TITLE OF FORM:

“Evaluation of the antioxidant activity of lutein/zeaxanthin early administered to premature newborns”

Date: 09/06/2017
Evaluation of the antioxidant activity of lutein/zeaxanthin early administered to premature newborns

TYPE OF STUDY: PILOT STUDY OF 50 (25 CASES + 25 CONTROLS) SUBJECTS RECRUITED

AORN Santobono-Pausilipon Napoli (center national project coordinator)

AOU Siena (coordinating center of doses of biomarkers of oxidative stress)

AOU Padova (center shared enrollment)

Coordinating Doctor:

Dr. Giuseppe De Bernardo: principal investigator

Prof. Giuseppe Buonocore: Coordinator of the gathering and dispensing samples

Dr. Daniele Trevisanuto: Coordinator of the collection cases

Dr. Mariangela Longini: Laboratory Coordinator

<table>
<thead>
<tr>
<th>Title of study</th>
<th>“Evaluation of the antioxidant activity of lutein/zeaxanthin early administered to premature newborns”</th>
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</table>
| Study Justification | During late years, there were many studies proving that lutein, a nutrient which is part of the carotenoids family, can be a real and important factor in preventing and protecting against numerous chronical diseases that affect millions of people in the entire world (such as in diabetic retinopathy in adult). The specialized literature, richer in information, is the one containing scientific studies and demonstrations which highlight and confirm the fact that lutein is able to reduce or slow down the risk of appearance of an ophthalmic pathology.  

Lutein is a polar soluble hydroxylate deriving from xantophylls that belong to the carotenoids family. The carotenoids are linear polyenes, *i.e.* hydrocarbons with conjugated double bonds, containing 40 atoms of charbon representing the highest class of phytochemical substances present in fruits, vegetables, human serum, breast milk, as well as human tissues.  

Carotenoids and their metabolites were largely studied in human serum and highlighted the physical and chemical characteristics through sophisticated
methods (HPLC, magnetic resonance spectroscopy, mass spectrometry, UV spectrophotometry, etc.). Through these studies there were identified until present time, 27 carotenoids and 8 metabolites in the human body. Generally, known carotenoids ingested, absorbed and metabolized by humans and found in serum are in number of 35. Lutein is the most important carotenoid present selectively in certain tissues of the human body, mainly at the level of the retina, macula (hence the name) and lens. In tissues and serum it is found together with a carotenoid dihydroxide, it isomer, zeaxanthin.

Lutein, usually ingested with other nutrients is partly eliminated directly through fecal matters (50-90%) and partially absorbed with the help of food fats. Incorporated in chylomicrons, it enters the blood flow where thanks to its liposolubility, it ties to the lipoprotein molecules (preferable HDL) and so it gets to different organs: liver, breast, colon, cervix, and ocularly, to the lens, iris and retina where, it selectively focuses in its central region (macula). In the cell, it is places in the entire thickness, from the exterior to the interior, in cellular membranes, tiding up to its polar terminal groups by its own membranes.

Lutein and zeaxanthin are present at the level of the umbilical cord and pass through the placental barrier; they are also present in the plasmatic ones, in breast milk and especially in the colostrum.

Concerning the way of administrating it, the lutein presents, through its specific characteristics, an elevated bioavailability after oral administration. The hematic levels of lutein, after providing nutrients rich in carotenoids, are increased with 67% from the 14% observed at beta-caroten.

Published studies proved that the chrono-active form of carotenoids is able to determine a slow release in the intestine offering a better absorption and reducing the necessity to be assimilated together with food.

Through interdisciplinary and coordinated studies, performed both in vitro and in vivo, there were identified different action mechanisms; particularly, it was demonstrated a defense mechanism of the tissue function by lutein, which is produced through the neutralization (quench) phenomenon of singlet oxygen and reactive oxygen species (ROS). This action provides the molecule with different activities: an antioxidant function, anti-inflammatory properties, properties which promote anti-tumoral effects, induction of detoxification enzymes and positive effect on proteins promoting the communication between joints (up-regulation).

Recently, there have appeared experimental and chemical data proving that the oxidative stress and harmful actions determined by ROS can play an important role in the pathogenesis of many neurological diseases as Alzheimer, Parkinson in adult and ROP and NEC in newborns.

This is due to the fact that the nervous system is characterized by membranes high in polyunsaturated fats, the first cellular compounds affected by ROS attack being the lipid peroxidation.

A similar mechanism can appear to certain ocular tissues (macula, lens, retina) which, containing high amounts of polyunsaturated fat acids, are more vulnerable than other structures with oxidative degradations induced by ROS.

