows, version 12.0 (SPSS, Chicago, IL). $P < 0.05$ was used to define statistical significance.

RESULTS

Cases and controls did not result statistically different as regards birthweight and gestational age (as expected for the inclusion criteria) and also as regards neonatal characteristics (Table I). No significant difference in comorbidities was observed between the two groups (Table II).

The prevalence of maternal hypertension was 16/36 (44.4%) among the cases and 16/43 (37.2%) among the controls (p=0.65), of premature membrane rupture was 7/31 (22.6%) among the cases and 3/32 (9.4%) among the controls (p=0.18).

As concerns feeding tolerance, the two groups were not different regarding the age when full enteral feeding was achieved, the age in days at the beginning of human milk fortification, the aspect and volumes of gastric residuals. In Table III results regarding feeding tolerance are shown. Mean highest residual as percentage of the previous feed is 69,2±47for the cases, and 65,2±37,2 for the controls (p=0,75). The total residuals as percentage of the 24 hours feeding are 29,42±29,32 among the cases and 25,18±25,00 among the controls (p=0,48). The weight growth was not significantly different in the two groups (Figure 1). Mortality was not significantly different between the two groups.

Table I. *Neonatal characteristics.*

	CASES (N=46)	CONTROLS (N=46)	P
Gestational age, wks, mean ± SD	28.26 ± 2.27	28.26 ± 2.27	1.00
Birthweight, gr, mean ± SD	968±236	984±230	0.75
Male sex, n(%)	18 (39.1)	20 (43.5)	0.83
Apgar score 1 min, mean ± SD)	5.04±2.74	5.26±2.52	0.69
Apgar score 5 min, mean ± SD	6.96±2.21	7.28±1.52	0.41
Caesarean section, n(%)	38 (82.6)	32 (69.6)	0.22

Table II. *Comorbidities.*

	CASES (N=46)	CONTROLS (N=46)	P
PDA, n (%)	23 (50)	22(48.9)	1.00
Surgery closing PDA, n (%)	4 (8.7)	4 (8.7)	1.00
CVO, days, mean ± SD	5.10±2.24	4.91±2.28	0.20
ROP, n (%)	10 (21.7)	10 (21.7)	1.00
IVH 3°-4° degree, n (%)	3 (10)	2 (7.1)	1.00
NEC, n (%)	1 (2.2)	4 (8.7)	0.36
NEC needing surgery, n (%)	1 (2.2)	0 (0)	1.00
PVL, n (%)	3 (6.7)	3 (6.7)	1.00
RDS, n (%)	35 (76.1)	35 (76.1)	1.00
BPD, n (%)	11 (25.0)	10 (22.2)	0.81

DISCUSSION

Growing clinical evidence has placed human milk feeding and the supply of donor milk as a basic right for preterm infants. Donor milk banking should be promoted, protected, and supported as an extension of national breastfeeding policies(29-30). Given the increasing available evidence on the benefits of donor human milk compared to formula, this type of milk is now routinely used in the centres where a milk bank is present.

A recent revised American Academy of Pediatrics policy statement encourages the practice of supporting mothers to express breast milk for their preterm infants, but also recommends to use "pasteurized donor human milk, appropriately fortified" if the mother's own milk is unavailable or in short supply (31). The latest statement, according to some Authors (32-33) is not fully supported by the evidence (34) and needs further studies to be confirmed.

In our study, no differences have been observed between the group fed mother's milk as a sole diet compared to the group fed donor milk as a sole diet, both in term of clinical outcomes and feeding tolerance and of growth. Regarding the latest, growth seems to be slightly superior in the mother's milk group during the first 20 days of life, a result that would confirm the one observed by Montjaux-Régis and coll in 2011 (33) even if in our study the result is not statistically significant. The main limits of the study are the small sample size and the fact that it is retrospective. A higher number of subjects might be useful to confirm or deny the result, while randomization is not possible due to ethical considerations.

In conclusion, we did not find significant differences in clinical outcome from mother's own milk compared

Table I. *Feeding tolerance in the two groups.*

	Cases (n=46)	Controls (n=46)	P
Day of exclusive enteral feeding (mean ±SD)	30,33±18,48	29,62±14,24	0,84
First day of fortification (mean ±SD)	15,64±2,18	15,70±2,14	0,93
Bloody residual, n(%)	4(8,7)	6(13)	0,74
Blood stained residuals, n(%)	16(34,8)	24(52,2)	0,14
Residuals >10cc, n(%)	10(21,7)	7(15,2)	0,59
2 Residuals >3cc, n(%)	26(56,5)	26(56,5)	1,00

with pasteurized donor milk. Only a slight and statistically not significant difference in growth could be observed, in favour of maternal milk.

Nevertheless, the maximum effort should always be put in supporting and promoting breastfeeding and donor milk used not only as an alternative to mother's milk but also as a breastfeeding promotion and support strategy. When a milk bank is available in fact, significantly less NICU neonates receive formula milk in the first weeks of life (35-36). In Italy, data from NICU patients show that exclusive breastfeeding at discharge is achieved for nearly 30% of neonates when banked milk is available during hospitalization and only for 16% of neonates when it is not. The use of human milk is therefore an instrument to promote breastfeeding in particular for sick and preterm newborns.

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HYPERBILIRUBINEMIA AND MANAGEMENT OF BREASTFEEDING

A. SOLDI[1], P. TONETTO[1], F. CHIALE[1], A. VARALDA[1], C. PEILA[1], G. SABATINO[2], L. OCCHI[1], F. GIULIANI[1], C. PERATHONER[1], G. PRANDI[1]

[1]*Neonatal Unit, University of Turin, Italy*
[2] *Neonatal Intensive Care Unit, University of Chieti, Italy*

Hyperbilirubinemia and jaundice are natural, physiological phenomena which are only to be expected in the neonatal period, within certain limits. The highest percentage of jaundice in breastfed newborns should be evaluated in connection with inadequate management of breastfeeding rather than a direct effect of breast milk. Breastfeeding is also linked to visible jaundice persisting beyond the first two weeks of life ("breast milk jaundice"), but the appearance of skin jaundice is not a reason for interrupting breastfeeding which can and should continue without any interruption in most cases. There have been numerous contributions to the literature which have rescaled the direct role of breast milk both in early jaundice and in the more severe cases of late jaundice. The reviewed guidelines for detection and management of hyperbilirubinemia underline how prevention of badly managed breastfeeding and early support for the couple mother-child are effective prevention measures against severe early-onset jaundice; furthermore, the breastfeeding interruption is no longer recommended as a diagnostic procedure to identify breast milk jaundice because of its low specificity and the risk to disregarding the detection of a potentially dangerous disease.

Skin jaundice appears in the first 5 days of life in about 60% of term or late preterm newborns, for whom mother's milk is the best feeding for its clinical (1) and biological (2)(3)(4)(5) properties. Hyperbilirubinemia is more frequent in breastfed than in formula fed newborns (6). Breastfeeding is linked both to a greater jaundice frequency and intensity in the first postnatal days ("breastfeeding jaundice") and to visible jaundice persisting beyond the first two weeks of life ("breast milk jaundice") (6)(7). After the first two weeks of life and sometimes up to 8-12 weeks after birth, jaundice is still visible in at least one third or half of breastfed newborns as a natural continuation of physiological neonatal jaundice, while late jaundice is practically absent in the artificially fed newborn (7).

The incidence of bilirubin encephalopathy (kernicterus) in the National Records in the U.S.A. and Europe shows that this rare but devastating complication of hyperbilirubinemia is far more frequent in the population of breastfed infants. In the U.S. records, only 2 newborns in 125 received supplements (8).

Early onset neonatal jaundice: "breastfeeding jaundice" or "not enough-breastfeeding jaundice"?

The reason for the link between breastfeeding and neonatal jaundice is not yet fully understood (9), but some factors may be involved, such as inadequate amounts of breast milk along with weight loss and slow intestinal peristalsis and late meconium emission as well as an increase in the enterohepathic circulation of bilirubin (6)(7). As regards bilirubin serum concentration, the optimally breastfed infant does not actually differ from the artificially fed infant in the first five days of life (6)(7).

The risk of inadequate milk introduction in breastfed infants is shown by greater weight loss in the early days of life compared with formula fed infants (7): at least 10-18% of infants who are fed by breast milk alone lose more than 10% of their neonatal weight. In breastfed newborns, that have physiological limitations in bilirubin metabolism, the reduced calorie intake compared with needs, even if some milk is supplied, causes an increase in serum bilirubin by intestinal absorption of unconjugated bilirubin due to poor meconium emission (6)(7)(8)(9)(10)

Key Words: hyperbilirubinemia, jaundice, breast milk jaundice, breastfeeding, inadeguate milk introduction

Corresponding Author: Dr. A. Soldi
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3135775
antonella.soldi@unito.it

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Table I. *Guidelines for jaundice treatment and monitoring.*

2010 - National Institute for Health and Clinical Excellence (NICE). Neonatal jaundice (Clinical guideline #98) (9). [www.nice.org.uk/CG98]
2010 - AMB Clinical Protocol#22: Guidelines for Management of Jaundice in the Breastfeeding Infant Equal or Greater than 35 Weeks' Gestation (6). Breastfeeding Medicine 2010;5:87-93.
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2007 - Canadian Paediatric Society. Guidelines for detection, management and prevention of Hyperbilirubinemia in term and late preterm newborn infants (35 or more weeks' gestation). Paediatr Child Health 2007;12:1B-12B

(11). Moreover, high bilirubin levels lead to lethargy with fewer feeds and less suckling efficacy which can thus cause further reduction in the ability to excrete bilirubin in stools (6)(11)(12). This may lead to redefine early jaundice in breastfed babies more appropriately as "breast non-feeding jaundice" or as "not enough breastfeeding jaundice" since it is not breastfeeding itself which determines jaundice but breastfeeding inadequacy (9). As a result, incidence of early jaundice has been suggested as an evaluation tool in support of breastfeeding by health care professionals (6).

The comparative lower incidence of jaundice in formula fed newborns might therefore be correlated with greater intestinal clearance of bilirubin (6), further supported by the presence of less absorbable lipids in formula milk to which the deconjugated bilirubin is bound in the intestine. This fact seems less evident with the new generation formulas in which the fat is more similar to the one in breast milk (9)(13).

The cases of neonatal jaundice owing to insufficient feed intake are to be identified and treated early in order to avoid, besides dehydration and malnutrition, reaching bilirubin neurotoxicity (6). In recent years, a number of guidelines and clinical protocols have been published on jaundice treatment and monitoring (Table I). All of them recommend special attention in the management of breastfeeding.

The guidelines underline how prevention of badly managed breastfeeding and early support for the couple mother-child are prevention measures against severe hyperbilirubinemia. The success of breastfeeding depends on the possibility of beginning in the immediate postpartum period with good colostrum intake which will enable meconium emission, skin to skin contact, and minimising separation between mother and child using the rooming-in service. The newborn should be allowed to suckle frequently, at least 8-12 times/24 hours and should not receive unnecessary water or formula

Table II. *Causes of extended unconjugated jaundice.*

- Hemorrhage, cephalohaematoma, polycythemia
- ABO or Rh factor incompatibility
- Red blood cells enzyme deficiency (e.g. glucose-6-phosphate dehydrogenase, pyruvate kinase)
- Erythrocyte membrane disorders (e.g. spherocytosis)
- Unsatisfactory amount of breast milk ("not enough breastfeeding jaundice")
- Breastfeeding ("breast milk jaundice")
- Genetic defects of the hepatic metabolism (e.g. deficit of uridine diphosphate glucuronosyltransferase, Crigler-Najjar syndrome types I and II)
- Galactosemia
- Hypothyroidism
- Intestinal obstruction
- Pyloric stenosis

supplements (water intake increases enterohepatic reabsorption of bilirubin) (6)(7)(8)(11)(12). Bilirubin concentration is inversely correlated with the number of feeds and indirectly correlated with the frequency of the supplements (7)(14). Increase in the number of feeds and reduction in water or glucose supplements mean an increase in breast milk, a reduction in weight loss as well as a decrease in bilirubinemia concentration owing to greater intestinal peristalsis with reduced enterohepatic circulation and increased excretion (6)(11).

Feed evaluation is the tool which allows optimization of latching on and milk intake. Moreover, it provides objective data to evaluate if milk intake is satisfactory along with proper monitoring of urine and evacuation as well as a daily weight check. The use of artificial milk (o donor milk if available) (15) supplements is to be restricted to cases of insufficient milk production even when adequate frequent latch proves to be ineffective (6)(12).

The guidelines define severe hyperbilirubinemia risk factors as follows: less than 38 weeks gestational age, appearance of jaundice in the first 24 hours of life, isoimmune haemolytic diseases or genetic causes such as glucose-6-phosphate dehydrogenase deficiency or hereditary spherocytosis, cephalohematoma or other significant bleeding, familial risk or belonging to particular ethnic groups such as Asiatics. The guidelines provide indications and strategies for accurate bilirubin monitoring and initiation of therapy whose aim is to make bilirubin encephalopathy (kernicterus) a "never event". Compared with serum dosage, the use of transcutaneous bilirubinometers allows frequent and non invasive bilirubin monitoring without separating mother and child. However, there are some limitations including the presence of pigmented skin, unfeasibility of use when phototherapy has already been initiated, poor accuracy at gestational age lower of 35 weeks. Serum dosage is advisable for determination in the first 24 hours of life and when deciding possible therapy (6)(9)(10)(11).

The appearance of skin jaundice is not a reason for interrupting breastfeeding which can and should continue without any interruption in most cases (6)(7)(11). During phototherapy, short pauses (up to 30 minutes) should be scheduled to allow for breastfeeding and taking care of the newborn, unless bilirubin values are near threshold values due to exanguinotransfusion or if the increase in serum levels are higher than 0.5 mg/day/hour. This pause pattern (up to 30 minutes every 3 hours) for breastfeeding has been demonstrated as not compromising the effectiveness of phototherapy (6)(9). Studies are currently underway to evaluate the effectiveness and tolerability of the different patterns of phototherapy withdrawal to allow frequent feeds (45 or 60 minute pauses on request/every hour/every 2 hours) (6). The use of optic fibre devices allows newborns to be breastfed without therapy withdrawal. Moreover, they do not need eyes pads to protect retina. One disadvantage, however, concerns the possibility of a lower peak of intensity compared with fluorescent lamps (11).

In rare cases of serious hyperbilirubinemia it might

be necessary to interrupt breastfeeding for intensive phototherapy or during exanguinotransfusion (6)(9)(11); when latch is not possible, milk expression is to be guaranteed to feed the newborn and to maintain lactation (6)(9)(12)(14). Although phototherapy increases insensitive loss of fluid, routine intravenous infusions are not necessary except for dehydrated or hypernatremic newborns or those who are unable to take adequate amounts of milk by tube feeding (9).

Breast milk jaundice. Even the mechanism of late jaundice is not completely clear. Several substances present in milk are involved in the pathogenesis: pregnan-3α-20β-diolo, progesterone metabolite and hepatic glucuroniltransferase inhibitor; inflammatory cytokine, above all interleuchine IL1β e IL6, with cholestatic effect; EGF (epidermal growth factor), which could cause reduced intestinal motility and increased bilirubin resorption. High levels of α-fetoprotein have been noted in the serum of newborns with breast milk jaundice, however the meaning of this is not understood (9)(16)(17).

Breast milk jaundice is by definition (16) the prolongation of unconjugated hyperbilirubinemia, in a breastfed baby with regular weight gain and normal production of urine and stools, with no signs or symptoms of disease and it is benign: the level of bilirubin does not exceed 12 mg/dl and decreases over time returning to normal values even if breastfeeding continues.

If the child is well and the bilirubin levels remain below the threshold value for phototherapy, no treatment is indicated. However, extended jaundice may also be a sign of disease and differential diagnosis is to be carefully evaluated (Table II), underlining that breast milk jaundice is a diagnosis of exclusion (6)(9)(16)(17).

In the past, breastfeeding interruption was proposed as a diagnostic procedure to identify breast milk jaundice but this procedure is no longer recommended because of its low specificity. In fact, the mere withdrawal of breast milk and the use of formula may itself determine the reduction in bilirubin levels regardless of the underlying cause of jaundice, providing falsely reassuring indications which might even lead to disregarding the detection of a potentially dangerous disease. Moreover, even if breastfeeding withdrawal is short, it places the newborn's ability to return to exclusive breastfeeding at risk, which might be uselessly harmful for the child and a source of worry such to discourage the mother. There have been numerous contributions to the literature which have rescaled the direct role of breast milk both in early jaundice and in the more severe cases of late jaundice. However, notwithstanding the WHO Recommendations on the duration of breastfeeding, breast milk withdrawal and its substitution with formula feeding has been and is still used as a therapy or even as a diagnostic tool in late jaundice. This approach is not recommended by the recent available guidelines since it is has an opposite effect on the continuation of breastfeeding.

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COMMUNICATION IN NICUS

A. COSCIA[1], C. MARTANO[1], F. CRESI[1], P. TONETTO[1], F. GIULIANI[1], M. FORNO[2], S. QUADRINO[2], M. MOMBRÒ[1], C. FABRIS [1]

[1]*Neonatal Unit, University of Turin, Italy*
[2]*CHANGE Institute, Turin, Italy*

In a Neonatal Intensive Care Unit (NICU) counseling should be a 'shared culture' for all the care givers: it should be developed by all the professionals, to face up to parents' needs of information, explanations, facility of decisions, finding of resources, agreement, help, reassurance, attention. The first essential aspect is the training in counseling skills, by periodic courses for all professionals of the department (physicians, nurses, and physiotherapists). In our department, a professional counselor is present, assisting the medical staff in direct counseling. The counselor's intervention allows a better parent orientation in the situation. A more effective sharing of these rules also facilitates the communication among parents and medical staff. Periodic meetings are established among the medical staff, in which the professional counselor discusses difficult situations to share possible communicative strategies. We wanted to have not only a common communicative style, but also common subjects, independent from the characteristics of each of us. Individuals are often faced with different situations. For every setting that we more frequently face in comm unication (for example the first interview with a parent of a very preterm infant) we have built an 'algorithm' that follows a pattern: (1) information always given; (2) frequent questions from parents; and (3) frequent difficulties in the communication. Counselling is also a tool to face some critical issue, such as the decision to open the department to parents 24 h on 24, or the promotion of mother's milk use in Very Low Birth Weight Infants (VLBWI).

In a Neonatal Intensive Care Unit the care is global, based on the newborn and on its family. This inseparable triad must be supported during the hospitalization both by the medical plan and the aware communication between the staff and those who receive the care.

"Not alone": a counselling program in NICU

The birth is physiologically accompanied by considerable expectations from parents, idyllic imagination and positive images. However, when something in birth goes wrong, as in the case of a very preterm birth, the abrupt change of perspective spreads a "grey" veil on the image that parents created. This "veil" makes it difficult to partially perceive ways of improvement. The arrival of a new newborn in the NICU leads to not only the infant's entry but the parents' too. It is a very delicate moment, rich in emotional tension: the father is usually the first one to be present at the door. He is very often alone, the entrance in NICU is disorientating, meeting with the baby and its image goes over every expectation. The father's and beyond the mother's reception is a crucial moment, the requested information is so vast, the risk to be misunderstood is very high. On one side, parents' precise requirements do exist, above all to understand what is happening, but also the necessity to get information (and therefore to recognize the right person to ask and to choose the questions). Besides, parents are asked to carry on an important task: to succeed in tolerating the wait (for examinations, checks, answers…) and therefore to bear the uncertainty, also preserving hope. This task is difficult to face up alone: for this reason the project related to counselling at our Intensive Care Unit is called "Not alone".

For these requirements, the difficult objectives of the medical staff are:

– To find the correct words

– To decide what to say

– Not to use a technical language but not to lose authoritativeness

– To complete the information given by others

Key Words: Counselling, communication strategies

Corresponding Author: Dr. A. Coscia
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3134437
alessandra.coscia@unito.it

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– To understand if parents have understood or if they need more information

– To inform without upsetting

– Not to delude, but also not to remove hope

Counselling arises from the need to satisfy these requirements. Therefore counselling addresses to people (as infant's parents in Intensive Care Unit) who are living a difficult or unexpected situation and they are able neither to modify nor to avoid it. They do not necessarily ask for a psychological intervention, but they have demand for information, orientation, someone who knows how to let them see the resources that they really have, to be able to find sustainable solutions and to face up to their emotions (1-4).

