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Solution of retinoids in vitamin E in the Di Bella Method biological multitherapy

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Abstract

OBJECTIVE: The aim of the liposoluble vitamin solution in the DBM formulation is to enhance the biovailability, the stability, the half-life and the therapeutic efficacy of the components without resorting to excessive or toxic doses.
METHOD: The DBM vitamin solution contains 0.5 grams of vitamin A palmitate ester, 0.5 grams of all-trans-retinoic acid (ATRA), and 2 grams of β-Carotene, in 1 000 grams of vitamin E acetate ester which stabilizes the other components, protecting them from oxidation. The solution is administered before meals at the dose of 90 to 150 mg per kg of body weight. The quantities have a determining value for a pharmacological result. No phenomena of overdose and/or toxicity have ever been detected in the tens of thousands of people who have used the solution with this formulation and dosage.
RESULTS: Thanks to the synergic effect of the components and their antioxidant, free antiradical, cell-membrane stabilizing, differentiating and cytostatic properties, this solution has constantly produced positive therapeutic responses. Favourable effects have been observed in the prevention and treatment of neoplastic diseases, as well as on immunity, physiological growth, trophism and functionality of tegumental, respiratory, digestive, urogenital and exocrine gland epithelia. A significant antidegenerative effect has also been observed in pretumoral stages. Studies have been published reporting 754 cases of various types of tumor which have greatly benefited from the use of this vitamin solution synergically and factorially reinforced by the other components of the DBM, such as Melatonin, Vitamins D and C, D2 receptor agonists, and GH inhibitors like Somatostatina and analogs.
CONCLUSIONS: In view of the documented results achieved, we believed it useful to provide the scientific community with the details of the formulation, preparation, posology, rationale, mechanism of action, biochemical, molecular and physiological bases, indications and clinical findings relating to the DBM liposoluble vitamin solution.

ORIGINAL ARTICLE

INTRODUCTION

The components of the DBM liposoluble vitamin solution are vitamin A palmitate ester, Betacarotene and All Trans-Retinoic Acid (ATRA), and the acetate ester of Alphatocopherol, formidable exergons used in quantities of fractions of milligrams.

The quantities have a determining value, providing a pharmacological result and not creating toxicity phenomena. They regulate anti-infection, anti-degenerative, anti-tumoral homeostasis and stabilize cellular membrane potentials.

In addition to protecting the cellular membrane, vitamin E carries out an anti-oxidising defence action. At the prescribed doses, the composition is designed never to reach accumulation or toxicity.

This mixture is important for elimination of free radicals, reducing their toxic effects and alterations to the microcirculation. The inflammatory states produced by free radicals can cause damage to various organs, including the respiratory system, leading in the long run to pulmonary foci, alteration of lung parenchyma, emphysema, altered bacterial response, immunitary complexes and the formation of intravasal clots. The elimination of free radicals together with the epithelioprotective and immunostimulating effect of retinoids solubilized in vitamin E leads to the reperfusion of ischemic organs and the improvement of the trophism functionality of organs and tissues.

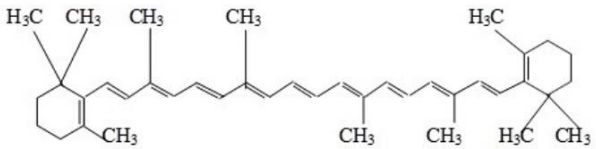
COMPONENTS

palmitate axerophthol	0.5 g
retinoic acid	0.5 g
betacarotene	2 g
D,L-alfa-tocopheryl acetate	1 000 g

STRUCTURE FORMULAS

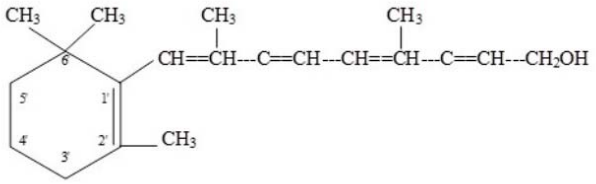
Betacarotene ($C_{40}H_{56}$)

β-β-carotene; *trans*-β-carotene; (*all*-E)-1,1'-(3,7,12,16-tetramethyl-1,3,5,7,9,11,13,15,17-octocanonaene-1,18-dyl9bis[2,6,6,-trimethylcyclohexene]; E160a.



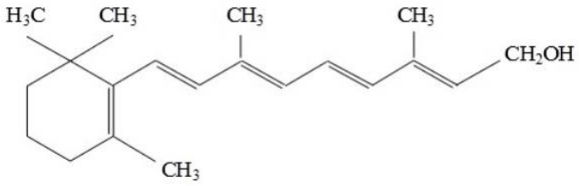
Vitamin A

Retinol or Axerophthol (A1) (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)-nona-2,4,6,8-tetraen-1-ol

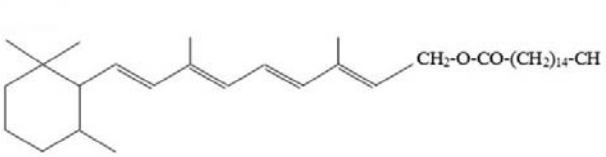


Axerophthol or Retinol

(2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona- 2,4,6,8-tetraen-1-ol

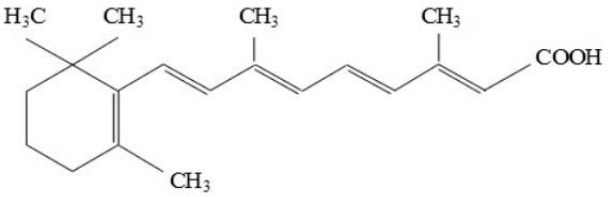


Retinyl palmitate ($C_{36}H_{60}O_2$)



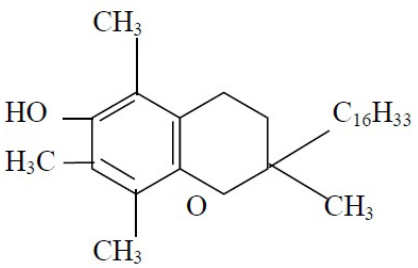
Trans-retinoic acid

3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-*all-trans*-tetraenoic acid, (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl) nona-2,4, 6,8-tetraenoic acid.