Due to the fact that carotenoids are amongst the most powerful antioxidants existing in nature, there are being developed new researches concerning the functional role of these substances in preventing neurodegenerative diseases in newborns. For the first time, few researchers have managed to prove that lutein and zeaxanthin have a protecting role against the photostress of the human macula. This demonstration was possible by isolation, identifying the chemical structure and measuring the concentration of lutein and zeaxanthin and their oxidation products from the eyes of the donors.
During the pregnancy, the percentage of fat acids of pregnant women’s plasma increases with approximately 51%. The increase refers mainly to the concentration of saturated (+ 57%) and monounsaturated (+ 65%) fat acids, but also polyunsaturated fat acids increase significantly.

Because these polyunsaturated fat acids are very sensitive to oxidation, the modification of their plasmatic levels influences the state of the antioxidant systems on the mother and subsequently to the fetus. Many studies have proved that the increase of the susceptibility to the peroxidation of polyunsaturated fat acids on pregnant women is accompanied by an equivalent increase of the tocopherol plasmatic concentrations which, immediately after birth, decrease sharply.

The plasmatic concentrations of antioxidants on newborns, comparative to the mother’s were smaller. In the umbilical cord, the levels of tocopherols and carotenoids are significantly smaller that the ones registered in the maternal plasma and the concentration of polyunsaturated fat acids on the newborn is significantly higher and a lot more increased than on the mother.

Furthermore, specific studies showed a growing interest towards the oxidative stress and oxygen reactive species which supposedly accumulate after birth. Many practices usually used in the delivery room, for example the drugs given to pregnant woman to ease her pain, the newborn’s extraction methods, the techniques to minimize body temperature decrease, blocking the umbilical cord and especially the use of oxygen to 100 % or a ventilated room (Room Air, RA) for newborns presenting asphyxia signs, do not always prove to be efficient, but they can also compromise the health of the newborn as a result of a significant increase of free radicals.

Some specific studies have compared the levels of free radicals, highlighted with particular markers, in the plasma of the umbilical cord of newborns with asphyxia treated 100% with oxygen or 21% with oxygen, comparative to a control group of children without asphyxia. The levels of free radicals were significantly increased immediately after birth in all three groups and grew at the two groups of newborns with asphyxia. In the group treated 21% with oxygen, these values decreased and at 28 days after birth, they have reached the same level of the newborns without asphyxia, whereas at the group treated 100% with oxygen the levels of free radicals still remains very high.

Thus, it results that a short exposure of the newborn to 100% oxygen is the cause of an extended oxidative stress state and a consistent increase of free radicals which seem to be involved in different diseases and pathologies during the first months of life, especially in the preterm infant increasing the incidence of ROP, IVH, BPD, NEC and infections significantly.

As a result, it is desirable that the newborn can increase the level of antioxidant protection to reestablish the oxido-reductive balance and to prevent the problems occurred from an extended exposure to high levels of free radicals and oxygen reactive species.

Oxidative stress, as well as in the case of age diseases, is considered one of the main factors which determine the deterioration of the retina. The attempt to maintain a correct balance between oxidant and antioxidant factors can help to prevent or reduce damage to the eyes or other tissues that occur in the newborn, especially preterm.

The premature birth is the most frequent cause of mortality, morbidity and disability. Premature babies have an extremely high risk to develop ocular or neurological lesions. The main complication at visual level that may appear is called retinopathy of prematurity, so called ROP. Oxidative stress is involved in
the etiology of this disease. In fact, premature babies, because of respiratory issues, are oftenly exposed to potentially damaging oxygen concentrations or to phototherapy with high blue light intensity. These therapeutic practices are sources of free radicals.

The lutein and zeaxanthin present in the macular pigment, due to their capacity to absorb blue light and their antioxidant action, could have an important role in protecting the eyes of the newborn against damages produced by intense light.

On adults, different elements suggest a protective action of lutein against photodamage, proved by numerous studies.

The studies performed on babies showed that the levels of carotenoids in the first four / six months of life are much reduced. This is due to the fact that the baby’s diet is based exclusively on milk, without any solid elements (as vegetables or green leaves), sole sources of this nutrient. Nevertheless, breast fed babies, in average, present high plasmatic lutein levels than babies fed with prepared milk.