Counselling is developed by all the professionals that intervene in that situation or that are involved to face up to those requirements of information, explanations, facility of decisions, finding of resources, agreement, help, reassurance, attention.

In medicine an important aim of counselling is to act as a "resiliency" factor, which supports parents' passage from the "victim's" to an "active" role. A shocking event (such as preterm birth) physiologically and inevitably produces parents' reactions as incredulity, followed by anger, mistrust and sense of guilt.

If parents are left alone to face up to such a situation, they will inevitably go to search for causes of what has happened and on this search they do not get over the situation. It is a faulty circle that departs from the emphasis on the past and it stops on the feeling of anger, impotence, report, with the parents' risk to take the role of victims looking for revenge and medical–legal consequences.

Instead, if parents are assisted with support, the passage to confront each other is more probable as the awareness of what has happened and the projection toward the future; they acquire an active role and become responsible in care.

A shared culture: counselling and its different aspects

The project "Not alone", put into work thanks to the collaboration of the institute of systemic-counselling "Change", contemplates various aspects, all to let counselling become a shared culture (5).

The first essential aspect is to form the ability of counselling through periodic courses for all professionals of the department (physicians, nurses, physiotherapists). Obviously, the extension of abilities of counselling is useful to other professionals (experts, advisors) and to other departments more frequently involved. In our department a professional counsellor is present assisting the medical staff in direct counselling. Parents are directly contacted in our a department, or on announcement of the medical staff or from the counsellor. Many couples accept the meetings proposed.

In a moment full of emotions, information is sometimes unclear; when a new life breaks into everyday life, the support of this figure, speaking about their living, helping parents to understand the situation, is a kind of well accepted help. The counsellor's intervention has also been helpful also for other professionals: psychologists, neuropsychiatrists, etc., when initially the necessity to be helped was denied.

The counsellor's intervention allows a better parent orientation in the situation. A more effective sharing of these rules also facilitates the communication among parents and medical staff.

Periodic meetings are established among the medical staff, in which the professional counsellor discusses difficult situations in order to share possible communicative strategies.

We wanted to have not only a common communicative style, but also common subjects, independent from the characteristics of each of us. Individuals are often faced with diverse situations. For every setting that we more frequently face in communication (for example the first interview with a parent of a very preterm infant) we have built an "algorithm" that follows a pattern: 1) information always given; 2) frequent questions from parents, 3) frequent difficulties in the communication.

The draft of the algorithm has been introduced then to all the professionals, who gave a vote from 1 to 4 to every point of the algorithm, according to their smaller or greater agreement with the proposed solutions. The most critical points have been discussed and we have arrived at a final solution.

The "case history of the communication"

To put the communication into effect would also need to record important moments, for instance the "case history of the communication", a tool that we are still studying: in fact it would be desirable to have the case history, a sheet dedicated to important communications that are absolutely to be shared with other professionals. Counselling for us has also been something more. The following phase has been to face the critical moments, the new steps.

In this case counselling becomes "element of development". The decision to open the department to parents 24 h on 24 has been an important moment in our reality.

When a baby is born, its parents bind it to the world. If during the care process there is the possibility of not separating them, the future behavior really changes: the newborn is stronger and the time of hospitalization decreases. To make it happen we must consider parents' life outside our Intensive Care Unit: some couples have got other children at home, the father usually works, moving in the city takes a long time. Based on these requests, the

Care group of our NICU proposed to the medical staff and nurses to open of the department 24 h on 24. In the months that preceded the NICU's opening, there have been a series of meetings in which the individual brought to the whole meeting own ideas and fears, absolutely justified. We must not forget that to be under the continuous surveillance of someone leads us to be judged, to lose own distances and perhaps the own "power". However, this introduction has not weakened the discussion, rather it has made it charming and rich of contents. The counsellor's presence and mediation allowed to find the resources in each individual and in the group and also the correct objective. The rules are written in a brochure that is given to parents by the counsellor that first welcomes them, and then are commented and discussed by the counsellor in the first meeting with parents: it is a questionnaire, including those questions that could be addressed by parents, and the relative answers.

Another important challenge in a NICU is the promotion of mother's milk use in Very Low Birth Weight Infants (VLBWI): this practice requires not only organization issues, but also an adequate staff attitude toward breastfeeding promotion and use of human milk, and specific optimal communication skills.

CONCLUSIONS

Learning communication techniques is essential to overcome differences among organization issues, adequate staff attitudes toward breastfeeding promotion and specific optimal communication skills, that depend on professional training background, professional interest and personal history (6).

Our group is constituted by imperfect professionals, but this group aims to learn and to look for new solutions for newborns and their families. We try to take advantage of an imperfect result, but we test and try every valid project for the health of those whom we care.

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METABOLISM AND BIOLOGICAL FUNCTIONS OF HUMAN MILK OLIGOSACCHARIDES

E. BERTINO[1], C. PEILA[1], F. GIULIANI[1], C. MARTANO[1], F. CRESI[1], P. DI NICOLA[1], L. OCCHI[1], G. SABATINO[2], C. FABRIS[1]

[1] *Neonatal Unit, University of Turin, Italy*
[2] *Neonatal Intensive Care Unit, University of Chieti, Italy*

It is well known that breastfeeding is beneficial both for its nutritional properties and for the presence of biologically active compounds. Among these, human milk oligosaccharides (HMOs), representing the third largest fraction of human milk, have been assigned important biological functions, such as prebiotic and immunomodulatory and antimicrobial effects. HMOs are synthesized in the mammary gland by glycosyltransferase enzymes and can be divided in core-oligosaccharides, sialo-oligosaccharides, fucosyl-oligosaccharides and sialo-fucosyl-oligosaccharides on the basis of their chemical structure. Glycosyltransferases enzymes are partially regulated by genetic mechanisms; according to the expression of secretory and Lewis' genes, it is possible to classify human milk in 4 different secretory groups. We hereby present a review of the current knowledge concerning HMOs, their metabolism and main biological functions.

Human milk oligosaccharides (HMOs), from a quantitative point of view, are the third component of human milk, after lactose and lipids [1]. They are carbohydrates composed of 3-10 monosaccharide units, and their content is 20-23 g/l in the colostrum and 12-14 g/l in mature milk [2].

Vaccine milk, the basic component of all formula milks, only contains a small quantity of oligosaccharides (<1g/l). Additionally, their chemical structure and composition are different from those contained in human milk [3]. With the use of modern analytical methods, several hundred HMOs have been identified [4]. However, it is important to emphasize that the main quantitative component of HMOs includes 20 to 25 oligosaccharides; the remaining oligosaccharides constitute only minimal amounts.

HMOs are synthesized in the mammary gland by the action of specific glycosyltransferases, through the sequential addition of galactose, fucose, *N*-acetylglucosamine (GlcNAc), and *N*-acetylneuraminic acid (Neu5Ac) to the lactose molecule. On the basis of their composition, HMOs can be classified as follows: core oligosaccharides, made of glucose, galactose, and GlcNAc, which represent the basic structures for the synthesis of more-complex molecules; [5] fucosyl-oligosaccharides, derived from the addition of ≥1 unit of fucose to the core; and [6] sialyl-oligosaccharides, derived from the addition of ≥1 unit of Neu5Ac to the core or to fucosyl-oligosaccharides. The presence of glycosyltransferases in the mammary gland is determined genetically; most of them are common to all women with the exception of fucosyltransferases, the activity of which is linked to expression of the secretor (*Se*) and Lewis (*Le*) genes [7]. The roles of the specific fucosyltransferases involved in different steps of HMO synthesis have been described [8].

Briefly, the fucosyltransferase encoded by the *Se* gene regulates the synthesis of HMOs containing fucose with an α-1,2 linkage; the *Le* gene promotes the synthesis of α-1,3 and α-1,4 linkages. Moreover, there are *Le* gene-independent fucosyltransferases that promote the synthesis of α-1,3-oligosaccharides that are present in all women.

Depending on the expression of *Se* and *Le* genes, both qualitative and quantitative compositions of HMOs are significantly conditioned. With reference to the presence or absence of specific fucosyl-oligosaccharides, 4 different groups of milk were hypothesized [7] and successively demonstrated [9]; group 1 milk (*Se*+/*Le*+; ≈ 70% of the European population) contains all types of fucosyl-oligosaccharides, with α-1,2, α-1,3, and α-1,4 linkages, group 2 milk (*Se*−/*Le*+; 20% of the population) does not contain α-1,2-fucosyl-oligosaccharides, because

Key Words: oligosaccharides; human milk; preterm infants

Corresponding Author: Prof. E. Bertino
Neonatal Unit, University of Turin
Via Ventimiglia 3, 10126 Turin, Italy
+39 011 3135775
enrico.bertino@unito.it

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of the lack of *Se* gene expression, group 3 milk (*Se*+/*Le*−; 9% of the general population) is characterized by the absence of α-1,4-fucosyl-oligosaccharides, because of inactivity of the *Le*gene, and group 4 milk (*Se*−/*Le*−; 1% of the general population) contains only α-1,3-fucosyl-oligosaccharides attributable to the expression of *Le*-independent fucosyltransferases.

Oligosaccharide metabolism

The breastfed newborn typically has a daily intake of several grams of HMOs (7-12 g/die).

A peculiar characteristic of these substances is that the chemical bonds linking the monosaccharide monomers of which HMOs are composed are resistant to hydrolysis catalyzed by enzymes present in the digestive tract (*e.g.*, lactase, saccharase-isomaltase, maltase-glucoamylase, trealase, amylase). Consequently, a significant portion of oligosaccharides ingested with mother milk passes without being digested through the small intestine, reaching the colon. Around 35-50% of the ingested HMOs can be found in the feces of breastfed newborns [10].

The remaining part (50-65%) follows different metabolic pathways (*i.e.*, partial transformation by intestinal bacteria or degradation by hydrolytic enzymes present in milk, such as fucosidase, N-acetyl-glucosaminidase, etc.). More recent studies suggest multistage HMO processing and degradation that depend on infant age, blood group and feeding regime [11]

Finally, another part is absorbed without being metabolized by the intestinal wall, as demonstrated by the presence of unmodified HMOs both in serum and in urine of breastfed newborns [12,13].

Physiological role

The precise mechanisms underlying the digestion and absorption of HMOs are still not completely understood. Undoubtedly, a certain amount is used as an energy source, but besides their nutritional function, oligosaccharides play a role in other important physiological processes.

Among physiological effects elicited by HMOs, the most important are:

- Prebiotic effect
- Inibition of bacterial adhesion
- Source of dietary fibers
- Source of fucose and syalic acid
- Immunomodulation

Prebiotic effect

According to the most recent findings, HMOs can be classified amongst prebiotic substances [14;15]. As a matter of fact, HMOs can assist the development of bifidogenic bacteria (such as *Lactobacillus bifidus*) in the intestinal tract of newborns, thereby protecting from gastrointestinal infections by lowering the intestinal pH and by creating a microenvironment unfitting for the proliferation of pathogenic bacteria [16].

HMOs also resist digestion [17] and reach the colon, where they selectively stimulate the development and the activity of *Bifidobacteria* and *Lactobacilli*, thus eliciting beneficial effects for the health of newborns [18;19;20], such as:

- Increase of nutrients and minerals absorption, in particular calcium and magnesium;
- Reduction of pathogenic bacteria proliferation;
- Stimulation of immune system;
- Regulation of the number of stool emissions.

Unmodified oligosaccharides reaching the colon may be subject to fermentation processes caused by resident bacteria [21], with concomitant production of gases and short-chain fatty acids (acetate, propionate and butyrate) which can be used as an energy source, have a trophic effect on mucoses and assist water reabsorption.

Inhibition of bacterial adhesion

It is a well-known fact that bacteria, viruses and toxins are able to produce their pathogenic effect on a host cell through the adhesion to specific receptors located on the cell membrane. These receptors are often represented by the glucidic residues of membrane glycoproteins and glycolipids.

Thanks to their glucidic structure, HMOs are able to act as "soluble receptor analogs" and to bind pathogen agents through a competitive mechanism, thus preventing their adhesion to cell receptors [22] and assisting their removal from the organism. This mechanism has been demonstrated both at gastrointestinal, respiratory and urinary level [23].

Clinical studies showed that newborns fed milk containing α-1,2 fucosyl-oligosaccharides (groups 1 and 3) experienced significant reductions in acute diarrheal episodes, particularly those attributable to *Campylobacter* and *Calicivirus* [24]. Antiadhesive antimicrobial effects may not be restricted to bacteria and viruses; they might also apply to certain protozoan parasites like Entamoeba histolytica, which causes amoebic dysentery or amoebic liver abscess [25]. Another mechanism through which oligosaccharides may play a protective function involves the glycome-modifying effect. When oligosaccharides come into contact with intestinal epithelial cells, they are able to change the specific cellular receptor structure, which leads to marked reductions in the adherence of pathogens.

Source of dietary fibers

HMOs may be considered as dietary fibers, since they reach undigested the colon and are eliminated through feces [26].

Source of fucose and syalic acid

Fucose, N-acetyl-glucosamine and syalic acid deriving from the hydrolysis of HMOs are readily available for the synthesis of biologically-active substances such as gangliosides and cerebral glycoproteins, involved in the central nervous system development [27].

In animal studies, the exogenous administration of Neu5Ac resulted in increased concentrations of brain gangliosides and glycoproteins, which are linked to improvements in learning performance [28]. Moreover, Wang et al [29] demonstrated higher brain ganglioside and glycoprotein sialic acid concentrations in breastfed infants, compared with formula-fed infants.

Immunomodulation

In addition to feces, oligosaccharides with the same pattern as in ingested milk have been found in urine of breastfed infants [12;13]. This finding, which is strictly related to high newborn intestinal permeability, demonstrates that a quota of oligosaccharides reaches the bloodstream. As a consequence, oligosaccharides may have an important role at systemic level, modulating the first step of the inflammatory process (*i.e.*, the adhesion of neutrophil leukocytes to the blood vessel wall through the link to selectin).

Since the HMOs chemical structure and conformation are similar to those of selectin glycoproteins, oligosaccharides can therefore bind to the circulating leukocytes and prevent their adhesion to the blood vessel walls [30]. Eiwegger moreover suggest that sialylated HMOs influence lymphocyte maturation and promote a shift in T-cell response toward a more balanced Th1/Th2-cytokine production and low-level immunity [31]. Sialylated HMOs also reduce IL-4 production in a subset of lymphocytes from adult patients with peanut allergy, which led to the conclusion that certain sialylated HMOs may contribute to allergy prevention [32].

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COW'S MILK PROTEINS IN HUMAN MILK

A. COSCIA[1], S. ORRÙ[2], P. DI NICOLA[1], F. GIULIANI[1], I. ROVELLI[1], C. PEILA[1], C. MARTANO[1], F. CHIALE[1], E. BERTINO[1]

[1]*Neonatal Unit, University of Turin, Italy*
[2]*CNR, Institute of Science of Food production, Turin, Italy*

Cow's milk proteins (CMPs) are among the best characterized food allergens. Cow's milk contains more than twenty five different proteins, but only whey proteins α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), and lactoferrin, as well as the four caseins, have been identified as allergens. Aim of this study was to investigate by proteomics techniques cow's milk allergens in human colostrum of term and preterm newborns' mothers, not previously detected, in order to understand if such allergens could be cause of sensitization during lactation. Term colostrum samples from 62 healthy mothers and preterm colostrum samples from 11 healthy mothers were collected for this purpose. The most relevant finding was the detection of the intact bovine alpha-S1-casein in both term and preterm colostrum. Using this method, which allows direct proteins identification, β-lactoglobulin was not detected in any of colostrum samples. According to our results bovine alpha 1 casein that is considered a major cow's milk allergen is readily secreted in human milk: further investigations are needed in order to clarify if alpha-1-casein has a major role in sensitization or tolerance to cow's milk of exclusively breastfed predisposed infants.

Cow's milk contains more than twenty five different proteins, but only whey proteins á-lactalbumin, â-lactoglobulin, bovine serum albumin (BSA), and lactoferrin, as well as the four caseins, have been identified as allergens (1). In particular, the casein fraction is composed of áS1-, áS2-, â-, and ê-casein, of which áS1-casein seems to be the major allergen according to IgE and T cell recognition data (2-4).

By now the detection of the above mentioned food allergens in human milk has been achieved by sandwich Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoblotting techniques. (3). ELISA and Immunoblotting techniques have the disadvantage of possible cross reactions with other proteins that could be present in the sample and don't take into account the possible chemical modification or proteolytic digestion of the proteins (5). Aim of this study was to investigate cow's milk allergens in human colostrum of term and preterm newborns' mothers by proteomics techniques in order to understand if cow's milk allergens could be a cause of sensitization established through lactation.

MATERIALS AND METHODS

Term colostrum samples were collected from 62 healthy mothers delivering at term (36 completed week of gestation).

Preterm colostrum samples were collected from eleven healthy mothers who delivered prematurely (between 25 and 30 weeks of gestational age).

All samples were collected from mothers following a non restricted diet including cow's milk and derivatives, just after breastfeeding their own babies.

Samples were gathered up until the 4th day after delivery and frozen and stored at -80°C immediately after collection. Before analysis samples were defrosted at room temperature. Two different pools of 245 mL were created, respectively one of Colostrum of mothers delivering Term infants (CT) and another one of Colostrum of mothers delivering Preterm infants (CP). Then in each pool (CT and CP) five complete protease inhibitor cocktail tablets were added.

The sensitive detection of less represented new types of proteins contained in the whey fraction was made through the introduction of an additional prefractionation step. This step theoretically increases the concentration of the most diluted proteins and simultaneously reduces the concentration of the

Key Words: cow's milk proteins, proteomics techniques, allergens

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Corresponding Author:
Dr. S. Orrù
sabrinaorru@yahoo.it

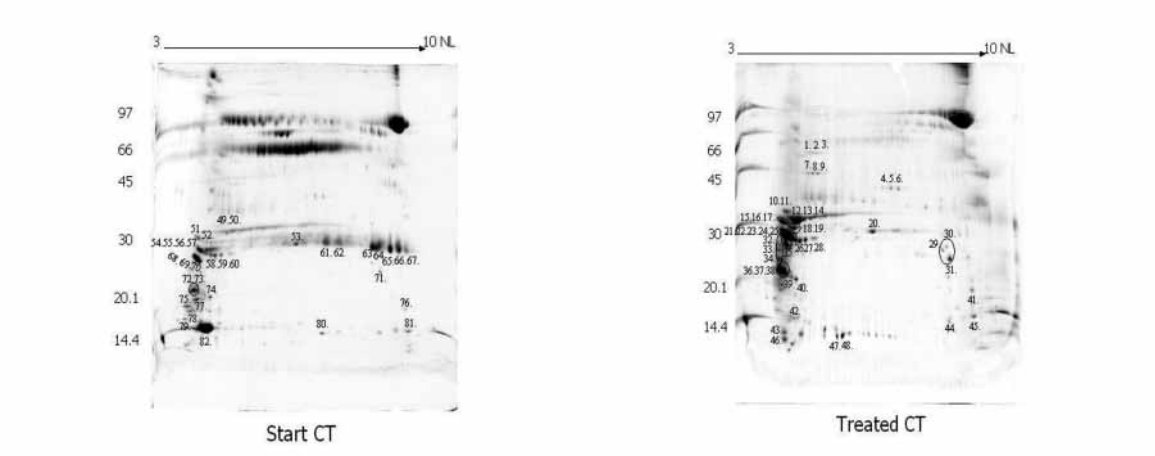

Fig. 1. *2D-Page map of 120 µg of protein in Start and Treated Term colostrum samples (CT).*

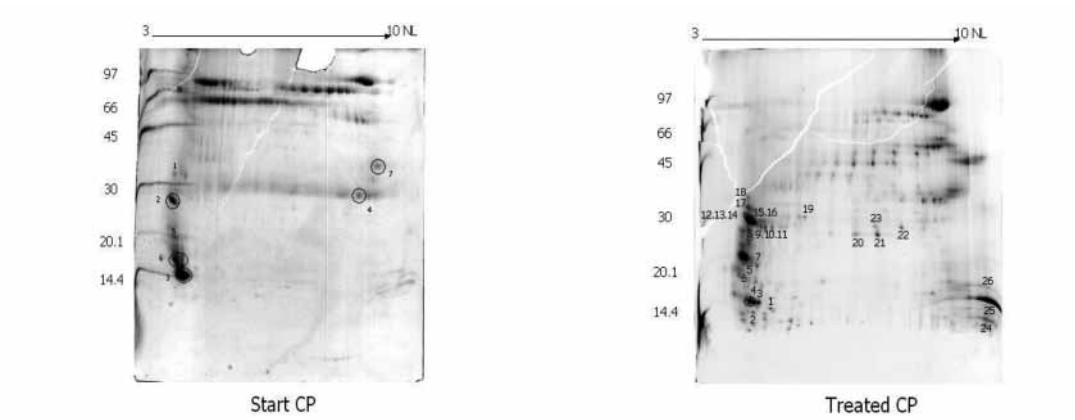

Fig. 1. *2D-Page map of 120 µg of protein in Start and Treated Preterm colostrum samples.*

proteins present at high concentration (Proteominer Treatment).

Subsequently each sample were subjected to the steps of proteomic techniques (loading on gel, image acquisition by proteomic imaging system, in-gel tryptic digestion of proteins and protein identification by tandem mass spectrometry).

RESULTS

The most important result achieved in this study is the detection in human colostrum of one of the major allergens found in bovine milk: the intact isoform of bovine alpha-S1-casein (spot n.39 in Fig. 1).

Also in the preterm colostrum sample, most of the proteins identified were different isoforms of caseins not previously reported in literature. (spot n.1 and 18 in Fig. 2 and Tab. I)

The identification of alpha-S1-casein, the bovine allergenic protein in human colostrum, was possible due to the higher sensitivity, the specificity and the reproducibility of the proteomics technique: for the first time such protein was detected using mass spectrometry based methods, that are more reliable than immunoassay methods, used in the past.

DISCUSSION

The most relevant finding in this study was the detection of the intact bovine alpha-S1-casein in human colostrum. In previous studies â-lactoglobulin, (6) one of the major cow's milk allergen, was detected intact in human milk (6-8) in estimated concentrations of nanograms per litre during their peak concentration by

Spot no	Sample Name	Protein name	UniProtKB/Swiss-Prot Entry name	UniProtKB /Swiss-Prot Accession number	Protein Mr	Protein p.I.	Protein sequence coverage	Peptide sequence
39	Treated CT	Alpha-S1-casein	CASA1_BOVIN	P02662	24570	4,98	14%	FFVAPFPEVFGK.E (38-49) EPMIGVNQELAYFYPEL FR.Q (148-166)
1	Start CP	Alpha-S1-casein	CASA1_BOVIN	P02662	24570	4,98	17%	HQGLPQEVLNENLLR.F (23-37) FFVAPFPEVFGK.E (38-49) YLGYLEQLLR.L (106-115)
18	Treated CP	Alpha-S1-casein	CASA1_BOVIN	P02662	24570	4,98	26%	HQGLPQEVLNENLLR.F (23-37) FFVAPFPEVFGK.E (38-49) YLGYLEQLLR.L (106-115) EPMIGVNQELAYFYPEL FR.Q (148-166)

Tab. 1. *Identification of Apha-s1-casein from start CP, treated CP and Treated CT.*

immunoassay based techniques. (6-8) Such techniques are very sensitive methods of detection of food allergens, yet they have the disadvantage of cross reacting with other proteins that could be present in the sample. (5) The problem of cross-reactivity is not negligible. Specific IgE (sIgE) for cow's milk proteins (CMP) have been reported to be present in blood sera of exclusively breast-fed infants: sIgE also for human milk whey proteins were found in the blood sera of atopic infants, and these sIgE strongly cross-reacted with the corresponding CMP (9). Moreover, immunoassay based methods focus on the detection of the intact protein and don't take into account the possible chemical modification or proteolytic digestion of proteins (5). On the contrary in our study, using ProteoMiner tecniques, â-lactoglobulin was not detected in preterm or in term human colostrum. In fact mass spectrometry methods have high sensitivity, specificity and reproducibility and using a prefractionation step they increase the concentration of the most diluted proteins and simultaneously reduce the concentration of proteins present at high concentration. For this reason they can be considered more reliable than immunoassay methods in the detection of hidden allergens at very low concentration. According to our results bovine alpha-1-casein could be considered the cow's milk allergen that is readily secreted in human milk. As a consequence this protein could be a cause of sensitization to cow's milk in exclusively breastfed predisposed infants

It is interesting to note some differences observed in preterm colostrum compared to term colostrum samples, in particular a higher concentration of bovine alpha-1-casein. A clear explanation of this observation is lacking. A possibility could be the different membrane permeability observed in mothers who delivered prematurely.

Further investigations are needed in order to clarify the clinical meaning of our findings in particular if alpha-1-casein has a major role in sensitization or in tolerance to cow's milk of exclusively breastfed predisposed infants. In fact the pathogenesis of food allergy is multifactorial, and it is also likely that the induction of oral tolerance is dependent on several conditions being met. For example, it might be important that food allergens be presented to the gastrointestinal tract in the context of breast milk, which contains numerous immunomodulatory and tolerogenic factors, such as oligosaccharides or TNF-â (10-12).

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HIGHER PROTEIN INTAKE STRATEGIES IN HUMAN MILK FORTIFICATION FOR PRETERMS INFANTS FEEDING. AUXOLOGICAL AND NEURODEVELOPMENTAL OUTCOME.

A. BIASINI[1], C. NERI[2], M.C. CHINA[1], F. MONTI[2], P. DI NICOLA[3], E. BERTINO[3]

[1]*Pediatric and Neonatal Intensive Care Unit, Bufalini Hospital Cesena, Italy*
[2]*Departmen of Psychology. University of Bologna, Italy*
[3]*Neonatal Unit, University of Turin, Italy*

Postnatal growth restriction and failure to thrive still remain a major problem in Extremely Low Birth Weight (ELBW) infants . The goal for the nutritional care of these infants is to achieve rate of growth similar to those of the fetus in utero at the equivalent gestational age. Human milk fortified remains the best food for all these preterms. Two groups of preterm of weight 580-1250 g and gestational age 23-32 wk, were fed with different protein intake in the human/maternal milk fortified (3,5 g Kg^{-1} per day and 4,8 g Kg^{-1} per day in the control and intervention group respectively).The feeding tolerance, intrahospital growth, neurological outcome and anthropometric data until 12 months of corrected age, were evaluated. The protein supplemented group (PSG) showed an intrahospital highter growth rate (mostly in head circumference, p 0,02, and length growth, p 0,04) only in the preterms with 580-980 g and 23-30 wk. In the same preterms, Griffith Development Mental Score at 3 and 12 months corrected age showed higher score than in the control group in the Performance (p 0,04) and Hearing/Language (p 0,03) items. The auxological evaluation in the postdischarge period showed in the PSG group mean z-score values for length higher than those in the control group at 9 (p 0,04) months of corrected age.

Extra uterine growth failure is still common in the Extremely Low Birth Weight (ELBW) infant during their hospitalization in Neonatal Intensive Care Unit (NICU) and it has been shown to be a negative prognostic factor for long term outcome (1). Preventing weight-loss with adequate nutrition is one of the most important goals for these neonates (2). The appropriate growth of lean body components, mostly the brain, but also the the development of fat mass, in neonates depends on their protein intake (3). Indeed, the degree of undernutrition and of growth inhibition account for some cognitive deficits present in a large proportion of preterm infants (4). The beneficial effects of human milk and of its enrichment are well-known, but the standard protein fortification does not fulfil the neonate's entire need for protein intake (5). We have tried to demonstrate that through the use of protein enriched milk, it is possible to maintain the advantages of breast milk without penalizing the preterm's growth. We aimed to improve the preterm's physical growth by maximizing the quantity of proteins in human milk according to the neonate's metabolic capacity derived by urea values.

MATERIALS AND METHODS

Two groups of preterm neonates of weight 580-1250 g and gestational age 23-32 wk, born between January 2010 and March 2011, were fed with maternal or bank milk of different protein contents from the first day of full enteral feeding with standard fortification. The end of the study was set at the time of discharge, transfer or when the baby was able to ingest > 50% of his prescribed quantity directly from the breast of his mother. Growth, biochemical indices of nutritional status, enteral intake, feeding tolerance, clinical histories and morbidity were assessed serially. The primary outcome variable was intrahospital anthropometric data growth (length was measured to the nearest cm using a length board) and the effects of the supplemented protein regime on the growth and neurodevelopment in the first year of age. Griffith development mental scales (GDMS) (10), Milani Comparetti and Amiel Tison neurological assessmernt

Key Words: growth, protein intake, human milk, preterm infants

Corresponding Author: Dr. A. Biasini
Pediatric and Neonatal Intensive Care Unit,
Bufalini Hospital Cesena
Viale Ghirotti 286, Cesena, Italy
+39 0547 352111
abiasini@ausl-cesena.emr.it

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Table I. *Anthropometric data growth in the ELBW of both groups.*

Data growth in neonates with *Weight g 580 – 980* *GA wk 23 – 30*	*Protein supplemen. group* *N 19*	*Control group* *N 13*	**P value**
Weight gain mean g/Kg/per day (SD)	19,5 (2,4)	18,1(1,0)	**0,05**
Length growth cm/wk (SD)	1,07 (0,25)	0,91 (0,2)	**0,04**
Head circumference Growth cm/wk (SD)	0,92 (0,1)	0,82 (0,1)	**0,02**
Milk volume intake ml/Kg/per day	160,2 (4,3)	163,1 (5,6)	**0,07**

corrected for gestational age were used in the clinical follow up. In both groups, about 60% of milk was provided by the infants' own mother and 40% was pasteurized donor milk from the hospital's milk bank. Adjustments to the level of fortification were made based on blood urea levels that were being monitored twice per week. Fortification level 1, that is the routine target in our unit (6), was achieved by increasing the amount of BMF (Aptamil) until 5 g/100ml and was set for the control group (CG: 27 newborn), whereas level 2 and 3 were obtained by graded amounts of protein (Protifar, Nutricia) only in the supplemented protein group (SPG: 34 newborn).The informed parent's consent was requested and acquired only for the latter sample. Every time the urea value was less than 40 mg/ml, fortification was yielded by one level. If the urea was 40 to 45 mg/dl without metabolic acidosis (pH > 7,30 and BE < - 4) no change in fortification was made. The max fortification in the CG was obtained by increasing BMF to 5% and was kept fixed around urea values of 45 - 50 mg/dl. The max fortification in the SPG instead, was reached with BMF 5% plus 1% of Protifar according to the tolerance of the neonate.

RESULTS

Assuming protein human milk content of 0,8-1,1 gr/dl and the prescribed diet volume intake of 160 ml Kg^{-1} per day the max protein intake should have been about 3,5 g Kg^{-1} per day in the control group whereas the extraprotein group in the level 3, would achieve a protein intake of 4,8 g Kg^{-1} per day. The energy intake group was 135 $Kcal^{-1}$ per day and 141 $Kcal^{-1}$ per day in the control and extraprotein group respectively. Anova ,t-test and χ^2 test were used for analysis, significance was accepted when $p < 0,05$. The growth speed of the SPG did not show any significant advantage versus the CG (weight g/Kg/day: 19,0 ± 2,2 versus 18,8 ± 2,1 ; head circumference growth cm/wk: 0,88 ± 0,17 versus 0,88 ± 0,35; length growth cm/wk: 1,04 ± 0,2 versus 0,88 ± 0,35) although exceeded the uterine one by 25% (+ 3,7 gr/Kg/die). The SPG showed significant higher levels of Urea mg/dl (32,9 ± 7,4) versus the CG (26,2 ± 14) p 0,02 and lower non significant Ph values (7,32 ± 0,03 versus 7,33 ± 0,03). The milk volume effectively given to the control group was slightly more in the CG than the PSG (162,7 ± 6,5 versus 160,4± 4,0) p 0,08. The tolerance to the the hyperproteic diet was quite accettable, metabolic acidosis and ipercreatinine were not checked more then

Table II. *Mean z-score values calculated with World Health Organization curves, at 3, 6, 9 and 12 months of corrected age (m.c.a.) in the two groups of preterms. Although the difference became statistically apparent only at 9 m.c.a. for the length (-0,85 versus -1,27 p 0,04 respectively), the overall values were always higher in the protein supplemented group (PSG).*

FOLLOW-UP	WEIGHT		HEAD CIRCUMFERENCE		LENGTH	
CHARACTERISTICS *Weight g 580-1250 GA wk 23-32*	***PSG*** *N =34*	***CG*** *N = 27*	***PSG*** *N =34*	***CG*** *N = 27*	***PSG*** *N =34*	***CG*** *N = 27*
3 m.c.a.	-0.95 (± 0.4)	-1.6 (± 0.6)	0.14 (± 0.47)	0.17 (± 0.64)	-1.07 (± 0.33)	-1.58 (± 0.87)
6 m.c.a.	-0.63 (± 1.2)	-0.83 (± 0.9)	+0.23 (± 0.49)	-0.13 (± 0.62)	-0.08 (± 0.46)	-1.4 (± 0.54)
9 m.c.a	-0.51 (± 1.2)	-0.68 (± 0.86)	+0.19 (± 0.47)	+0.19 (± 0.62)	**-0.85 (± 0.55)**	**-1.27 (± 0.55)**
12 m.c.a.	2.07 (1.47)	-3.22 (1.69)	0.33 (1.23)	-0.14 (1.42)	-0,63 (0,28)	-0,50 (0,30)

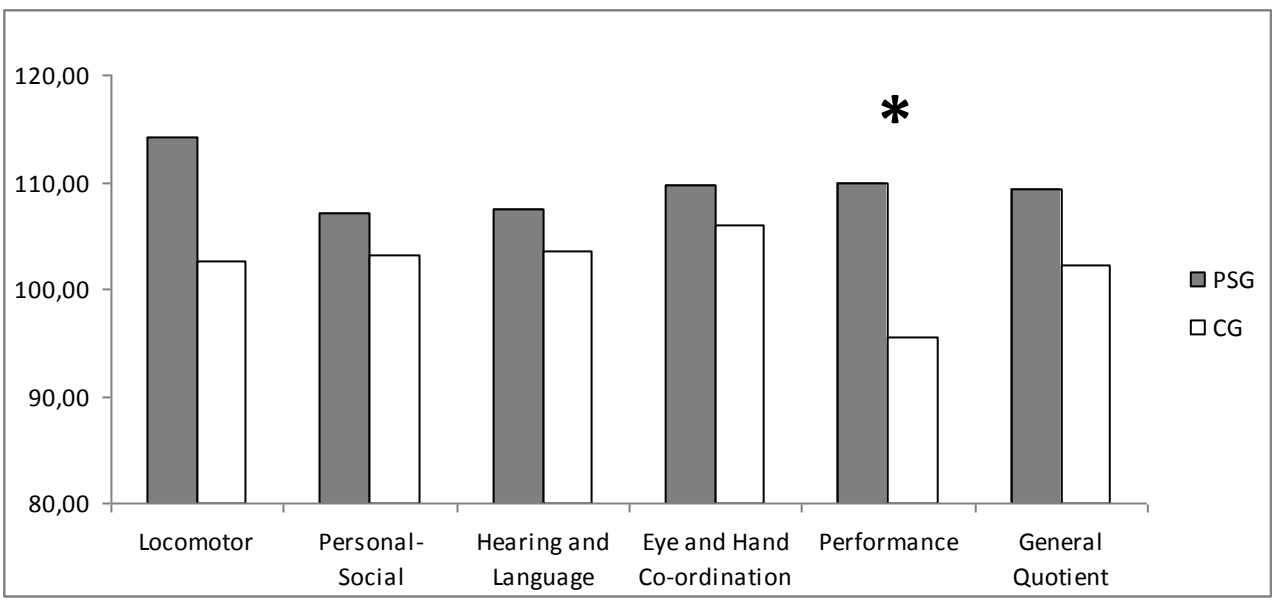

Fig. 1. *GDMS at 3 months' corrected age in ELBW with covariate CRIB; significant different between groups is showed in the performance items (95,5 versus 109,8 p 0,04) .*

previous time. The PSG had a higher CRIB score (2,6 ± 2,1 versus 1,7 ± 1,1 p 0,02), lower neonatal weight g (939 ± 196 versus 1012 ± 163 p 0,08) and gestational age week (27,8 ± 2,3 versus 28,4 ± 0,81 p 0,11) than the CG and this, with the moderate protein regime intake of the control group, could have blunted the statistical significance of the analysis. Although the neonates in the control group were primarily more mature and their milk intake was slightly larger, their growth rate was minor than the other's. We performed a second analysis in the ELBW neonates with birth weight 580-980 g and GA 23-30 weeks of both groups: the protein supplemented neonates yielded a clear advantage in length (p 0,04) and head circonference (p 0,02) growth, whereas the difference in weight gain was smaller (p 0,05). Also in this second analysis the actual volume of milk taken by the control group was bigger

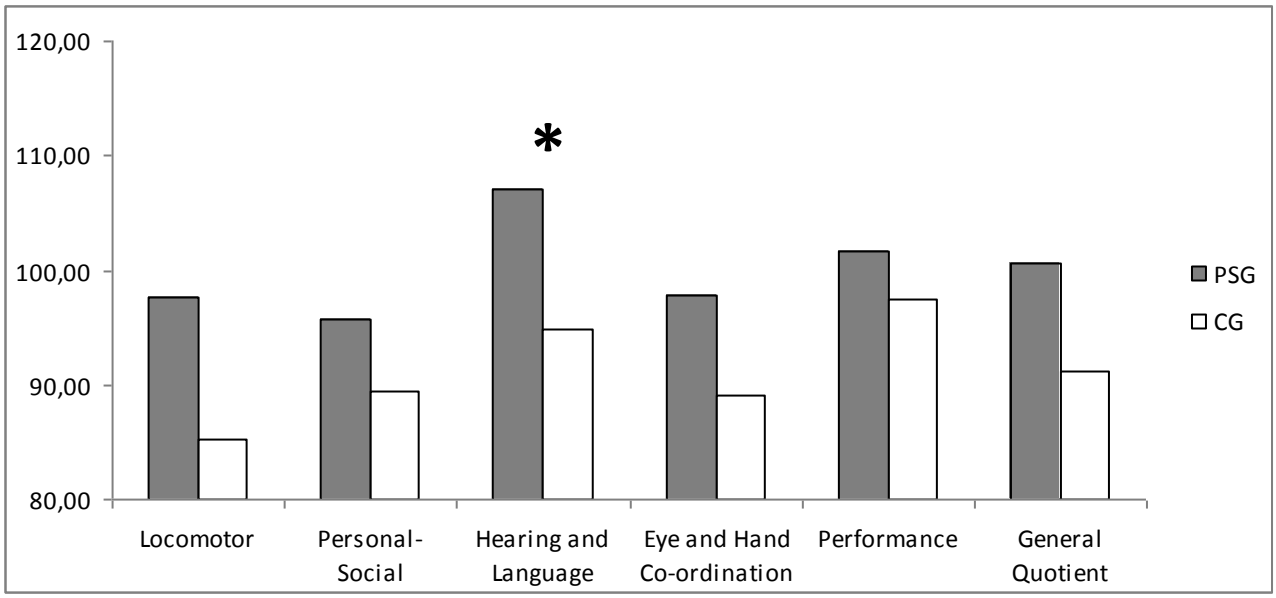

Fig. 2. *GDMS at 12 months' corrected age in ELBW with covariate CRIB; significant different between groups is showed in the Hearing and Language items (107,04 versus 94,83 p 0,03) .*

than that taken by the protein supplemented one (Table I). The evaluation of the anthropometric data until 12 months of corrected age showed that the PSG had a catch-up growth better than the control one : the weight, length and head circonference mean z-score values, were always higher for the protein supplemented regime although the difference became significant for the height only at 9 (-0,85 versus -1,27 p 0,04) months of corrected age in the experimental and control group respectively (Table II). Neurologic outcome was defined by the presence or the lack of any of the following neurological impairment (NI): Cerebral Palsy (CP), index score < 80 at GDMS, deaf/hearing loss requiring amplification in both ears or bilateral blind. MRI was performed a 40 week of gestation in 25/34 and 24/27 in the PSG and CG respectively; clearly pathologic images were showed in one neonate in the control group who demonstrated NI later. He was the only one with NI between all neonates of both groups. GDMS at 9 months' corrected age didn't showed any significant difference between two groups although the protein supplemented one expressed higher score; at 3 and 12 months' corrected age the evaluation, made with covariate CRIB, demonstrated a significant advantage in the performance (p 0,04) and in the hearing and language subscale (p 0,03) for the ELBW neonates fed with protein supplemented regime (Figure 1,2,). Also considering the entire preterm group,the protein supplemented neonates scored better than control in the Hearing/Language items (106,06 versus 98,97 p 0,03).