Different milk formula for newborns found at present time on the market are not enriched with this type of carotenoids, thus their content of lutein and zeaxanthin is very low, exciting certain formulae which are not traded in Italy prepared using egg mixes. Breast milk, is thus the only source of lutein for the newborn before ablactation, and breast feeding proves to be of considerable importance as primary source of these micronutrients for the newborn, proper development and visual function protection. Taking into consideration the correlation between the lutein in the blood and breast milk and the reduction of its levels, similar to all carotenoids, in milk, after 6 days from birth, there is already an important contribution of nutrients high in lutein during breast feeding. Such diet enriched in lutein is particularly important especially for the mothers of premature babies or babies having a small weight when born. In fact, premature babies and underweight babies need more nutritive substances essential to a fast growth, because they have not benefit from the contribution of highly nutritive and energetic substances transferred from their mothers during the last weeks of pregnancy. Also, the gastrointestinal and renal functions which are not completely developed reduce the absorption and withhold of important micronutrients, amongst which important antioxidants that protect the newborn from the exposure to high level of free radicals produced excessively at birth and several times as a result of the resuscitation techniques used. Breast feeding is important for the antioxidant contribution to the protection of the newborn and definitely the nutritional state of the mother has subsequently an essential part because it influences the nutrition of the newborn, especially concerning certain solvable nutritive elements, such as lutein and zeaxanthin. It should be emphasized that in the literature are already present searches and results with the use of lutein / zeaxanthin in the newborn.

The recent Gong work has evaluated the role of lutein / zeaxanthin comparing the data obtained from various studies, including those of Romagnoli, Dani and Manzoni. Furthermore the RCT analysis of Rubin on the subject, it can be concluded that lutein / zeaxanthin are well tolerated and well absorbed from preterm infants also after oral administration. The extremely interesting result that has emerged although not statistically significant (probably due to the small sample) is that supplementation with lutein / zeaxanthin reduced the incidence and severity of ROP.

Our protocol is born from the idea that given the interesting results of earlier work is considered important to deepen a dosage of at least 1 ml / kg equal to 0.5 mg of lutein and 0.05 mg of zeaxanthin.

It is important to assess the value of the key markers of oxidative stress biological
antioxidant potential (BPT) and total hydroperoxide (TH) during and after treatment.

Already in a previous work (S. Perrone, M. Longini) has demonstrated a reduction in radicalemia in term infants, during and after administration of lutein / zeaxanthin by determination of the BTP and TH.

Preparation based on lutein ans zeaxanthin have never reveled on humans negative or harmful effects after administration, or to the gastrointestinal or sistemis level. In recent studies there were not reported adverse phenomena after administrating 20 mg/day of lutein or zeaxanthin for a period of 6 months, or interactions with other liposolvable nutritive elements.

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<th>Justifying the non-commercial character of the study</th>
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<td>This study is based on the administration of a dietary supplement of lutein/zeaxanthin. It is included in the category of a spontaneous/non-commercial pilot study, because it is born from the need of private health care of identifying a natural antioxidant product which may reduce the severeness of the diseases related to the prenatal period, limiting the suferance of newborns and their families, by reducing simultaneously the charges in SSN pregnancy which arise from the standard treatments and short-term and medium to long-term rehabilitation. It was chosen the product LUTEIN ofta 0,5 gocce, because Sooft Company proved to be able to supply this drug without any charges, also signing a statement that certifies the fact that the scientific results of the research will remain at the disposal of the scientific community and that the management of the intellectual property protection activity concerning any invention that may derive from the research is made according to the law in force and mainly to Art. 65 of the Executive Decree no. 30 from 10 February 2005 – „The Code of Industrial Property”. In conclusion the results of the research will remain available for the Scientific Community and shall not be used for commercial purposes or profit.</td>
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<th>The registration and trading status of the product participating at the study</th>
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<td>The Registers of the Ministry of Health concerning non-pharmacological products. The product is registeres in the Register of dietary supplements on the web site of the Ministry of Health (<a href="http://www.ministerosalute.it/alimenti/dietetica">http://www.ministerosalute.it/alimenti/dietetica</a>) and classified with the following code: 905619452, according to the executive decree no. 111 from 27 January 1992. These products are submitted to the European Directive concerning food, according to the Executive Decree no. 169 from 21/05/2004, and to the European Directive 2001/20/EC concerning drugs, transposed to Italian level through the executive decree 211 from 24/06/2003. Ingredients: solution of 5% Lutein and 2,25% Zeaxanthin with excipients (Corn starch, glucose, potassium sorbate, xanthan gum, citric acid). Description: LUTEIN ofta 0,5 gocce is a dietary supplement based on lutein and zeaxanthin. The lutein and its isomer, zeaxanthin, are two carotenoids highly spreaded in nature, which cannot be synthetized by the human body. They are normally absorbed through food rich in such carotenoids (vegetables with green leaves, with big leaves as turnips and spinach, and fruits such as apricots and kiwi) and through breast milk. After absorption, the lutein and zeaxanthin are distributed to all tissues, but they are selectively accumulating in the eyes. Mainly, they are focusing in the retina and macula lutea, the latter being the place where clear images are formed, whose development is complete around the 4th or 5th month of life. The lutein and zeaxanthin are the only pigments present at the level of macula lutea and have a protection role. They contribute and maintain the functionality and integrity of the eye’s sensitive structures (retina, iris, lens), preventing or reducing the damages caused by free radicals. In specialized literature is largely described their high antioxidant potential.</td>
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</table>
activity and the advantages of a consumption or supplement. Lutein and zeaxanthin pass through the placental barrier, they are also present in the colostrums and breast milk and it is believed that they take part to the embryogenesis of the retina and to the development of visual functions during the first weeks of life.