DISCUSSION AND CONCLUSION

We demonstrated that the max advantages of the protein supplemented regime were acquired by the premature baby with extremely birth weight and gestational age ≤ 30 weeks. A greater efficiency of protein deposition and protein gain had been previously demonstrated, when the weight and gestational age are low (7). In our study the rate growth gain in these neonates regarded more the head circumference and the length than the weight, and this confirms that the growth was mostly that of the lean body mass. When the neonates reached 1100 - 1200 g , their tolerance to higher volume increased and became 165 – 168 ml Kg^{-1} per day. In our NICU the daily target growth velocity of the weight and circumference are ≥18 g / Kg per day and ≥ 0,8 cm / per week respectively. Closely monitoring the rate of in-hospital growth once birth weight has been regained, is of crucial importance: if those rates falter, the dietary protein/energy ratio should be increased and this was done appropriately. The mean weight at the end of the study in PSG and CG was 1780 ± 86 versus 1731 ± 99 (p 0,02) respectively; this low mean

weight of both groups is evidence of early autonomy in breast feeding: at this time the advantages of breast feeding exceed any enrichment practices and the baby, to achieve the same growth rate which he had on the hyperproteic diet, must compensate by ingesting higher quantities of milk for its lower energy. 83,7 % of the study infants were discharged home with partly maternal milk and 64 % with exclusively maternal milk. The critical point remains when leaving the baby entirely to maternal breast feeding. This usually happens at 35-36 gestation wk and is accompanied by an expected slow growth rate. Could this be too big of a penalty for the baby's brain growth? Nutrients and growth factors probably continue to strongly regulate the brain development until 42 wk (8). Can we trust that the brain plasticity at 35-36 wk outweighs its vulnerability to minimal nutrient repletion? The neurodevelopment follow up is still in progress for all the babies of the study and partial results of GDMS are showing a higher score in the ELBW of the protein supplemented group with respect to those in the control one. The significant difference was found only at 3 and 12 months' corrected age; later, probably, other variables would be considerated. We also demonstrated large limits of tolerance to the protein intake during the stable growth phase and the urea values driving the adjustable fortification, may be safely increased to 45 mg/dl. As we visualized a clinical advantage in the PSG the study was stopped and we started giving the same increased protein intake to all the extremely low birth weight prematures admitted to our NICU (11). The benefit of this intrahospital nutritional regime with hight protein intake most likely lasts after discharge from the NICU, through the first year, suggesting an early window of opportunity to positively influence the growth potential of these infants, whereas other nutritional postdischarge management showed a scarce evidence of improved growth (9). We believe, like others authors, that the ELBW must be routinely fed ,with the human/maternal milk enriched by high levels of proteins as soon as possible. Although further studies for the implementation of this strategy could be performed, we believe that the benefits of using the method have been proven substantially enough, and further evidence may be unnecessary.

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EARLY NEUTRAL PREBIOTIC OLIGOSACCHARIDE SUPPLENTATION REDUCES THE INCIDENCE OF SOME ALLERGIC MANIFESTATIONS IN THE FIRST 5 YEARS OF LIFE

S. ARSLANOGLU[1,], G.E. MORO[1,2], G. BOEHM[3], F. WIENZ[3], B. STAHL[3], E. BERTINO[4]

[1]*Italian Association of Human Milk Banks, Milan, Italy*
[2]*Center for Infant Nutrition, Macedonio Melloni Hospital, Milan, Italy*
[3]*Danone Research, Friedrichsdorf, Germany*
[4]*Neonatal Unit, University of Turin, Italy*

Background: A mixture of neutral prebiotic oligosaccharides has been shown to reduce the incidence of atopic dermatitis (AD) and allergy associated symptoms during the first 2 years of life. Objective: To evaluate if this protective effect against allergy lasted beyond the intervention period until 5 y of age. Methods: In a prospective, double blind, placebo-controlled fashion, healthy term infants at risk of atopy were fed either a prebiotic-supplemented (0.8 g/100 ml scGOS/lcFOS) or placebo-supplemented (0.8 g/100 ml maltodextrin) hypoallergenic formula during the first 6 mo of life. Following this intervention period, follow-up continued until 5 y of life. The present study evaluated (i) the cumulative incidence of allergic manifestations during 5 y, and (ii) the prevalence of allergic and persistent allergic manifestations at 5 y. Monitored allergic manifestations were AD, recurrent wheezing, allergic rhinoconjunctivitis and urticaria. Results: Ninety-two children (50 in placebo group, 42 in intervention group) completed the 5-y follow-up. The 5-y cumulative incidences of any allergic manifestation and atopic dermatitis were significantly lower in the scGOS/lcFOS group (30.9, 19.1 %, respectively) compared to placebo group (66, 38 %, respectively) ($p<0.01$ and <0.05). Children in the scGOS/lcFOS group tended to have a lower incidence of allergic rhinoconjunctivitis, and allergic urticaria (4.8 vs 16% for both manifestations, $p=0.08$). There was no difference in the cumulative incidence of recurrent wheezing. With regard to the prevalences at 5 y, intervention group had significantly lower prevalence of any persistent allergic manifestation and rhinoconjunctivitis (4.8, 2.4 %, respectively) compared to placebo (26, 14 %, respectively) ($p<0.01$ and $=0.05$). Prevalence of persistent AD tended to be lower in the intervention group (2.4 vs 12 %, $p= 0.09$). Although intervention group had 75% reduction in the prevalence of persistent wheezing (4.8 vs 14 %), no significance was shown. Conclusion: Oligosaccharide prebiotics (scGOS/lcFOS), when started early in life have a protective effect against allergic manifestations in high risk infants. The protection lasts beyond infancy until 5 y of life, for AD and allergic rhinoconjunctivitis. Long-term follow-up studies in larger populations are warranted to evaluate the potential preventive effect of this mixture on asthma. Abbreviations: lcFOS: long chain fructo-oligosaccharides, scGOS: short chain galacto-oligosaccharides, HO: human milk oligosaccharides.

Over the past several decades, allergic diseases have become more frequent in the Western societies, but also in the developing countries. (1). The progression of infant allergy to atopic diseases such as atopic dermatitis (AD), allergic rhinoconjunctivitis, and finally asthma is defined as "pediatric allergic march" (2). Several strategies have been developed to avoid the onset of the allergic march. Most recently strategies based on the modification of the intestinal microbiota have been established (3-7).

Intestinal microbiota acquired during the early postnatal period is one of the essential factors for the appropriate development of immune system in terms of immune regulation and induction and maintenance of tolerance to environmental and self antigens (4,8). The composition of the gut microbiota differs between healthy and allergic infants and in countries with a high and low prevalence of allergies (9-13). These differences were observed within the first week of life preceding clinical

Key Words: prebiotics, oligosaccharides, atopic dermatitis, allergy prevention, immunomodulation

Corresponding Author: Prof. S. Arslanoglu
Italian Association of Human Milk Banks, MilanVia Libero
Temolo No 4, 20126 - Milan, Italy
+905321553002
asertac@tiscali.it

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symptoms, suggesting their causative role in allergic disorders (9,13). This evidence provided a strong rationale for modifying the gut microbiota and thereby modulating the immune response in infancy. Both probiotics (live microorganisms that, when administered in adequate amounts, confer a health benefit on the host) (14), and prebiotics (selectively fermented dietary ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits) (7) can modulate early development of intestinal microbiota (15-20) and have been proposed for the prevention of allergic disorders.

Human milk oligosaccharides (HMO) represent the third largest component in human milk (21-24 g/L in colostrum and 12-14 g/L in mature milk), having both the prebiotic potential and the direct interaction with the immune cells (21-26). A specific prebiotic mixture of 90% short chain galactooligosaccharides (scGOS) and 10% long chain fructo-oligosaccharides (lcFOS) developed to mimic the functional characteristics of HMO (27) has been shown to produce an intestinal microbiota in formula fed infants similar to that found in breast fed infants (5).

Recently, the authors showed that early supplementation of infant formulas with this specific mixture of neutral prebiotic oligosaccharides in infants at high risk for atopy reduces the incidence of AD, allergic urticaria and recurrent wheezing beyond the 6 mo-intervention period up to 2 y of age (28,29). The present paper reports the results of 5 y follow-up of the same cohort aiming to monitor if this allergy prevention effect is long-lasting. To answer this question we evaluated (i) the cumulative incidence of allergic manifestations during 5 y, and (ii) the prevalence of allergic and persistent allergic manifestations at 5 y.

METHODS

Study Design

The original study was a randomized, double-blind, placebo-controlled trial as described elsewhere (28,29). Briefly, healthy term infants with a parental history of atopy received either prebiotic supplemented (8 g/ L scGOS/lcFOS) or placebo supplemented (8 g/ L maltodextrin) hypoallergenic formula during the first 6 mo life (28). To evaluate the possible long-term protective effect of the prebiotic intervention on allergy, follow-up studies were performed until the age of 2 (29) and 5 y. The present study reports the results of the 5y follow-up of this study group.

Subjects

Healthy term infants with a parental history of atopic eczema, allergic rhinitis, or asthma in either mother or father were eligible for the original study as described earlier (28). Out of 134 infants who completed 2y study (29), parents of 95 infants gave consent to participate to the 5y follow-up.

The study protocol was approved by the Ethical Committee of the Macedonio Melloni Hospital, Milan, Italy. Informed written consent was obtained from parents.

Nutritional Intervention

Nutritional intervention was for the first 6 months of the study and explained in detail in the original paper (28). Infants whose mothers started formula feeding within the first two weeks of life were randomly assigned to be fed by one of the two study formulas. The recipe of both formulas was based on a hypoallergenic formula with extensively hydrolysed cow's milk whey protein (Aptamil HA, Milupa Italy). In the intervention group, this formula was supplemented with 8 g/L scGOS/lcFOS (IMUNOFORTIS, Danone, The Netherlands), and in the placebo group the same formula was supplemented with 8 g/L maltodextrin (Glucodex 12, Roquette, Germany).

Five-year Follow-up Protocol

Data were collected throughout (i) follow-up visits, (ii) diaries written by parents, and (iii) telephone calls by trained personnel.

Follow-up visits consisted of a detailed physical examination, evaluation of the growth and structured interviews by the study physicians. These visits had been scheduled at 1,2,3,4,5,6,9,12,18, and 24 mo of age until 2 y of age (29). After 2 y visits were performed yearly up to 5 y. Any sign or symptom related to allergy (AD, wheezing episodes, urticaria, and after 2y allergic rhinoconjunctivitis) were recorded. Unscheduled visits were done when the investigators were contacted by the family for the occurrence of symptoms related to allergy.

Diaries and telephone calls were used to collect the data between two scheduled visits and to increase the compliance. Parents were instructed to record allergic symptoms, clinic visits, tests, physician's diagnosis, prescription of medications and were asked to contact the investigators and bring these reports and medical documents at each visit.

The children with persistent symptoms at 5 y were sent to a pediatric allergist for confirmation.

Endpoints and Definitions

Endpoints

Allergic manifestations.

Main endpoints of interest were

- cumulative incidence of allergic manifestations during 5y-follow-up, and
- prevalence of allergic and persistent allergic manifestations at 5 y of age.

We evaluated also

- prevalence of allergic manifestations at 5 y in relation to the presence of AD within the first 6mo of life to assess if early AD occurrence has played a role for persistent allergic manifestations.

Monitored allergic manifestations were *AD, recurrent wheezing, allergic rhinoconjunctivitis, and urticaria*.

Growth.

Body weight and height were measured at each clinic visit by experienced nurses, and the growth data were assessed at 2 y and 5 y of life. Birth data were derived from the birth records.

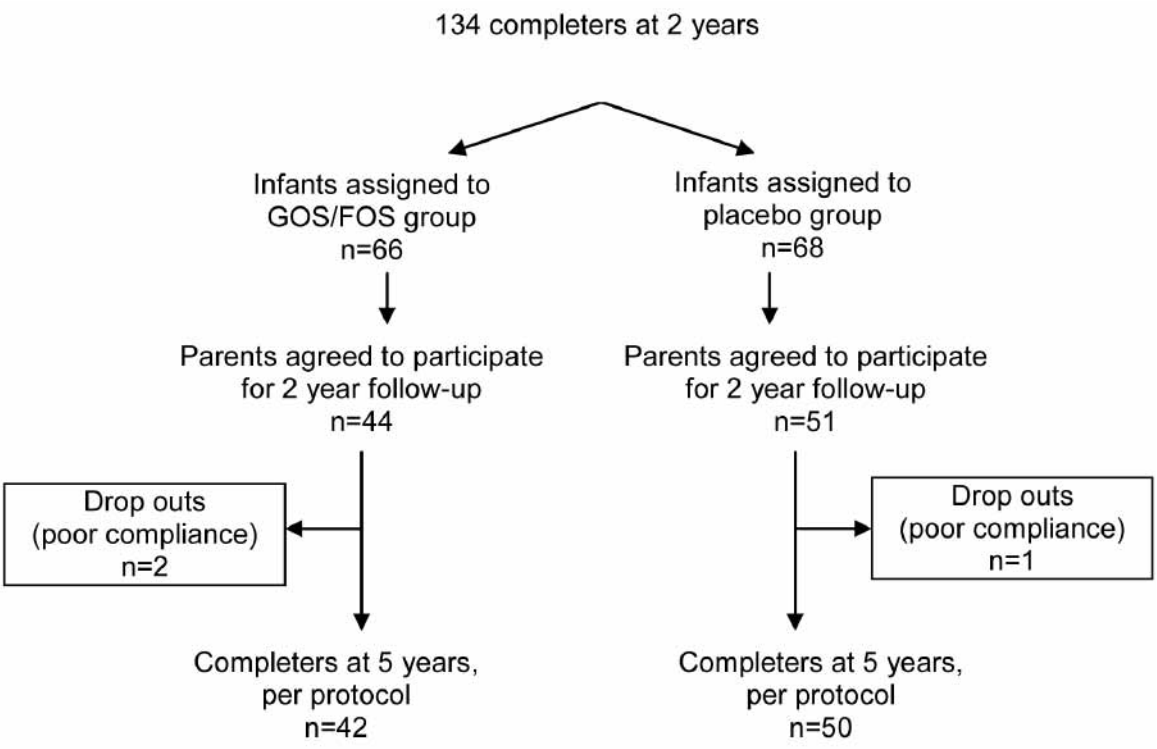

Fig. 1. *Flow chart showing the disposition of the subjects throughout the study (Poor compliance: poor compliance to follow-up protocol).*

2. Definitions

Cumulative incidence during 5 y (35)

Number of cases in the cohort who ever acquire an allergic manifestation during 5 y-folow-up

Number of all cases who completed 5 y follow-up

Prevalence at 5y (35)

Number of cases in the cohort who have an allergic manifestation at 5 y

Number of all cases who completed 5 y follow-up

An allergic manifestation was defined persistent if started before 3 y of age and was still present at 5 y. This definition has been adapted from the definition used for persistent wheezing (36). If the child had more than one manifestation at 5 y, presence of only one manifestation was considered for calculation of "any manifestation".

Atopic dermatitis (AD) was diagnosed according to the criteria described by Harrigan and Rabinowitz (30) and Muraro et al (31). After 2 y the diagnosis of AD was confirmed in the presence of an itchy skin condition with a minimal duration of 4 weeks if there were following features: involvement of the cheeks, or forehead and outer limbs, general dry skin in the past year. Recurrent wheezing was defined as 3 or more physician-diagnosed wheezing episodes during each follow-up period (31). Allergic urticaria was defined as (32) 2 or more episodes of itching eruptions or swelling with typical appearance seen by a physician and provoked by the same allergen. Allergic rhinoconjunctivitis was considered as repeated physician diagnosed nasal and ocular symptoms (itching,sneezing, increased secretion, and nasal blockage) not related to common cold or infection (33,34).

Statistical Analysis

Randomization and sample size calculation were described in the original study (28). The power calculation had been done to detect any difference for the incidence of AD during the first 6 mo intervention period. Therefore, this 5-y follow-up study is significantly underpowered and can be considered as an explorative study. The analyses were performed on the cases who completed 5y follow-up.

One-way ANOVA and t-tests were used to compare continuous variables between two treatment groups. Fisher's Exact test was performed for the categorical data analysis. Statistical significance was set at the 5 % level of probability. Odds ratios were used when prevalence of the allergic manifestations at 5y were expressed in relation to the presence of AD at 6 months.

Statistical analyses were performed using the SPSS 14.0 software for Windows (SPSS Inc, Chicago, IL).

RESULTS

Parents of 95 infants out of 134 2-y follow-up completers agreed to participate in this study. Ninety-two children (50 in the placebo, 42 in the scGOS/lcFOS

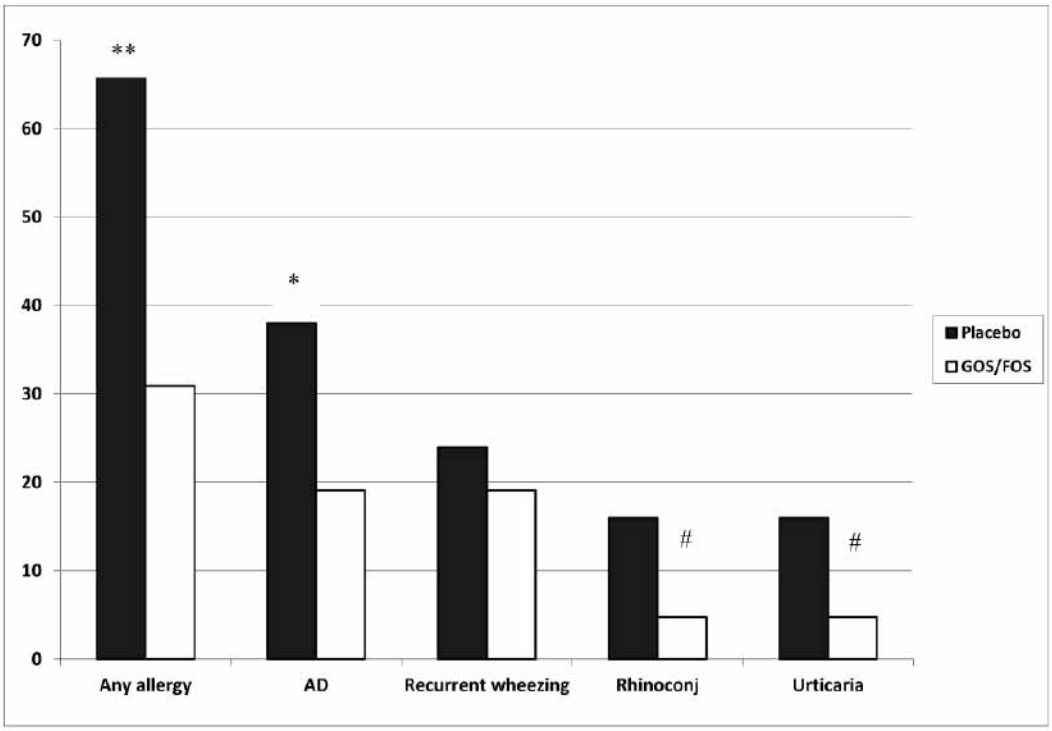

Fig. 2. *Cumulative incidence of allergic manifestations during 5-y follow-up period in the scGOS/lcFOS and placebo groups (n=50 in pacebo group, n=42 in scGOS/l cFOS group) Different from placebo, *p<0.05., **p<0.01 Tendency to be different, # p=0.08*

group) completed the 5 y follow-up period (Figure 1). Baseline characteristics and demographic data of the completers were similar in placebo and in scGOS/lcFOS groups (Table I).

Cumulative incidences of allergic manifestations during 5y

The 5-y cumulative incidences of any allergic manifestation and atopic dermatitis were significantly lower in the scGOS/lcFOS group (30.9, 19.1 %, respectively) compared to placebo group (66, 38 %, respectively) (p<0.01 and <0.05). Children in the scGOS/lcFOS group tended to have a lower incidence of allergic rhinoconjunctivitis, and allergic urticaria (4.8 vs 16% for both manifestations, p=0.08). Cumulative incidence of recurrent wheezing did not show a significant difference between the groups (19.1 and 24 %, respectively) (Figure 2).

Prevalences of allergic and persistent allergic manifestations at 5 y

At 5 y of age children in the intervention group had significantly lower prevalence of any allergic manifestation, any persistent allergic manifestation and rhinoconjunctivitis (4.8, 4.8, 2.4 %, respectively) compared to placebo (28, 26, 14 %, respectively) (p<0.01, p<0.01, and =0.05). Prevalence of persistent AD tended to be lower in the intervention group (2.4 vs 12 %, p= 0.09). At 5 y only 4.8 % of children had persistent wheezing in the scGOS/lcFOS group, compared to 14 % in the placebo group. But this reduction in the intervention group was not statistically significant (Figure 3).

Final situation of the children who developed AD within 6 mo in comparison with those who did not (prevalence of persistent allergic manifestations and rhinoconjunctivitis at 5 y)

Fifty % of the cases with AD at 6 mo in the placebo group continued to have allergy in terms of AD, persistent wheezing, and rhinoconjunctivitis at 5 y compared to 16.7 % in the intervention group (p<0.05). The children who had AD during the first 6 mo in the whole group developed more persistent allergic manifestations and allergic rhinoconjunctivitis at 5 y of life (Table II). Prevalences of any persistent allergic manifestation, AD, and allergic rhinoconjunctivitis at 5 y were significantly higher in AD-6mo(+) children (40, 30, 25 %, respectively) in the whole group, compared to AD-6mo(-) ones (9,7, 1.4, 4.2 %,

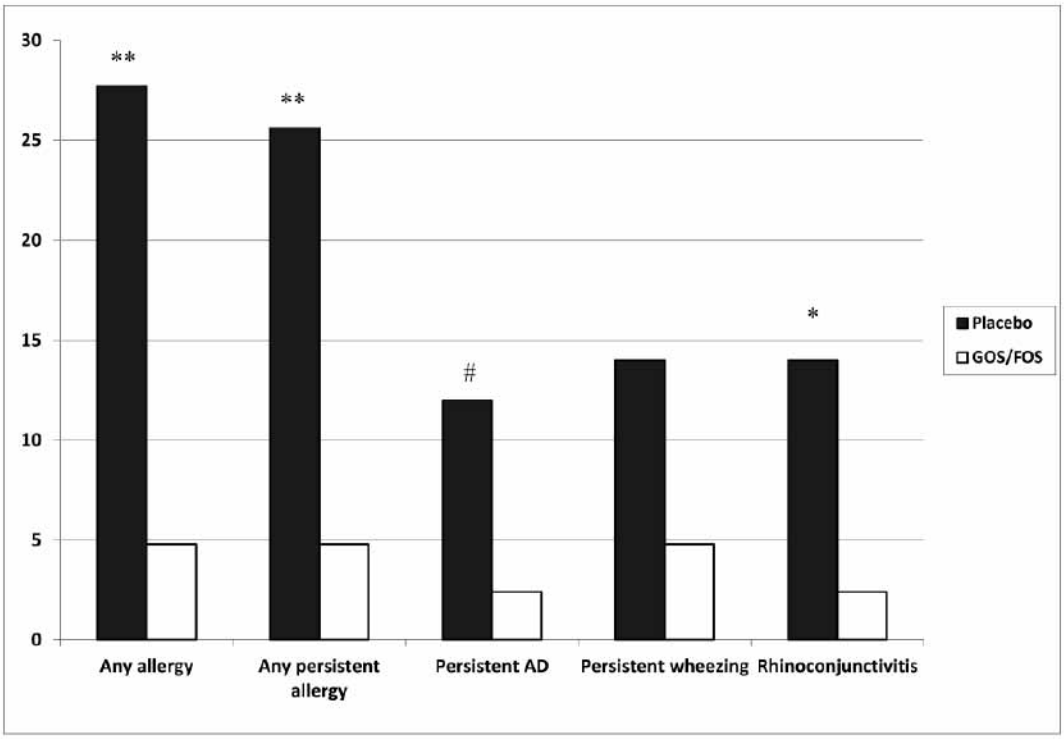

Fig. 3. *Prevalence of allergic manifestations at 5 year in the scGOS/lcFOS and placebo groups (n=50 in pacebo group, n=42 in scGOS/l cFOS group) Different from placebo, *p=0.05, **p<0.01 Tendency to be different, # p=0.09*

respectively) (p<0.01, p<0.0001, p=0.01 with odds ratios 6.2, 30.4, 7.7 respectively). AD-6mo(+) children tended to have also more persistent wheezing at 5 y (20 vs 5.6 %, p=0.065 with odds ratio 4.3)

Growth

During the follow-up, growth expressed as mean body weight and length at 2 y and 5y was found to be adequate and similar in the placebo and intervention groups (data not shown).

DISCUSSION

The present follow-up study demonstrates that neutral prebiotic oligosaccharide supplementation (scGOS/lcFOS) early in life continues to be protective against some allergic manifestations up to 5y in infants at high risk for atopy. In the same group of infants, we had previously shown that this mixture reduced the cumulative incidence of AD, recurrent wheezing, and allergic urticaria during first 2 y of life (29). For the current 5-y follow-up study, we addressed both "*the occurrence of allergic manifestations during 5y-period (cumulative incidence)",* and the "*the presence of allergic and persistent allergic manifestations at 5 y (prevalence)".*

During 5y-period the cumulative incidence of AD was significantly reduced in the intervention group, and there was a tendency of reduction for rhinoconjunctivitis and urticaria. At 5y of age 26 % of the children in placebo group still had persistent allergic manifestations, whereas in the intervention group only 4.8 % had (p<0.01), corresponding to a reduction by over 80 %. The reduction of prevalence in the intervention group was significant for rhinoconjunctivitis, and there was a tendency of reduction for persistent AD. Although the prevalence of persistent wheezing was reduced by 75 % in the prebiotic supplemented group (4.8 vs 16 %), the difference was not statistically significant mainly because of the fact that the follow-up became underpowered to detect any difference. Five-year follow-up was not planned at the start of the study, and the parents were informed about it at the end of 2 y; which is why only 95 parents out of 134 two-y completers accepted to participate (resulting in 31.3 % drop-out rate with respect to 2-y completers). The study was powered only to detect any difference for AD during 6 mo.

The most important findings deriving from this study are (i) 50 % reduction of the 5y-cumulative incidence

Table I. *Baseline characteristics and demographic data of completers for 5 year follow-up*

	Placebo	scGOS/lcFOS
	n= 50	n= 42
Gender (male/female ratio)	1.1	0.8
Birth weight* (grams)	3328 ± 461	3248 ± 479
Mother' s age* (y)	32.7 ± 4.6	34.4 ± 3.7
Atopy in the family (%)		
Only maternal	58.0	57.1
Biparental	22.0	23.8
Only paternal	20.0	19.0
Number of births*	1.4 ± 0.8	1.4 ± 0.7
Vaginal delivery (%)	64	66.7
Maternal smoking (%)	18.0	16.7
Age at onset of day care* (mo)	13.1 ± 4.1	11.7 ± 4.2
Furred pets at home (%)	24.5	26.2

**Values are mean + Standard deviation*

of AD in the intervention group (38 vs 19 %) (ii) ~80% reduction in the prevalence of the *persistent* allergic manifestations at 5y . Atopic dermatitis is a chronic, inflammatory skin disease with a rising prevalence in western societies. The peak incidence is in the first year of life (37). Although it has a clearance rate of 40% at 2 years and 65% in adolescence, AD can be the starting point of the "pediatric allergic march", followed by subsequent allergic diseases such as rhinoconjunctivitis asthma (2,38,39). In a large German prospective birth cohort (37) 50% of children with atopic dermatitis had allergic airway disease by the age of 5 years. Also our own data in this paper indicates that occurrence of AD within 6 mo increases the likelihood of the persistence of allergic manifestations at 5 y (odds ratios for the prevalence of any persistent manifestation, AD, and persistent wheezing are 6.2, 38.2, 4.3).

In fact, in our 5-y study, the significant reduction of the AD occurrence (57 % reduction at 6 mo, 50% at 2 y) (28,29) in the intervention group has been accompanied by a marked reduction (~80%) in the prevalence of the persistent allergic manifestations later on. These two findings coupled together imply that there is a role for prebiotics in allergy modulation. The decreased prevalence of rhinoconjunctivitis in scGOS/lcFOS group at 5 y is another clue supporting this claim. Besides, there is plenty of evidence pointing at rhinoconjunctivitis as the predictor for later asthma development (40). We speculate that 75 % reduction in persistent wheezing in the intervention group, though not significant, is worth considering a possible preventive effect of this oligosaccharide mixture on asthma and warrants further randomized trials in larger populations with a longer follow-up powered to detect asthma at school age.

Table II. *Prevalence of the persistent allergic manifestations and rhinoconjunctivitis at 5y in relation to the presence of AD at 6 months (placebo-intervention groups combined)*

Allergic manifestation at 5y (%)	AD-6mo (+) n= 20	AD-6mo (-) n= 72	Odds ratio (95%CI) p values
Any persistent allergic manifestation (%)**	40	9.7	6.2 (1.9-20.3) 0.003
Persistent AD ***	30	1.4	30.4 (3.4-272.8) 0.0
Persistent wheezing (%)#	20	5.6	4.3 (1.0-18.8) 0.065
Allergic rhinoconjunctivitis (%)**	25	4.2	7.7 (1.7-35.6) 0.01

**Values express the percentage of the children having these allergic manifestations at 5 years of age in the placebo-intervention groups combined. Different from placebo, *p<0.05, **p<0.01, ***p<0.0001, #p=0.065 AD-6mo(+): the group of children who developed AD within the first 6 months of life AD-6mo(-): the group of children who did not have AD within the first 6 months of life*

To our knowledge this is the first study showing the long-term effectiveness of prebiotic oligosaccharides on primary prevention of allergy in children. But, our findings on primary prevention of AD has been recently confirmed up to 1 y of age in infants at low-risk for atopy. This large multicentric trial conducted in 7 centers from 5 European countries has shown that neutral+acidic oligosaccharide supplementation of infant formulas reduces the cumulative incidence of AD by 40 % up to 1 y of life (41).

Regarding the probiotic approach as primary prevention strategy for allergy, the pioneer Finnish study demonstrated that administration of L. rhamnosus GG for 1 mo before and 6 mo after birth was associated with a significant reduction in the cumulative incidence of eczema during the first 7 y of life (42-44). Another Finnish study conducted with a mixture of 4 probiotics and prebiotics reported a similar but more modest preventive effect on eczema and atopic eczema (45). However this effect, lasted up to 5y of age only in children delivered by caesarean section (46). Other studies using different probiotics for primary prevention of AD in infants at high atopy risk yielded variable results (47-51) .

Although we can not extrapolate the specific mechanism through which this long-lasting prevention on allergy occurred, modification of intestinal flora is very likely to be the principle way by which this immune modulation has been orchestrated. The relationship between the intestinal microbiota composition early in life and later occurrence of allergic cases further supports this interpretation (9,13). In fact, dendritic cells of the intestinal mucosa play an important role in the differentiation of regulatory T cells (Treg) which are known to be important in the development of immune tolerance (3). Alterations in Treg functions are associated with the development of

allergic diseases (52,53). And evidence indicate that the gut microbiota acquired during the early postnatal period is essential for the proper development of Treg and Th1/th2 balance (54). The previous studies with term and preterm infants indicate that scGOS/lcFOS mixture has prebiotic activities, producing an intestinal microbiota similar to that of breast fed infants (15-19). Recent data shows that this modification lasts beyond the intervention period (55). Also during the 6 mo intervention period of this follow-up study, it has been shown that supplementation with this mixture resulted in a significant increase in the number of bifidobacteria compared with the placebo group (28).

However, any potential direct effect of the studied prebiotics on the immune cells, and target receptors like lectins (21, 56,57) can not be excluded. HMO have a very complex structure, and apart from their bifidogenic effects, are known to inhibit the adhesion of pathogens on the epithelial surface, interfere with leukocyte recruitment to the inflammation sites, inhibit cell-cell interactions of immune cells via selectins, DC-SIGN, galectins and other target molecules (23-25, 58), and directly affect cytokine production and T-cell activation (21). Recently,an in-vitro study provided the first evidence for the transfer of synthethic scGOS/lcGOS and pectin-derived acidic oligosaccharides through an epithelial monolayer. Thus, it is possible that the studied mixture has been transferred through intestinal epithelial cells, and interacted directly with immune cells (59).

CONCLUSION

In conclusion, breast milk is the gold standard nutrient in the first 6 mo of life and is recommended also for the primary prevention of allergy. The marked reduction in the occurrence of AD and rhinoconjunctivitis with prebiotic oligosaccharides leads us to consider utilization of prebiotics as a primary prevention strategy for formula-fed infants. Long-term follow-up studies in larger populations are warranted to evaluate the potential preventive effect of this mixture on asthma.

Author disclosure

S. Arslanoglu, G. E. Moro, S. Rizzardi, M. Coppola, no conflicts of interest; G. Boehm is the Director of Infant Nutrition Research of Danone. B. Stahl and F. Weinz are from Danone Research Center. This company provided the scGOS/lcFOS mixture utilized in this study.

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AMENDMENT TO 2010 ITALIAN GUIDELINES FOR THE ESTABLISHMENT AND OPERATION OF A DONOR HUMAN MILK BANK

S. ARSLANOGLU[1,2], E. BERTINO[2,4], P. TONETTO[2,4], G. DE NISI[2,3], A.M. AMBRUZZI[2], A. BIASINI[2,5], C. PROFETI[2,6], M.R. SPREGHINI[2], G.E. MORO[2]

[1]Department of Neonatology, Dr. BehcetUz Children's Hospital, Izmir, Turkey
[2]Italian Association of Human Milk Banks, Milan, Italy
[3]Neonatal Unit, S. Chiara Hospital, Trento, Italy
[4]Neonatal Unit, University of Turin, Italy
[5]Pediatric and Neonatal Intensive Care Unit, Bufalini Hospital Cesena, Italy
[6]Neonatal Intensive Care Unit, A. Meyer Pediatric Hospital, Florence

The present paper is an amendment to the recent Italian Guidelines of human milk banking published in 2010. Working Group on Guidelines (Panel) of the Italian Association of Human Milk Banks (AIBLUD) states, in accordance with the European Union Comission's Amending Directive of January 2011, that the hard plastic feeding bottles used in the collection, storage and pasteurization of the human milk should be Bisphenol A (BPA) free. Until new evidence are availablepolycarbonate feeding bottles should not be used for collection, storage and pasteurization of human milk. The papersummarizes the former and current European Commission Directives and shows the related amending changes to the 2010 Italian Human Milk Banking Guidelines.

The present paper is an amendment to the recent Italian Guidelines of human milk banking published in 2010[1]. The Working Group on Guidelines (hereinafter "Panel") of the Italian Association of Human Milk Banks (Associazione Italiana Banche del Latte Umano Donato=AIBLUD) feels the need to state an important change in milk storage practices. This amendment refers to the "Part3.3. Milk Containers" of the"Section 3: Procedures for collection and storage of the milk"[1].

AIBLUD Panel states, in accordance with the European Union Commission's Amending Directive of January 2011, thatthe hard plastic feeding bottles used in the collection, storage and pasteurization of the human milk should be Bisphenol A (BPA) free. Until new evidence are available. polycarbonate feeding bottles should not be used for collection, storage and pasteurization of human milk.

On 28 January 2011 European Union Commission (EC) published an Amending Directive as regards the restriction of use of BPA in plastic infant feeding bottles[2]. Table I summarizes the former EC Directive[3], the development stages for the Current EC Directive, and the current Directive[1].

Table II shows the related amending changes to the 2010 Italian Guidelines[1] for Section 3: Procedures for collection and storage of the milk, Part 3.3. Milk Containers.

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Key Words: milk banking, storage, feding bottles, Bisphenol A, polycarbonate bottles

Corresponding Author: Prof. S. Arslonoglu
Italian Association of Human Milk Banks, MilanVia Libero Temolo No 4, 20126 - Milan, Italy
+905321553002
asertac@tiscali.it

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Table I. *The former and current European Commission (EC) Directives as regards the use/restriction of BPA and polycarbonate feding bottles and the development stages of the current Directives2*

1. **August 2002 EC Directive[3]:**Commission Directive 2002/72/EC authorised the use of 2,2-bis(4-hydroxyphenyl) propane(Bisphenol A=BPA), as monomer for the manufacture of plastic materials and articles intended to come into contact with foodstuffs in accordance with the opinions of the Scientific Committee on Food (SCF)and the European Food Safety Authority (EFSA) .
2. **March 2010[4]:**The Danish Government informed the Commission and the Member States that it has decided to apply the safeguard measures and to temporarily ban the use of BPA for the manufacture of plastic materials in contact with food intended for children aged 0-3. This decision has been based on a risk assessment by the National Food Institute at the Technical University of Denmark that evaluated a comprehensive study on animals exposed to BPA in low doses monitoring the development of the nervous system and the behaviour in newborn rats.

   European Commission asked EFSA give its opinion on the subject.
3. **July 2010[5]:** French Government informed the Commission, and the Member States, that it has decided to apply the safeguard measures to temporarily ban the manufacture, import, export and placing on the market of feeding bottles containing BPA based on the opinions of French Food Safety Authority (AFSSA) and National Institute of Health and Medical Research (INSERM).
4. **September 2010:** EFSA Panel Opinion[6]: Based on the comprehensive evaluation of recent human and animal toxicity data, no new study could be identified, which would call for a revision of the current tolerable daily intake (TDI) of 0,05 mg/kg bodyweight per day.This TDI is based on the NOAEL of 5 mg/kg b.w./day from a multi-generation reproductive toxicity study in rats, and the application of an uncertainty factor of 100, which is regarded as conservative based on all information on BPA toxicokinetics. However, in a minority opinion one Member of the Panel concluded that the effects observed in certain studies raised uncertainties which may not be covered by the current TDI which should therefore be considered temporary until more robust data becomes available in the areas of uncertainty.The Panel noted that studies conducted on developing animals have suggested other BPA-related effects of possible toxicological relevance, in particular biochemical changes in brain, immune-modulatory effects and enhanced susceptibility to breast tumours. These studies had many shortcomings. The relevance of these findings in relation to human health cannot be assessed at present. The Panel will reconsider the current oinion if any new data become available.
5. **January 2011 Current EC Directive[2]:**Until further scientific data are available to clarify the toxicological relevance of some observed effects of BPA, in particular as regards biochemical changes in brain, immune-modulatory effects and enhanced susceptibility to breast tumours, the use of BPA in the manufacture and placing on the market of polycarbonate infant feeding bottles should be temporarily banned. The Authority has a mandate to monitor new studies to clarify these endpoints.Member States shall adopt and publish, by 15 February 2011 at the latest, the laws, regulations and administrative provisions necessary to comply with this Directive. They shall forthwith communicate to the Commission the text of those provisions.