As a result, the lutein and zeaxanthin are essential to the health of the eye, both on adults and children during their first hours of life. Thus, the absorption of these substances proves to be necessary in situations of necessity growth or in case of deficient intake, both during the aging period of the ocular structures responsible for the visual function (macula), and during their conservation period.

The lutein and zeaxanthin do not present contraindications and can be taken at any age.

Use: 
*LUTEIN ofta 0,5 gocce* is indicated in situations of necessity growth or in case of deficient food intake of its components on different age groups.  
*LUTEIN ofta 0,5 gocce* can be useful especially in preventing macular or retina damages.

### Study Objectives

**Main objective**  
To evaluate if the administration of pharmacology doses of Lutein/Zeaxanthin micronutrients in water (*LUTEIN ofta 0,5 gocce* – Neox) is able to reduce the level of radicalemy and at the same time to elevate the antioxidant power of premature newborns (gestational age: ≤ 32 weeks and / or weight at birth: ≤ 1500 grams).

### Study sketch

Pilot study, non-commercial, with food supplement, treated vs. control with a ratio 1: 1 double-blind. The identical vials, corresponding to placebo and active product, have an alphanumeric code without meaning for the operator, but that identifies the traty from control, in an anonymous document kept by the Statistical. The document will be placed in a sealed and anonymous envelope and it will be kept open by the statistician at the time of data analysis.

### People participating at the study and their number

**Inclusion criteria**
- Newborns with a weight ≤ 1.500 grams and/or gestational age ≤ 32 weeks;  
- Male and female newborns;  
- Newborns whose parents want to sign the information form – informed consent.

**Exclusion criteria**
- Informed consent is not signed  
- Infants of birth weight ≥ 1.500 gramms and/or gestational age > 32 weeks  
- infants hospitalized after 36 hours of life  
- Infants with ophthalmologic disease at randomization  
- infants with severe malformations

**Number of patients about to be registered**
It is expected to register a total number of 50 newborns divided in two groups:
- Experimental group A: 25 newborns treated;  
- Control group B: 25 newborns treated.

**Randomization**
Subsequently enrollment patients will be randomly divided into Group A and Group B throught a web system that allows the randomization into three different recruitment centers.
Multicenter pilot study. At the study will participate three Italian experimental centres: Naples, Siena and Padova. The centre from Naples will be the national coordinator and the center from Siena will handle in a centralized way the laboratory results. The center from Padova will help to recruit an adequate number of cases.

BAP and TH are two bio-markers with different hypothetical changes clearly different. For this reason we will use the study method proceeds by the following online site http://clincalc.com/stats/samplesize.aspx (Rosner B. Fundamentals of Biostatistics. 7th ed. Boston, MA: Brooks/Cole; 2011). The inserted reference values are for BAP 2361 (±466) µmol/L and for TH 156.75 (±64.0) Ucarr (Perrone S., Longini M. et. Al). The difference for BAP that we hypothesize to find is an increase of at least 500 µmol/L which is approximately 20% of the control data. By setting $\alpha=0.05\%$ and $\beta=0.1\%$ with a power of 90% the number of samples required is $25 + 25 = 50$. For the TH assume a decrease of 50 Ucarr which is approximately 30% of the data of the control. By setting $\alpha=0.05\%$ and $\beta=0.2\%$ with a power of 80% also in this case the required number of samples is $25 + 25 = 50$. However $25 + 25$ is the minimum number. Taking into account any ongoing study waivers (parents may request to discontinue treatment at any time) which go beyond statistical calculations. So in case of necessity we will recruit a number of infants to reach the minimum number according to the design of the protocol ($25 + 25$).