Table II. *Amendment to 2010 Italian Guidelines1: Section 3. Part3.3. Milk containers*

	2010 Italian Human Milk Banking Guidelines[1]	**Changes considering the 2011 EU Commission's Amending Directive**
3.3.Introduction	Glass and hard plastic **(polycarbonates, polypropylene)** or soft plastic (polyethylene) containers are currently available on the market.	Glass and **Bisphenol A (BPA) free hard plastic(polypropylene)** or soft plastic (polyethylene) containers are currently available on the market.
Recommendation 3.3.1.	It is possible to use both glass and hard plastic containers (A*). **Monouse hard plastic containers** are preferable to glass containers, as the latter are considered to have a potential risk for operators (wounds due to cutting) as well as for infants (micro particles of glass in milk) (√).	No change **Monouse BPA free, hard plastic** containers are preferable to glass containers, as the latter are considered to have a potential risk for operators (wounds due to cutting) as well as for infants (micro particles of glass in milk) (√).
Recommendation 3.3.2.	Soft polyethylene bags are not recommended (B*).	No change
Evidence to recommendations 3.3.1.	No relevant differences have been observed in the stability of some constituents of human milk (leucocytes, total immunoglobulin, IgAs and hydrosoluble constituents) stored in glass or in rigid plastic (polypropylene) containers. **The use of polycarbonate containers did not lead to a substantial contamination thatcan be considered dangerous, although there have been concerns regarding bisphenol A. But evidence show that severe conditions prepared to simulate the worst-case scenario, such as high temperatures for extended periods of time, repetaed dishwashing and boiling increase the migration of bisphenol A from polycarbonate baby bottles to water or food simulants. These data suggest the utilization of monouse hard plastic containers.**	No change **BPA is used as monomer in the manufacture of polycarbonate plastics.Polycarbonate feeding bottles is the main source of exposure to BPA for infants[7].Evidence show that severe conditions prepared to simulate the worst-case scenario, such as high temperatures for extended periods of time, repetaed dishwashing and boiling increase the migration of bisphenol A from polycarbonate baby bottles to water or food simulants[8,9]. On January 28, 2011, European Union Comission published an Amending Directive as regards the restriction of use of BPA in plastic feeding bottles. Until further scientific data are available to clarify the toxicological relevance of some observed effects of BPA, in particular as regards biochemical changes in brain, immune-modulatory effects and enhanced susceptibility to breast tumours, the use of BPA at the manifacturer and polycarbonate infant feeding bottles at the markethave been temporarily banned. This directive has been adopted by the Member States[2].**
Evidence to recommendations 3.3.2.	Soft polyethylene bags reduce some components of human milk (significant loss of lipids and liposoluble vitamins), they are also difficult to be sealed, easy to be contaminated and to be broken.	No change

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UPDATE OF ADJUSTABLE FORTIFICATION REGIMEN FOR PRETERM INFANTS: A NEW PROTOCOL

S. ARSLANOGLU[1,2], E. BERTINO[2,3], A. COSCIA[3], P. TONETTO[2,3], F. GIULIANI[3], G.E. MORO[2]

[1]*Department of Neonatology, Dr. BehcetUz Children's Hospital, Izmir, Turkey*
[2]*Italian Association of Human Milk Banks, Milan, Italy*
[3]*Neonatal Unit, University of Turin, Italy*

Preterm infants fed fortified human milk in standard fashion receive less protein than they need due to customary assumptions. Protein is limiting for growth and neurocognitive development,and shortfalls of proteinare not acceptable. "Adjustable fortification" regimen has been proven as an effective way to provide adequate protein intakes and appropriate growth in this group of infants. Italian Association of Human Milk Banks (AIBLUD) has promoted and implemented this Adjustable fortification regimen in neonatal intensive care units (NICUs) with success. This paper presents an update of "Adjustable fortification" regimen; a new protocol already utilized in several italian NICUs.

The objective of human milk fortification is to increase the concentration of nutrients to such levels that at the customary feeding volumes preterm infants receive amounts of all nutrients to meet their requirements. Protein is limiting for growth and neurocognitive development, which is why shortfalls of proteinare not acceptable (1-3). However preterm infants fed fortified HM receive less protein than they need due to ''customary assumptions'' (4). "Adjustable fortification" of HM has been proven to be an effective fortification strategy in providing the preterm infants with adequate protein intakes resulting in appropriate growth (weight, lenght and head circumference gain) (5,6). Italian Association of Human Milk Banks (AIBLUD) has promoted and implemented this Adjustable fortification (ADJ) regimen in several neonatal intensive care units (NICUs) with success. The present update defines a new, and more practical ADJ protocol.

The new "adjustable fortıfıcatıon"protocol

- ADJ of HM starts with Standart Fortification (STD).
- Once full-strength STD is established, twice weekly blood urea nitrogen (BUN) measurements are performed.
- Based on the BUN levels, the necessity to add extra protein (a protein supplement) is evaluated and adjustments are done.

In the following part you will find three tables explaining in detail the updated ADJ Regimen:

1. The nutritional products and the metabolic marker usedfor ADJ Regimen (Table I)
2. When and how to start human milk fortification (Table II)
3. How to proceed to Adjustable Fortification of HM (Table III)

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Key Words: adjustable fortification, fortification, individualized fortification, preterm infant, human milk

Corresponding Author: Prof. S. Arslonoglu
Italian Association of Human Milk Banks, MilanVia Libero Temolo No 4, 20126 - Milan, Italy
+905321553002
asertac@tiscali.it

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Table I. *The nutritional products and metabolic marker used for Adjustable Fortification Regimen*

Nutritional Products	
1. **Multicomponent HM Fortifier (HMF):** eg. FM85 (Nestle), BMF (Danone) in Italy 2. **Protein supplement (PS)**	
The Metabolic Marker on Which Adjustment is Based	
Blood Urea Nitrogen (BUN)	<10 mg/dl, increase the fortification to the next level 10-16 mg/dl, no change >16 mg/dl, decrease the fortification level

Table II *When and how to start HM fortification*

When	How
Start fortifying HM with a multicomponent HMF when milk volume reaches 70-80 ml/kg/d	1.Start with half-strength, procede to full-strength fortification with HMF in a few days (2-3 days) monitoring the tolerance or 2. Start directly with full-strength fortification with HMF Full strength fortification with HMF is Standard Fortification (Level 0)

Table III. *How to proceed to Adjustable Fortification*

Supplementation per 100 ml of HM	Levels of Adjustable Fortification and Quantity of HMFs and Protein to be Added					
	-2	**-1**	**0 Standard**	**+1**	**+2**	**+3**
Multicomponent HMF*						
FM85	2.5 g	3.75 g	**5 g**	5 g	5 g	5 g
or						
BMF (former Eoprotin)	2 g	3 g	**4**	4 g	4 g	4 g
Protein supplement**						
Amount of protein to add	-----	-----	-----	0.4 g protein	0.8 g protein	1.2 g protein

**The amount of fortifier to be added to 100 ml of HM has been given for 2 specific multicomponent HMFs utilized in Italy*
***The amount of the protein supplement to be added to 100 ml of HM has been given as amount of protein without specifying a certain protein supplement.*

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EARLY ENTERAL FEEDING WITH HUMAN MILK FOR VLBW INFANTS

G. DE NISI[1,2], M. BERTI[2], M. DE NISI[3], E. BERTINO[4]

[1]*Italian Association of Human Milk Banks, Milan, Italy*
[2]*Neonatal Unit, S. Chiara Hospital, Trento, Italy*
[3]*Department of Epidemiology, Trento, Italy*
[4]*Neonatal Unit, University of Turin, Italy*

In a NICU early enteral feeding is usually possible only when the newborn clinical conditions permit it. Because of the frequent need of umbilical/central catheters, they usually start with parenteral feeding and/or with minimal enteral feeding (trophic feeding). This kind of management is even more frequent in VLBWIs, in which the risk of NEC is very high. In this work we describe a model of early enteral exclusive feeding (EEEF) based on the use of banking human milk followed by mother milk. In the Centre of Neonatology of Trento, as in other Centers, the newborns weighing less than 750g or with a GE <27 weeks, are treated with parenteral nutrition and minimal enteral feeding. The newborn weighing 750–1249g and with GE >26 weeks define a group in which we find critical neonates, who can not be treated with enteral feeding, and neonates whose clinical conditions permit EEEF. In particular, in a period of 16 years (1994-2009) in Trento, 308 newborns weighing 750-1249 g and GE >26 weeks were admitted. The 90,9% has been treated with prenatal steroids, the 91,9% was inborn, the 96,1% survived. In the 59,1% of the cases (175) we gave EEEF. We could continue with a complete EEEF in the 40,2% of the total (119 cases). The characteristics of these neonates and our centre management, based mainly on early use of banking human milk and mother milk, are detailed described.

Feeding VLBWI is always associated to the fear of non adequate nutrition with consequent non adequate growth (1). Because of a presumed association between feeding and necrotizing enterecolitis (NEC), many preterm infants are not treated with enteral feeding during the first days of life. All this brought to extensive use of parenteral nutrition (PN), with complications as scarce growth, colostasis, osteopenia and sepsis. In this way small volumes of human milk or formula (Minimal Enteral Feeding - MEF) are frequently used to stimulate the intestinal development in order to minimize these complications(2,3).

MEF refers to small milk volumes (less than 24 ml/kg/day) given to parenteral nutrition and to stimulate the gut; enteral feeding volume increases after prespecified interval, typically 7-14 days (4).

Therefore there is interest for early enteral nutrition in the preterm infant, but there are some debated points: the choice of substrate, the timing of first feeding, the method of feeding, the rate of feeding advancement, the risk of NEC.

About the choice of substrate, only the exposure to enteral food can consent the maturation of intestinal peristalsis in the VLBWI(5). The use of human milk rather than formula accelerates the timing gastric emptying and increases the gastric tolerance(6).

About the risk of NEC, the meta-analysis of McGuire (4), even if on old studies, remains still valid: human milk is better than formula in the prevention of NEC. The early introduction of trophic feedings shortened the time to reach full feedings without increasing the risk of NEC(7). The only variable that very clearly impacts the risk of NEC is the type of feeding. Human milk provides strong protection against sepsis, NEC and death(8,9,10) . The protective effect against NEC is also preserved in donor milk despite the heat treatment which donor milk undergoes(11,12,13) . Therefore the ideal food for VLBWI is the preterm human milk. It is necessary the maximum effort to promote fresh mother milk and human milk banking.

Key Words: VLBWI, enteral feeding, human milk

Corresponding Author: Dr. G. De Nisi
Neonatal Unit, S. Chiara Hospital, TrentoLargo Medaglie d'Oro 9, 38122 - Trento
+ 39 0461 903111
giuseppe.denisi@apss.tn.it

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About the timing of first feeding, the Cochrane review[14] concludes that early feeding does not give significant effects on growth, NEC, mortality or discharge age. Some benefits are seen in the more numerous trial: few days of PN, few cases of residuals and sepsis.

About the method of milk administration there are studies (15,16) that show a better gastric tolerance with intermittent bolus rather than continuous gastric infusion. The Cochrane review (17) concludes that the method of continuous nasogastric gives a significant delay of the full enteral feeding, rather than the method of gastric intermittent bolus, but the two methods do not involve difference in growth, in NEC incidence or hospitalizing length.

About the fast or slow advancing of feeding there are few controlled studies (18,19): they show the feasibility of increasing of enteral feeding by 20-25 ml/kg/die from the 1st to the 6th day, up to 140-180 ml/kg/die after 8 days. In ELBWI it has been reported the Scandinavian clinical practice (20) of increasing the enteral feeding by 20 ml/kg/die since the 1st day of life. A randomized trial (14) about "slow" versus "fast" increase of volumes on 185 VLBWI did not show significant correlation in the NEC incidence, neither in neonates with weight <1000 grams.

On the other hand another study (21) advises against the quick increasing of feed volume, that would give a bigger incidence of NEC, but the authors start with late feeding, with scarce using of human milk and with excessive increase of feed, however associated to prolonged PN.

A real early full enteral feeding in VLBWI seems possible and with good effect, without increasing the risk of NEC. This nutrition management falls within a global care context that must plan: prenatal steroids, "in uterus" transport, neonatal emergency transport, care in delivery room, minimal handling and human milk banking.

These management factors are present in Trentino and permitted this kind of nutrition.

In a study of 2005 (22) feeding for the VLBWI is introduced in a very careful way: to initiate feedings on day 2-3 of life when possible (a concept based on personal opinions); but to initiate feedings when breast milk is available (without considering the utility of human milk banking). Another meta-analysis (23) presents a comparison among seven studies with different opinions about feeding type (breast milk or formula), about timing to start feeds ("as soon as possible"; 2-3-4-8 days) and volumes without specifying some important points, as definition of "feed intolerance", plan of action for gastric residuals, policy for umbilical catheters.

On the other side the Norwegian Centers (24) calculated the relative risk of future late-onset septicemia if full enteral feeding with human milk (also bank's milk) is not established within 8-9-10 days of life. This study was done on a great number of infants <1000 grams. All the centers had their own human milk bank; they usually started within a few hours after delivery, with 1-2 milliliters of milk every 2-3 hours, increasing by 0,5-1 milliliter every 6-8 hours as tolerated.

MATERIALS AND METHODS

Trento experience

Our NICU is a tertiary care one in the town of Trento (pop. 100,000), in Northern Italy. The catchment area (Province of Trento) is all mountainous (6200 Km^2 - 520,000 inh.). There are 7 outside district hospitals with as many maternity units (4 without pediatric facilities): all VLBW and newborns with moderate to severe pathology are admitted to the regional hospital of Trento; distances range 20-60 km.

Neonatal Population is about 5200 newborns per year**,** of which 30% are born in the regional hospital; 50-55 VLBW per year, the vast majority admitted to our NICU. Vital statistics figures are in the lowest range among the other Italian regions: from 1994 to 2009 we admitted n. 718 VLBW infants without lethal malformations with in-hospital mortality rate 9,3%.

Our perinatal care has a regionalized-centralized (area-based) organization. Assisted neonatal transport has been performed by our referral NICU since 1973 (8,000 transports so far); >90% of the VLBW infants are inborn; maternal steroid prophylaxis is being approaching the 90% in the last years; surfactant prophylaxis is routinely given to <29 weeks infants in delivery room. Our NICU has 8 intensive care beds, 10 doctors, 25 nurses; pediatric and neonatal surgery available in the hospital, accommodation for 4 nursing mothers and a Human Milk Bank.

Good background situation and prenatal care (high incidence of maternal steroids and inborn), give us in many cases fit and healthy VLBWIs from the beginning. We think that in most cases we should better let the baby manage on his/her own, avoiding unnecessary stress and interventions. On the other hand, monitoring and follow-up were expanded, so as *minitouching* wouldn't turn into carelessness.

Early Exclusive Enteral Feeding protocol (EEEF)

Since 1993 our NICU has employed the early exclusive enteral feeding (EEEF) protocol. This protocol, which is targeted to specific VLBWI conditions, is based on the exclusive administration of human milk, either from the mother or from a donor, to VLBWI weighing 750-1250 grams and with GA >26 weeks.

This class of newborns is important cut-off for us, because for the neonates <27 weeks or <750 grams we use early parenteral nutrition with minimal enteral feeding, as other Centers.

From 1993 we use this feeding protocol:

WHO: VLBW >750g and >26w;

NO: asphyxia, RDS severe, hypotension, sepsis

YES: intubation-CPAP (only selected cases)

WHAT: Preterm donor Bank Milk and/or own mother's milk

HOW: Start at 2 hrs of life; <1000 g: 12 meals/day; >1000 g: 8 meals/day

Table I. *Comparison of some clinical otutcomes between Trento area (2006-2010) e Vermont Oxford Network (VON) (2010).*

	Trento	VON	O R	IC 95% RR		
Number of cases	**267**	**54983**				
inborn	82%	86%	0,72	0,67	0,78	*
prenatal steroids	92%	77%	3,33	3,29	3,36	*
cesarean section	82%	71%	1,82	1,76	1,87	*
multiple births	22%	28%	0,73	0,50	0,96	*
congenital anomalies	4%	5%	0,74	0,13	1,35	
intubation in delivery room	36%	51%	0,54	0,38	0,70	*
surfactant prophylaxis	29%	32%	0,88	0,69	1,06	
RDS	54%	74%	0,42	0,31	0,53	*
pneumothorax	1%	4%	0,37	-0,61	1,34	
nasal CPAP	61%	68%	0,73	0,63	0,82	*
convenzional ventilation	40%	63%	0,40	0,25	0,54	*
CLD after 28 days	11%	47%	0,14	-0,19	0,48	*
CLD after 36 weeks	7%	31%	0,18	-0,24	0,60	*
PDA	16%	37%	0,34	0,07	0,61	*
Indomethacin	4%	19%	0,18	-0,40	0,76	*
Ibuprofen	12%	11%	1,14	0,82	1,46	
NEC	1%	6%	0,24	-0,73	1,21	
IVH	10%	26%	0,33	-0,02	0,68	*
1° grade	6%	11%	0,48	-0,01	0,97	*
2° grade	1%	6%	0,24	-0,73	1,21	
3° grade	1%	4%	0,27	-0,85	1,40	
4° grade	2%	5%	0,44	-0,36	1,23	
Cystic PVL	2%	3%	0,62	-0,25	1,49	
ROP screening	84%	74%	1,78	1,73	1,83	*
ROP positives	13%	33%	0,30	-0,04	0,64	*
ROP severe grade (III -IV)	2%	7%	0,24	-0,73	1,21	
discharged on human milk	88%	45%	9,15	9,10	9,19	*
deceased	8%	14%	0,52	0,11	0,94	*

* *statistically significant*

HOW MUCH: First day: 3-5 mL per meal. Daily increments: 2-3 mL per meal

Fluid Intake: I°-II°-III° day: 30-50-70 ml/Kg/die; V° day: 100 ml/Kg/die; X° day: 150 ml/Kg/die.

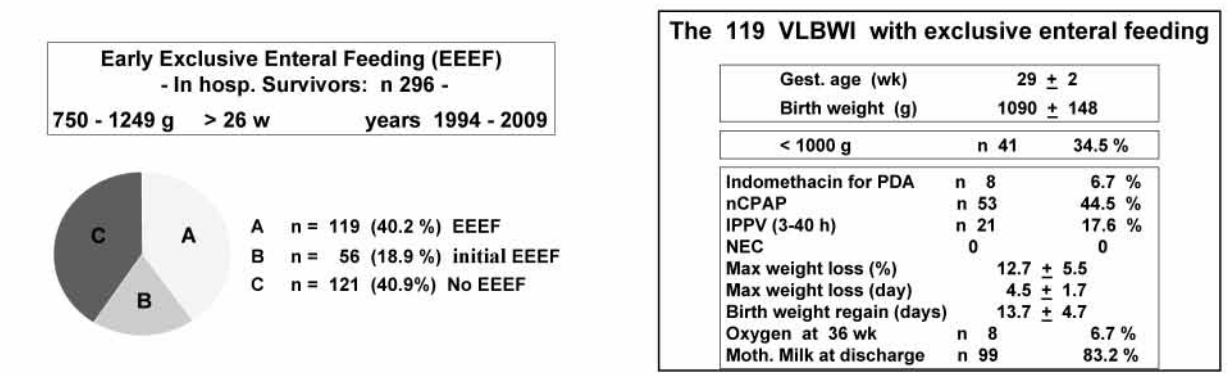
Feeding protocol is *flexible and individualized*, based on clinical conditions and feeding tolerance of infant (3). Monitoring of real fluid intake and fluid output is mandatory. Every newborn has an electronic table where many data are collected: weight, weight loss, amount of meals with residuals, amount of infusion and parenteral nutrition, diuresis, calorie needs, water needs, sodium, plasma osmolality, urea, creatinine. In this way we count the precise amount of milk taken by this newborn.

About gastric residuals, we know that milky residuals in the day of life are frequent in preterm infants, without correlation to risk of NEC (25). In our department we studied 42 VLBW infants treated with exclusive enteral feeding with preterm human milk: the residuals increased in the 2nd-3rd day of life, but in the following days they remained 1-3 millilitres, in spite of the increasing amounts of meals. In order to prevent potential residuals we assume the indications of the literature (26) and we prefer prone positioning for our babies after meal.

About NEC correlated to feed's fast advancements, in a randomized trial of "slow" vs "fast" feed advancements (19) the incidence of NEC was not significant in all the weight-subgroups.

In a period of 16 years (1994-2009) in Trento, 308 newborns weighing 750g-1249g and GE >26 weeks were admitted. The 90,9% has been treated with prenatal steroids, the 91,9% was

Fig. 1. *Early Exclusive Enteral Feeding (EEEF) in VLBWI from 1994 to 2009*



inborn, the 96,1% survived. In the 59,1% of the cases (175) we gave EEEF. We could continue with a complete EEEF in the 40,2% of the total (119 cases). In particular, we treated without infusion 41 cases of neonate with BW <1000g. The nCPAP and IPPV did not avoid the EEEF. The maximum weight loss was about the 13%, at the 4-5th day of life. The BW regain there was at the 13-14th days of life. Mother milk at discharge was in the 83,2% of the cases. (Fig. 1)

RESULTS

In the Table I you can see many differences statistically significant. In particular, there are indicators for the management of Trento which the high prevalence of antenatal steroids and the low prevalence of RDS, ventilatory support and PDA, as well as a low percentage of IVH and ROP. About the mortality rate there is a comforting result in respect of VON (8% vs. 14%), as well as the very high percentage of discharged with breast milk: 88% vs. 45% of the VON. This figure is justified by the presence of the Human Milk Bank in Trento and a special support and promotion of breastfeeding mothers.

DISCUSSION AND CONCLUSION

We think that this feeding protocol with a regionalized-centralized perinatal care is an important factor reducing NEC in VLBW infants and support the mother-neonate bond. This management must be associated to an greater use of prenatal steroids, to a less aggressive surfactant approach and to availability of human milk banking, factors that may also affect other clinical outcomes.

From a comparison with VON (Vermont Oxford Network) (27) we demonstrated significant lower figures on chronic IPPV, parenteral nutrition, infusions, transfusions. In every birth weight (BW) group a significantly higher proportion of VLBWIs was discharged on breastfeeding in Trento compared with VON.

A more recent comparison with VON confirm these differences (Table I). The comparison of data and results with VON is of great interest because the data of Trento refer to a well-defined basin, than area based, and therefore statistically comparable with those macro.

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TOLERABILITY OF DONKEYS' MILK IN 92 HIGHLY-PROBLEMATIC COWS' MILK ALLERGIC CHILDREN

G. MONTI[1], S. VIOLA[1], C. BARO[2], F. CRESI[3], P.A. TOVO[1], G. MORO[4], M.P. FERRERO[5], A. CONTI[2], E. BERTINO[3]

[1]*Department of Paediatric and Adolescence Science, Regina Margherita Children's Hospital, Turin, Italy*
[2]*CNR, Institute of Science of Food Production, Turin, Italy*
[3]*Neonatal Unit, Department of Paediatric and Adolescence Science, University of Turin, Italy*
[4]*Italian Association of Human Milk Banks, Milan, Italy*
[5]*Laboratorio Analisi, Koelliker - Missionari della Consolata Hospital, Turin, Italy*

Objective: Not exclusively breastfed children with cows' milk allergy (CMA) require a formula or other alternative food, but past and present guidelines differ concerning the best choice. Our aim was to investigate the clinical tolerability, palatability and nutritional adequacy of donkeys' milk (DM) in children with proven CMA. It was important to identify a CM replacement for these children, highly problematic from the feeding standpoint, in spite of their age. Study design: A prospective study was conducted on 92 children with CMA, diagnosed through a CM elimination diet, followed by double-blind, placebo-controlled food challenge (DBPCFC) unless contraindicated. Maternal milk was unavailable and current CM substitutes could not be used. Moreover, 89% were affected by multiple FA, and subjected to very restricted diets. Within 3 months after the last CM challenge, DBPCFC for DM was performed. CM or DM skin prick test and sIgE determination preceded the CM or DM challenge, respectively. Native electrophoresis and immunoblotting were used to identify CM and DM cross-reactive proteins. Z-scores of weight and length/stature for age were calculated at DM food challenge (T_0) and during DM assumption. Results: 83 children (90.2%) liked and tolerated DM, at challenge and during follow-up, with increased Z-score for weight and length/stature and improved nutritional parameters. Bovine beta-lactoglobulin was identified as the cross-reacting protein among the DM allergic patients. Conclusions: DM was found to be a valid alternative foodstuff, in terms of clinical tolerability, palatability and nutritional adequacy, in subjects with CMA who were highly problematic from the feeding standpoint.

Treatment of cows'milk allergy (CMA) hinges upon the complete elimination of cows'milk proteins (CMP) from the diet. In non-breastfed infants, in children under two-three years of age, and in children above 2-3 years with CMA associated with allergies to other staple foods, it is mandatory to find a substitute formula or other alternative food [1].

The selection of a formula depends on the allergy syndrome to be treated. Current cows' milk (CM) substitutes are soy-protein-based formulas (SF), extensively hydrolized formulas of either CM proteins (eHF) or rice proteins (RHF), and amino-acid formulas (AAF). There is no agreement concerning the best choice, in recent [1-3] nor previous guidelines [4,5]. All these substitutes have an acceptable nutritional value, although the long-term effects in infants have yet to be studied [6,7]. Moreover, many questions remain open concerning the high concentrations in SF of phytate, aluminium and phyto-estrogens (isoflavones). The chief limitation upon the use of eHF, RHF and AAF consists of their extremely unpleasant taste [8-10]. For these reasons, alternative milks from other mammalian species have been considered [9, 11-19]. Equine milks (mares' and donkeys' milk) were the object of clinical studies on very small series [11,13,14], and recently on larger series, in vivo [18,19] or both in vivo and in vitro [9], with high rates of tolerability. This prospectic study investigated the efficacy of donkeys' milk (DM) in terms of clinical tolerability, palatability and nutritional adequacy, in

Key Words: cows' milk allergy, alternative milk substitutes, donkeys' milk

Corresponding Author: Dr. G. Monti
Department of Pediatric and Adolescence Science, Regina Margherita Children's Hospital, TurinPiazza Polonia 94, 10126 Turin, Italy
+39 011 3135257
giovanna.monti@email.it

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an even larger series of children with proven CMA, highly problematic from the feeding standpoint, and identified any CM and DM cross-reactive proteins.

MATERIALS AND METHODS

Subjects and study design

Between 1 February 2004 and 1 July 2009, 92 children were recruited (55 boys; age-range 7.5-121.5 months, median 19.5, mean 27.3) with proven CMA, for whom maternal milk was unavailable and current CM substitutes could not be used.

Table I shows age and symptoms at first observation. At recruitment, 82/92 children (89%) suffered from allergy to other foods (mainly eggs, wheat, and fish).

Unless contraindicated [1], the diagnosis of CMA was made on the basis of a CM elimination diet (2-4 weeks), followed by double-blind, placebo-controlled food challenge (DBPCFC). Before food challenge (FC), skin prick tests (SPT) were done for CM, and sIgE for cows' milk proteins (CMP) were determined. At recruitment, the parents of 8 children (mean age 28 months) preferred to avoid current CM substitutes, but rather to try DM directly; the parents of a further 3 children (mean age 16 months) who were already taking SF wished to try DM. Of the remaining 81/92 children, 53 already at first observation presented GI symptoms such that it was inappropriate to use SF [1,3], whereas the other 28 (30.4%) presented an allergy to SF between first observation and recruitment, confirmed by positive DBPCFC. SF could therefore not be used for any of these 81 subjects, and it was also impossible to use either eHF, RHF or AAF: 7/81 children did not tolerate eHF and refused to take RHF or AAF, because of their unpleasant taste; the remaining 74/81 categorically and systematically refused to take either eHF/RHF or AAF, some immediately at the first attempts, others after taking those formulas for variable periods of time (12 months on average). It was thus proposed to the parents of these 81 children that they participate in the study using DM to replace CM.

Ethical approval was obtained from the local Review Board, and informed parental consent was given. DM was obtained from a certified organic farm where donkeys are raised outdoors and fed exclusively with organic foodstuffs.

Skin tests

CM or DM SPT were performed before CM or DM challenge, respectively with fresh CM, or with fresh DM, as described elsewhere [20].

Determination of specific IgE

Serum levels of CMP-sIgE and DM proteins (DMP)-sIgE were determined by the automated Pharmacia CAP system FEIA (Phadia & Upjohn Diagnostics, Sweden) on blood samples taken during the SPT.▪

Food challenge

Both CM and DM food challenge were performed following Monti et al. [9]. The FC for DM was performed during a period of 0.5-3 months after the last FC for CM, using fresh DM after heating to 70° for 2 min.

Follow-up

The study design included clinical and auxological follow-up, at DM food challenge (T_0), and after 1 month (T_1), 2-3 (T_2), 4-6 (T_3), 7-12 (T_4), 13-18 (T_5), 19-24 (T_6), 25-36 (T_7), and 37-48 months (T_8) of DM consumption. The last evaluation was defined as T_{end} and corresponded to the moment when the child stopped ingesting DM. Auxological evaluation was performed following Monti et al. [9]. The child's diet was appropriately balanced depending on requirements by age by a dietician. The parents kept a food diary, with particular reference to daily DM consumption. Resolution of the CMA was assessed periodically during follow-up. For the 92 children, at T_0 and after 6-12 months (T_c) of DM ingestion, parental consent was requested to evaluate the following nutritional parameters: blood count, iron status (serum iron, transferrin, saturated transferrin, ferritin), calcium-phosphorus balance (calcium, phosphorus, alkaline phosphatase, vitamin D), protein balance (serum prealbumin, IGF-1), lipid balance (total cholesterol and HDL, triglycerides, apolipoproteins A1 and B, and their ratio).

Statistical analysis

Z-scores of weight (WA) and length/stature (LA) for age were calculated from the formula $Z = x- |X| / |SD|$, taking the Gardner and Pearson growth curves as reference for children up to 24 months and the Tanner curves after 2 years of age, to evaluate changes in anthropometric parameters independently of age. The differences in Z-score between T_0 and T_1 and between T_0 and T_{end} were evaluated by the t-test for paired data. Z-score differences between check-ups were analyzed with the ANOVA test for repeated measures. A post hoc analysis was performed through the Bonferroni multiple comparisons test to establish the significance of differences between check-ups. The difference in blood-chemistry parameters between T_0 and T_c was evaluated through the t-test for paired data, or the Wilcoxon test for paired data, as appropriate. Significance was set at $p < 0.05$. Statistical analysis was performed with the Stat□ 5.5 software (StatSoft, Inc).

Native Electrophoresis and Electroblotting

For native electrophoresis, skimmed DM was mixed with boric acid-borax buffer 0.2M, pH 8.4, "Sample buffer" (0.5M Tris-HCl pH 6.8, 0.5% (w/v) Bromophenol blue and glycerol) and water (1:1:1) and brought up to 200 µl. Each gel was loaded with 150µg of total protein, following Bolt et al. [21]

Immunostaining

Immunolabeling was performed according to Natale et al. [22] with serum from 17 patients: of these, 12 only had FC positive to CM, 5 also had FC positive to DM.

Mass spectrometry

DMP were identified by MALDI-TOF (Ultraflex II TOF/TOF, Bruker) after reduction and alkylation according to Fortunato et al. [23]. The proteins were then digested in gel with trypsin, following Bertino et al [24].

RESULTS

Table I reports the outcome of SPT for CM and sIgE

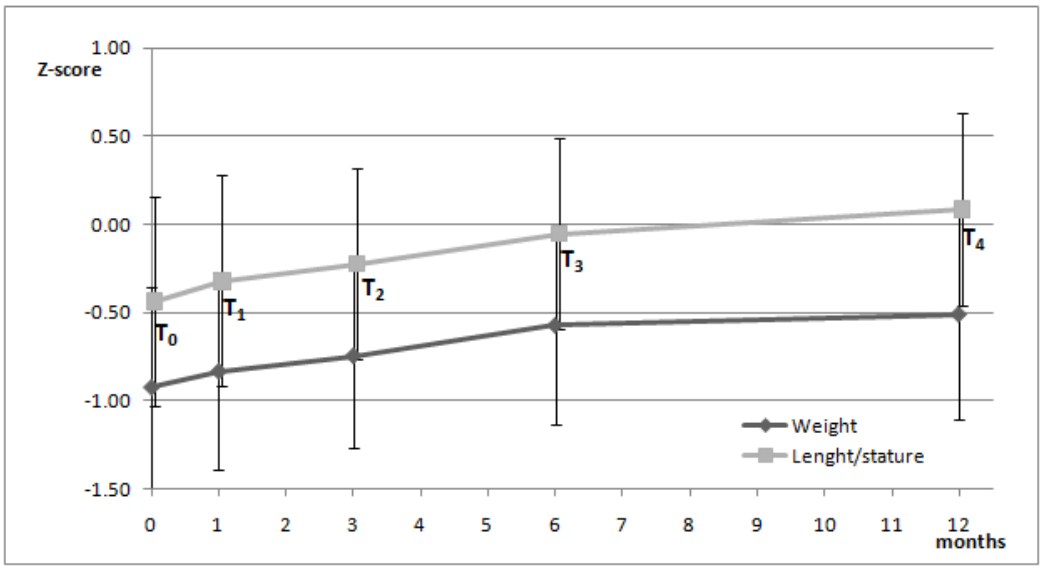

Fig. 1. *Weight and length/stature Z-score variations in the first year of uninterrupted DM feeding.*

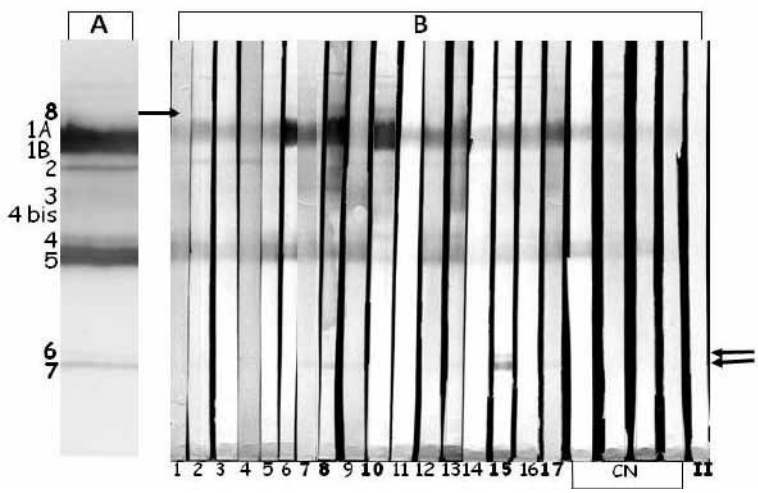

Fig. 2. *Native Electrophoresis and Immunoblotting. A = Native blotting on NC membrane of fresh donkeys' milk proteins stained by Sypro Ruby blot staining; B = Native Immunoblotting using as primary antibody sera from 17 different patients. In bold type, patients with oral challenge positive for both donkeys' and cows' milks (patients 7, 8, 10, 15 and 17); the remaining 12 patients were only allergic to cows' milk proteins. Secondary antibody: alkaline phosphatase-conjugated goat anti-human-IgE. CN = negative controls, sera of four non-allergic subjects; II = secondary Ab control, immunoblotting without primary Ab.*

levels for CMP, as well as age and clinical symptoms at the last CM food challenge, prior to enrolment. As patients with a recent history of anaphylaxis, or in whom a severe allergic reaction had occurred immediately after CM exposure and with positive CM IgE tests should not be challenged [1], CMA was confirmed with DBPCFC in 79/92 children. Sixty-nine children (75%), with SPT and/or sIgE positive for CMP, had an IgE-mediated CMA.

83/92 children (90.2%) both liked and tolerated DM at the challenge and for the entire duration of follow-up. DM was tolerated by 20/23 (87%) children with non-IgE-mediated CMA and by 63/69 children (91.3%) with IgE-mediated CMA. In particular, all 11 children with prior anaphylaxis due to CMA tolerated DM, as did both

Table I. *Clinical characteristics of each patient*

	FIRST OBSERVATION		PbP CMP	CMP sIgE (KU/L)			CMP CHALLENGE		PbP DM	DM sIgE	DM CHALLENGE	
N	AGE (months)	SYMPTOMS	(ø mm)	CAS	ALA	BLG	Age (months)	Symptoms	(ø mm)	(KU/L)	Age (months)	Symptoms
1	37	AD, DPE	0 (NEG)	0,09	0,09	0,09	40	G, AP (e)	0 (NEG)	0,09	43	-
2	9	AD, FTT	15 (POS)	11,80	10,40	4,32	19	AD (l)	0 (NEG)	1,41	20	-
3	38	CD, FTT	2 (NEG)	0,31	2,60	0,19	38	D (e)	0 (NEG)	0,37	40	-
4	54	**AN (CMA), AD, AP**	8 (POS)	0,69	24,64	0,01	-	not performed	0 (NEG)	0,01	58	-
5	12	U/A, FTT	6 (POS)	0,46	0,04	0,58	13	U/A (i)	0 (NEG)	0,01	14	-
6	29	DPE	0 (NEG)	0,02	0,01	0,03	30	D (l)	0 (NEG)	0,01	33	-
7	8	AD, severe IC, CD	8 (POS)	1,41	0,34	0,67	9	U, D, NS (i)	0 (NEG)	0,09	10	-
8	6	AD, FTT	8 (POS)	2,94	0,00	0,08	10	U (i)	0 (NEG)	0,19	13	-
9	120	AD, GERD, AP, FTT	3 (POS)	0,09	0,09	0,09	121	AP (e), WL (l)	0 (NEG)	0,09	121,5	AP (l)
10	2	DPP	11 (POS)	4,76	3,45	1,72	34	D, AP (i)	3 (POS)	0,09	36	NS, D (i)
11	4	AD, DPP	0 (NEG)	0,09	0,09	0,09	15	AP (e), AD (l)	0 (NEG)	0,12	16	-
12	50	AD, U/A, DPP*	0 (NEG)	0,06	0,20	0,31	50,5	AP (e), DPP (l)	0 (NEG)	0,05	51	-
13	43	FTT	0 (NEG)	0,11	0,02	0,46	45	D, WL (l)	0 (NEG)	0,06	48	-
14	2	AD	10 (POS)	2,93	0,09	1,86	32	U, A (i)	2 (NEG)	0,07	34	-
15	4,5	severe IC, CD	3 (POS)	0,36	0,44	0,56	19,5	U/A (i)	0 (NEG)	0,07	20	-
16	10	AD, FTT, important U/A (CMA)	7 (POS)	6,05	0,09	0,03	-	not performed	0 (NEG)	0,09	10	-
17	10	AD	0 (NEG)	0,02	0,14	0,01	11	U (i)	8 (POS)	5,6	12	NS, OS, V, U (i)
18	94	AD, CD, AP, FTT	0 (NEG)	0,06	0,08	0,07	96	AP, D (e)	0 (NEG)	0,04	97	AP, D (e)
19	3	AD, GERD	14 (POS)	3,04	0,08	0,05	-	**not performed - AN (accidental exposure) at 45 m**	0 (NEG)	0,05	47	-
20	15,5	GERD, FTT	2 (NEG)	0,23	0,98	0,76	16	V (i)	0 (NEG)	0,1	16,5	-
21	16	AD, AN (EA)	1 (NEG)	0,60	0,09	2,16	16	AD (l)	0 (NEG)	0,05	17	-
22	9	GERD, generalized important U (CMA)	4 (POS)	2,25	1,23	5,32	-	not performed	0 (NEG)	0,02	10	-
23	4	AD, FTT	15 (POS)	12,20	0,09	0,09	8	AD (l)	0 (NEG)	0,13	10	-
24	58	AD, DPE	0 (NEG)	0,09	0,09	0,09	59	D (e), AD (l)	0 (NEG)	0,01	59,5	-
25	43	GERD, DPE*	5 (POS)	1,57	1,09	0,32	45	GERD (l)	0 (NEG)	0,33	48	GERD (l)
26	7	AD	15 (POS)	17,40	0,08	0,07	-	not performed – generalized U (i) and NS (i) (accidental exposure) at 17 m	4 (POS)	1,51	19	-
27	5	AD	0 (NEG)	0,09	0,09	0,09	10	AD, D (l)	0 (NEG)	0,01	12	-
28	56	**AD, AN (CMA)**	3 (POS)	0,83	0,10	0,03	81	U, V, NS (i)	0 (NEG)	0,01	82	-
29	16	AD, DPE	2 (NEG)	0,13	0,17	0,44	17	V, D, ER (i)	0 (NEG)	0,12	18	-
30	5	AD	8 (POS)	8,71	0,01	0,19	22	U (i)	0 (NEG)	0,02	23	-
31	50	AD, AN (EA)	7 (POS)	0,14	0,02	0,07	51	U (i)	0 (NEG)	0,01	52	-

children suffering from severe CMA-FPIES. Of the 7 children who were intolerant of eHF, only one was also intolerant of DM. Nine children (9.78%) were positive at the DM challenge. Clinical symptoms, none of which were life-threatening, are detailed in Table I. Only 4/9 were sensitised to DM (SPT and/or sIgE positive). A further 14/83 children (16.8%) were sensitised to DM, but none reacted at the FC. 10/11 children with prior CMA anaphylaxis had negative DM IgE tests.