**Products administered:**

LUTEIN oftə 0,5 gocce, containing a solution of 5% Lutein and 2,25% Zeaxanthin with excipients (Corn starch, glucose, potassium sorbate, xanthan gum, citric acid) (group A)

Placebo solution with unique excipients (Demineralised water, potassium sorbate, xanthan gum, citric acid) (group B)

**Experimental Group:**

Group A (25 newborns) will be treated with LUTEIN oftə 0,5 gocce, (1 ml per Kg equal to 0,5 mg of lutein and 0,05 of zeaxantin), divided in two additional administrations during the day from the standard hospital treatment foreseen. The first dose will be given within 36 hours of life, the least to 30th day of life.

**Control Group:**

Group B (25 newborns) treated with Placebo solution (additionaly to the standard hospital treatment foreseen. The first dose will be given within 36 hours of life, the least to 30th day of life.

The product will be administered by adding it in the milk (human or formulae), or in a small quantity of glucose, either orally or by gavage.

The administration of the product (LUTEIN oftə 0,5 gocce) starts within the first 36 hours of life and continues with daily administration for 30 days.
laboratory investigations foreseen. The second semester will be used to study and analyse the results achieved.

<table>
<thead>
<tr>
<th>Procedures</th>
<th><strong>Methodology:</strong></th>
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<td>When born, premature newborns that are comply with the inclusion parameters will be introduced and separated in two random groups (A and B). All newborns, during the observation period, will be submitted to the blood collection (1 ml) from the umbilical cord and peripheral (at the same time with the routine collections) on which there will be performed blood gas analysis and oxidative stress markers (TH and BAP). All registered newborns will also be submitted to the verification of their history concerning the examination and collection, in view of evaluation the presence or absence of ROP, NEC, IVH, BPD and infections.</td>
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<td><strong>Collection Time</strong></td>
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<td>• T0, basic at birth, before begining the treatment: blood collection from the umbilical vein.</td>
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<td></td>
<td>• T1, first check, to 15 days after initiation of treatment: peripheral blood collection in view of verifying it before the Lutein/zeaxanthin administration.</td>
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<tr>
<td></td>
<td>• T2, second check, at 30th day since the begining of treatment: peripheral blood collection in view of verifying it before the Lutein/zeaxanthin administration.</td>
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The parents will be informed concerning the experimental procedure and the checks schedules. It will be required the written consent of the parents. The protocol shall be submitted to approval to the local ethics commission.

**Evaluation Parameters:**

**Efficiency:**
a) Evaluation of oxidative stress biomarkers in the peripheral blood of the premature newborn before and after the treatment.

**Tolerability:**
There will be transcribed on the data sheet possible adverse events which may be observed by the doctor.

**Evaluation Scale**
a) Evaluation of the oxidative stress: by deremining the oxidative stress biomarkers and registering them in the data sheet.
b) They must be indicated:

**Demographic Characteristics**
- Sex
- Gestational Age
- Weight (with percentile)
- Mode of delivery
- Preeclampsia
- Premature rupture of membranes
- Using steroids before birth
- Using antibiotics before birth
- Use of drugs or harmful substance before birth

**Clinical features**
- Apgar score
- Use of surfactant
- Umbilical catheter
- CVC location and duration
- Intubation
- Mechanical ventilation (procedures and time)
- Supplementation of O2
- Incidence of infections
- Use and type of antibiotics
- Use and type of antifungal
- Use and type of post-natal steroids
- Use and type inotropic
- Use of NPT
- Duration of treatment in NICU

*Nutritional Characteristics:*
- Oral feeding start time
- Exclusive oral feeding start time
- Fluid volume
- Intake calories (protein, carbohydrates and fats)
- Use of maternal and /or human milk

*Other therapies associated and/or permitted*
During the trial period it is not permitted any product administration or antioxidant activities (anti-radicals) or products which contain even a sole component having this result, excepting the protocol.

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<th>Basic examination</th>
<th>At birth, as it is foreseen by newborn protocols, the babies will be submitted to several medical examinations.</th>
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<tr>
<td>Control examination</td>
<td>From the Birthing Unit, the newborns will be transferred to intensive care rooms where they will be submitted to multiple control examinations.</td>
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<tr>
<td>Follow-up examinations</td>
<td>As it is foreseen in neonatal protocols.</td>
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<tr>
<td>Statistical Analysis</td>
<td>The Student t test for independent data is the type of analysis that will be used to assess statistically significant differences between the parameters recorded in the two groups (treated and control). The Student t test is the method of choice for analysis as it allows to evaluate the values of a continuous random variable measured in not very many samples (25 subjects per group). It will be using the statistical package SPSS system.</td>
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</tbody>
</table>

REFERENCES
Lutein: description, pharmacokinetics activity and characteristics, mechanism of action, safety and tolerability


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Lutein in Neonatology


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SIGNATURES FOR APPROVAL

Signature 9-6-2017
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