DM consumption

At T_{end}, the period of DM consumption in the 83 children was 1-69 months (mean 15.48 months, median 12). Note that eight patients were only recruited 1-2 months before the end of the study. DM consumption was 200-900 ml/day depending on age (mean and median 300 ml/day). The reasons why children dropped out of the study were: 23/83 acquired a tolerance of CM, after a mean period of DM ingestion of 13 months; in a further 8 cases DM was suspended due to its high cost, after a period of at least 12 months ingestion; a further 4 children (mean age 45 months) no longer wanted to drink DM, after a mean period of ingestion of 15.5 months.

Growth-related data

For the 83 children (49 boys) who regularly and uninterruptedly ingested DM for up to 69 months, the duration of follow-up enabled statistical elaboration to be applied to growth figures for these cases. Table II reports auxological values at T_0 and T_{end}.

55/83 subjects (35 boys) participated in all the scheduled check-ups, at least until T_4. In these patients, negative Z-score values at recruitment, for weight and for length/stature respectively, were found in 44/55 (80.0%) and 36/55 (65.5%) subjects, and a significant increase in Z-score was recorded both for weight ($p<0.001$; $F=17.52$) and for length/stature ($p<0.001$; $F=9.29$) during the entire period of evaluation (Fig. 1).

Nutritional parameters

For 24 children, parental consent was obtained to evaluate nutritional parameters at T_0 and at T_C (median 8 months). Table III shows the parameters for which a statistically-significant difference was found.

Native Electrophoresis and Immunoblotting

Figure 2A shows the separation of DMP in native

Table II. *Growth-related data*

	T_0 Mean (DS)	T_{end} Mean (DS)	Statistical significance
Weight (Kg)	11.00 (3.10)	13.34 (3.09)	
Length/stature (cm)	82.51 (11.64)	91.55 (10.38)	
Z-score for weight	-0.95 (1.15)	-0.57 (1.19)	$p < 0.01$*
Z-score for length/stature	-0.42 (1.15)	0.01 (1.13)	$p < 0.01$*

** t-test for paired data*

Table III. *Nutritional parameters*

	T_0 Mean (DS) Median (IQR)	T_c Mean (DS) Median (IQR)	Statistical significance
Hemoglobin (g/dl)	11.93 (0.99) 11.80 (1.55)	12.43 (0.95) 12.50 (1.00)	p = 0.008*
Serum iron (µg/dl)	76.78 (43.36) 73.00 (39.50)	89.55 (35.25) 80.00 (42.50)	p = 0.006#
Saturated transferrin (%)	20.78 (9.50) 18.00 (9.50)	23.78 (9.71) 23.00 (13.50)	p = 0.005#
1,25-$(OH)_2$ vitamin D (pg/ml)	73.39 (15.06) 71.20 (14.85)	64.37 (15.69) 65.00 (13.65)	p = 0.019#
Serum prealbumin (mg/dl)	17.62 (4.29) 18.00 (4.50)	19.14 (3.81) 19.00 (6.50)	p = 0.039*

** t-test for paired data*
 # Wilcoxon-test for paired data

conditions. The proteins in the numbered bands were identified by MALDI-TOF MS and are listed in Table IV. Bands 6, 7 and 8, highlighted in bold type in Fig. 2A, are specific to the subjects allergic to DM; (nos. 7, 8, 10, 15 and 17; in bold in Fig. 2B).

Through native electrophoresis, the recognition reactions of the IgE of allergic subjects toward the conformational epitopes of the DMP were pointed up. The five patients allergic to DM (nos. 7, 8, 10, 15 and 17) showed specific reaction bands (bands 6, 7 and 8 in Fig. 2A) that were not found in subjects who were only allergic to CM.

DISCUSSION

The results of this prospective study show the high tolerability (90.2%) of DM in a series of 83 children with proven CMA, larger than previous series. This result confirms those of two recent studies [18,19] on smaller series, in which DM was tolerated respectively by 25/26 (96%) and by 24/25 (96%) recruits. Unlike our series, which included subjects with severe CMA (prior anaphylaxis, acute severe FPIES, children with GI symptoms with malabsorption syndrome and FTT), the children enrolled in the smaller studies only suffered from a mild or moderate form of CMA.

The high percentages of tolerability of DM observed in our subjects, both with IgE-mediated and with non-IgE-mediated CMA, were not dissimilar, suggesting that tolerability of DM is independent of the type of allergic reaction to CMP.

All of the 11 patients with prior anaphylaxis to CM tolerated DM at the FC and during the follow-up. However, in a previous study including five patients with prior anaphylaxis to CM [9] , only one tolerated DM at the challenge. At present no further data are available

Table IV. *Identification of the proteins in bands 1A to 8 in Fig. 2 by MALDI TOF and MALDI TOF/TOF mass spectrometry.*

Band	pI	MW kDa	Protein (Accession number)	Maldi Mass spectrometry Identification matching peptides (coverage %)
1A (fig.2A)	5.78 6.02	25511 25305	Beta-casein precursor (Q9GKK3 Horse) Alpha-s1 casein (Q8SPR1 Horse)	6 (21) 4 (13)
1B (fig.2A)	5.78 6.61	25511 76141	Beta-casein precursor (Q9GKK3 Horse) Serotransferrin precursor (Transferrin) (P27425 Horse)	6 (21) 8 (19)
2 (fig.2A)	6.02 8.32	25305 75420	Serum albumin precursor (Q5XLE4) Lactotransferrin precursor [fragment] (Lactoferrin) (O77811 Horse)	26 (45) 7 (15)
3 (fig.2A)	-	-	Not identified	-
4 (fig.2A)	4.85	18500	Beta-lactoglobulin I precursor (variant B)	18 (81)
4B (fig.2A)	4.79	18528	Beta-lactoglobulin I precursor (P13613)	6 (42)
5 (fig.2A)	4.85 6.61	18500 76141	Beta-lactoglobulin I precursor (variant B) Serotransferrin precursor (Transferrin) (P27425 Horse)	13 (67) 11 (25)
6 (fig.2A)	6.61 4.70	76141 18530	Serotransferrin precursor (Transferrin) (P27425 Horse) Beta-lactoglobulin-2 (Beta-LG-2), (Beta-lactoglobulin II, minor monomeric) (P19647)	11 (29) 7 (52)
7(fig.2A)	4.70	18530	Beta-lactoglobulin-2 (Beta-LG-2), (Beta-lactoglobulin II, minor monomeric) (P19647)	7 (52)
8 (fig.2A)	5.78 6.61	25511 76141	Beta-casein precursor (Q9GKK3 Horse) Serotransferrin precursor (Transferrin) (P27425 Horse)	6 (21) 8 (19)

concerning the tolerability of DM in subjects with prior anaphylaxis to CM; thus, although the results of the present study are promising, the use of DM in these subjects should be contemplated with considerable caution, and the introduction should always be in a hospital setting. Data concerning the tolerability of DM in subjects with severe FPIES due to CMA are also good, but no conclusion may be drawn in this connection, considering the small number of cases.

DM was tolerated in our 83 patients not only at the FC, but for the entire follow-up (mean 15.48 months, median 12), which was sufficiently long to exclude the onset of clinical reactions consequent on primary sensitization to DM.

Of the nine children (9.78%) who were positive at the DM challenge, in no case did DM induce severe systemic reactions of the immediate type at the FC, in line with previous findings [9,18,19] and it caused immediate reactions in only two of our patients out of nine who were positive at FC.

Specific allergometric tests for DM had poor predictivity on both the outcome of the DM oral challenge and the onset of immediate clinical reactions; in particular, the high number of false-positive DM IgE tests might be due to the presence of sIgE primitively directed against epitopes of CMP cross-reacting with epitopes of DMP, without however being responsible for clinical reactions against the latter. DM IgE tests, on the contrary, showed good negative predictivity for the outcome of the DM oral challenge; this was particularly true in the 11 patients with prior anaphylaxis to CM, 10 of whom were not sensitized to DM and none of whom reacted at the FC with DM.

Our patients were highly problematic from the feeding standpoint, since most of them (89%) were affected by multiple FA, leading to the exclusion of staple foods (e.g. egg, wheat, fish) and obliging them to follow a particularly restrictive and monotonous diet: this made it of vital importance to identify a replacement foodstuff for CM, in spite of their age. Unfortunately, current CM substitutes (SF, eHF, RHF and AAF) could not be used. In this connection, the patients recruited for this study were particularly demanding with regard to the palatability of foods, presumably due to their age. DM was found to be a valid alternative including from the standpoint of palatability: its pleasant taste made DM immediately acceptable to all our patients.

Weight and length/stature-gain were evaluated in terms of Z-score. This method evaluates changes in anthropometric parameters associated with the introduction of DM independently of age. The great majority of subjects had negative weight and length/stature Z-scores at enrolment, and showed a significant improvement during the study period (Table II). In particular, the group of patients who completed the follow-up of 12 months presented increases in weight and length/stature Z-score at each checkup after the introduction of DM, as Fig. 1 shows.

The nutritional parameters also showed encouraging results (see Tab. III), although these were available for a smaller number of patients; the diets of all 24 children were particularly restricted, despite their being balanced by the dietician. Diet did not undergo any particular changes between T_0 and T_C, except for the addition of DM. These results, which are in agreement with those of another recent study [19], may in part be attributed to the reported nutritional characteristics of DM. [25,26].

Native electrophoresis of the DMP revealed three peculiar bands present only in the immunoblotting experiments of DM allergic subjects. These immunoreactive bands contained donkey β-casein and β-lactoglobulin II. The lack of pure DM standard proteins made it impossible to perform inhibition experiments with these two cross-rective proteins. This result is interesting in the light of the fact that, for human β-casein, the existence of a conformational epitope cross-reactive with the sIgE for bovine β-lactoglobulin has been reported [27], making it plausible that the only CM protein that can stimulate the production of IgE cross-reactive with the DMP is β-lactoglobulin.

CONCLUSION

It was of great importance to be able to use a palatable and well tolerated substitute foodstuff for our 83 children; despite their restricted diet, DM helped these children to achieve correct growth in terms of height and weight, and normalized the blood-chemistry parameters evaluated, or maintained those parameters within the normal range. A final but no less important aspect is the psychological advantage of being able to use "proper" milk as an alternative to CM, The children either consumed DM as such, or in the form of derivatives (e.g. ice-cream) or of other milk-based foods (e.g. cakes, puddings, home-made biscuits).

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RELATIONSHIP BETWEEN SLEEP/WAKEFULNESS AND GASTROESOPHAGEAL REFLUX IN SYMPTOMATIC NEWBORNS.

F. CRESI[1], E. LOCATELLI[2], E. MAGGIORA[1], P. DI NICOLA[1], E. CESTER[1], G. SABATINO[3], C. MARTANO[1], F. GIULIANI[1], E. BERTINO[1]

[1]*Neonatal Unit, University of Turin, Italy*
[2]*Department of Paediatric and Adolescence Science, Regina Margherita Children's Hospital, Turin, Italy*
[3]*Neonatal Intensive Care Unit, University of Chieti, Italy*

The state of sleep/wakefulness is well known to influence esophageal acid exposure and the number of acid refluxes whereas it is uncertain whether the same is true of the non-acid refluxes that predominate in the newborns. To investigate the relationship between sleep/wakefulness and refluxes 45 newborns with gastroesophageal reflux symptoms were studied with combined multichannel intraluminal impedance and pH monitoring. We found that sleep/wakefulness influenced acid and weakly acidic reflux frequency (awake 2.6±0.8; asleep 2.1±1.1; p=0.006). A negative correlation was found between sleepness periods and the mean reflux duration for both acid (R=0.55;p<0.001) and weackly acidic (R=0.51;p<0.001) refluxes. This finding may raise some concerns about the over-prescription of antacid drugs in newborns.

The understanding of the factors that influence gastroesophageal reflux (GER) has a clinical importance and is essential for the correct interpretation of diagnostic data. The state of sleep/wakefulness is known to influence esophageal acid exposure and the number of acid refluxes(1,2), whether or not the same happens with the non-acid refluxes that predominate in the newborns is still uncertain. Combined multichannel intraluminal impedance and pH monitoring (MII/pH) is a new technique that accurately identifies GER episodes irrespective of their acidity and has already been used in newborns to assess the effects of drugs(3,4), thickening agents(5) and feeding strategies(6). It was employed in the present study to evaluate the relationship between GER and sleep/wakefulness periods in the newborns with symptoms indicative of GER.

PATIENTS AND METHODS

24-hr MII/pH monitoring was performed on term newborns, totally or partially formula fed, referring consecutively to our Neonatal Care Unit for regurgitations (>6/day any amount or >once/day half the feed). The exclusion criteria were treatment with medications with GI effects, and evidence of infection, metabolic, or CNS disease.

A single-use, flexible MII/pH probe with 7 metal electrodes placed at 1.5 cm was inserted transnasally into the esophageal lumen after a 3-hr fasting and connected to the impedance-voltage transducers/recording device (Sleuth System; Sandhill Scientific Inc., Highlands Ranch, CO, USA). The impedance signal was measured bipolarly between paired electrodes forming 6 impedance channels. A reflux episode was defined as a decrease of impedance starting in the most distal channel (channel 1), extending proximally to the second channel or further and followed by an increase in impedance to baseline values (6). A pH-sensitive antimony electrode situated on the probe at channel 1 was used to read the pH value of the refluxate. X-ray guidance was used to position the probe with the pH-sensitive tip 2 cm above the lower esophageal sphincter. MII/pH identified GER episodes and illustrated their duration, level and pH. The duration of an episode was defined as the time, in seconds, between its onset at the 50% drop in impedance from baseline relative to nadir and bolus exit at the 50% recovery point from nadir to baseline recorded at channel 1. Its level was defined as the proximal extent reached by the refluxate, shown by the

Key Words: gastroesophageal reflux, wakefulness and sleep periods, multichannel intraluminal impedance, newborns.

Corresponding Author: Dr. F. Cresi
Neonatal Unit,University of TurinVia Ventimiglia 3, 1026
Torino, Italy
+39 0113135781
francesco.cresi@unito.it

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Table I. *Variations of reflux features during sleep and wakefulness.*

		Awakeness	Asleepiness
Acid	Episodes/hour(no.)	0.4(0.3)	0.7(0.7)*
	Level (no.of channels)	3.7(1.0)	3.5(0.9)
	Duration (sec.)	25.4(21.6)	24.4(13.7)
	pH	3.0(0.7)	2.5(0.9)*
Weakly acidic	Episodes/hour(no.)	2.7(1.1)	1.1(0.6)*
	Level (no.of channels)	3.3(0.5)	3.3(0.6)
	Duration (sec.)	22.9(9.8)	21.7(12.4)
	pH	5.4(0.2)	5.1(0.3)*

Data presented as mean (SD).
*T-test for paired values: *p<0.05 compared with awake period.*

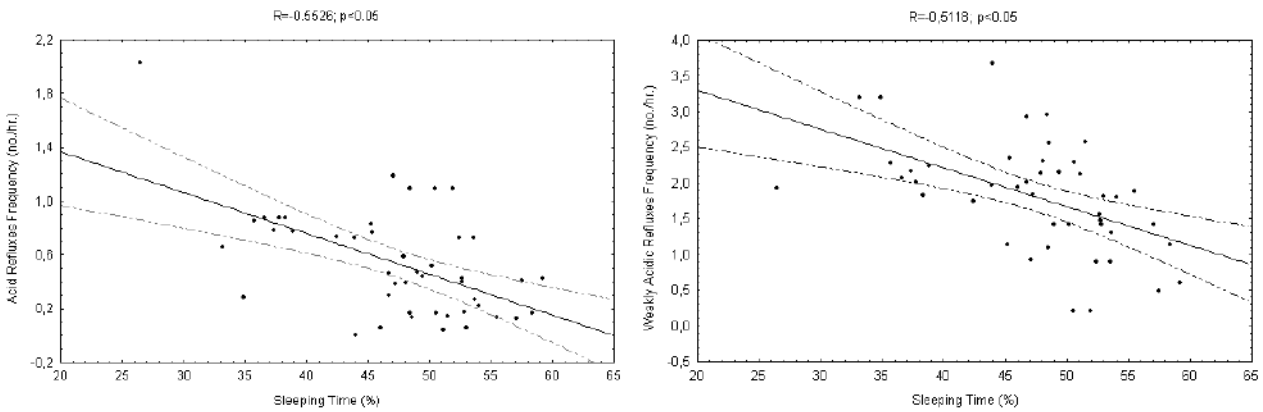

Fig. 1. *Relationship between reflux frequency and sleep duration.*

number of channels sequentially involved in the temporary impedance decrease. The pH was defined as the lowest value reached during the reflux. Refluxes were classified as acid (pH<4) and non-acid (pH≥4). The patients were kept supine in their cots during the examination. They were all bottle-fed every eight hours with 25 ml/Kg of the same formula. Sleep/wakefulness states were noted on the MII/pH device every 15 min by the parents or the nurses. Reflux episodes were divided into "asleep" and "awake" episodes.

Differences were evaluated with the t-test for paired values with p<0.05 as the significance cut-off. Pearson linear correlation was used to test the relationship between reflux features and sleeping duration. Statistical analysis was performed with the Stat 5.5 software (StatSoft, Inc.,

Tulsa, OK, USA). The local Ethics Committee approved the research protocol. Written consent was obtained from the parents.

RESULTS

Forty-five neonates (24 M, 21 F) aged 17±7 days and with a mean weight of 3874±365 g and body length of 53.2±3.6 cm were studied. On a total of 1135.2 hours of MII/pH monitoring 209.6 were discarded owing to the presence of artifacts, feeding times and temporary changes of position. 2155 episodes were observed during the remaining 925.6 hours. Their mean frequency per patient was 2.4±0.7 per hour: awake 2.6±0.8, asleep 2.1±1.1 (p=0.006). The mean reflux level was 3.5±0.5 channels. The mean duration was 22.8±7.5 seconds. The mean pH was 4.6±0.8. There were no differences in level, duration and pH between awake and asleep episodes.

GER characteristic Data for the asleep and awake episodes are reported in Table I.

A negative correlation was found between sleepiness periods and the mean reflux duration for refluxes acid

(R=0.55;p<0.001) and weackly acidic (R=0.51;p<0.001) (figure 1).

DISCUSSION

The reflux frequency decreased during the sleepiness periods suggesting a reduction of Transient Lower Esophageal Sphincter Relaxations (TLESR) according to the hypothesis of an enhanced vagal-tone that improves Lower Esophageal Sphincter (LES) control through cholinergic stimulation during sleep(7). The increased reflux frequency observed in the awakeness periods was primarily due to non-acid refluxes. This finding shows that MII/pH is more accurate than pH-metry alone to study GERD in the first weeks of life(8), in agreement with the hypothesis that the pH-metry alone is not sufficient to study GER in newborns because of the prevalence of weakly acidic reflux events due to the frequent consumption of milk, which buffers gastric acidity.

The mean reflux duration of both acid and non-acid refluxes was inversely correlated with the total sleeping time. It was reported that weakly acidic reflux events are stressful for infants, and the discomfort they cause is similar to that induced by acid reflux events(9). We speculate that the refluxes duration, but not reflux pH, might be a relevant stressful factor in newborns that induce a reduction of sleeping time.

In conclusion, our data disclose a relationship between sleep/wakefulness and refluxes in newborns with GER symptoms. Both weakly acidic and acid reflux events have been shown to be equally responsible for reduction of sleeping time. If our results are confirmed in a larger population, the diagnostic and therapeutic methods currently applied to infants with GERD should be revisited, as weakly acidic events would also deserve appropriate therapy.

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