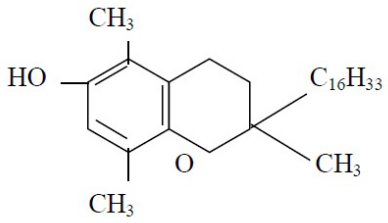


Vitamin E

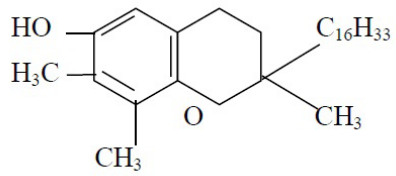
α-tocopherol (5, 7, 8 trimethyltocol)



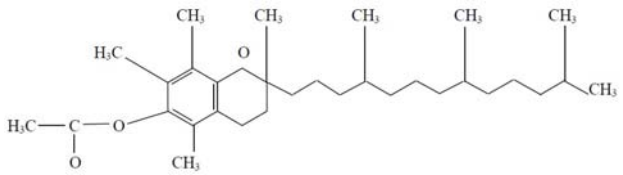
β-tocopherol (5, 8-dimethyltocol)



γ-tocopherol (7, 8-dimethyltocol)



D,L-α-tocopherol acetate
3,4-Dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-benzopiran-6-ol acetate; (2RS, 4'R,8'R)-2,5,7,8-Tetramethyl-2-(4',8',12'-trimethyltridecyl)-6-cromanile acetate.



TECHNOLOGY

The raw materials must be of maximum purity and stored at the correct temperature. Once the container has been opened, their storage requires the introduction of inert gases (e.g. nitrogen) and observance of manufacturer and pharmacopeia indications.

Trans retinoic acid and Betacarotene are in solid form, while Vitamin A Palmitate (at room temperature) and alfa Tocopherol acetate are liquid and highly viscose. They can be mixed together to achieve molecular level dispersion. The solution can be prepared by gentle stirring. Stirring must be performed using an efficient temperature controller, to be selected according to the size of the batches to be prepared. The preparation methods differ slightly depending on the apparatus used, although the general principles are similar. Anhydrous acetone and ethyl alcohol can be used as solubilisation coadjuvants of retinoic acid and betacarotene. In this case, it is necessary to ensure that they are completely eliminated under a nitrogen flow.

Nitrogen

In all cases, whether using acetone or alcohol, nitrogen must be used at the end of processing to eliminate these substances. Cylinders of top purity pharmaceutical nitrogen can be purchased from suppliers of therapeutic oxygen and/or other gaseous substances. The nitrogen contained in these cylinders is pressurised at 200 atmospheres; in order to use the nitrogen, the cylinder must be fitted with a flow reducer equipped with two gauges (to measure the pressure inside the cylinder and the discharge pressure). The discharge pressure can be varied by means of the fine adjustment control on the reducer. The nozzle is connected to a tube which has an in-line porous septum filter. The purpose of the filter is to trap the metal impurities coming from the walls of the cylinder, which could be carried along by the flow of gas: these impurities would be a potent oxidation catalyser and therefore harmful for the stability of the product.

It should be borne in mind that, except for vitamin E acetate, these substances are sensitive to:

1. light
2. air (oxidative alterations)
3. certain temperatures

Light

Particular care must be taken in preventing exposure of the substances to direct sunlight (light with radiation in the ultraviolet spectrum). It is thus necessary to work using opaque receptacles and in red-light rooms.

Air

The components are all sensitive to oxidation by oxygen in the air: the most sensitive component is certainly Beta-carotene, due to the presence of an extensive π system, which also gives it its typical brick red colour. Since tocopherol acts as a radical scavenger, it can capture the oxygen in the air, forming a labile bond with the unpaired electrons of the oxygen molecule and partly protecting Beta-carotene from oxidation: it is however possible to prevent this bond from occurring. This can be done by exploiting the greater solubility of nitrogen in lipids with respect to oxygen, saturating the tocopherol with nitrogen before adding the Beta-carotene and the other retinoids.

Temperature

It should be noted that in the absence of oxidants, the resistance of the substances to thermal degradation is relatively good and in any case depends on the processing time. It is advisable not to exceed a temperature of 40 °C during processing.

Preparation method according to Italian decree dated 16-06-98 no.186, published in the official gazette dated 17-06-98 no.139.

Preparation of 1 000 g of retinoid solution con acetone or ethyl alcohol 95 °C as organic solvent. The technician must work under a hood wearing gloves, mask and goggles, at room temperature.

Step 1: Weigh 0.5 g of trans-retinoic acid, which must be reduced to VERY FINE powder, and then dissolved in a mortar by adding the organic solvent in DROPS;

still working in the mortar, add 2 gr of Beta- carotene in organic solvent. (N.B. the remaining powder must be stored COLD and under nitrogen).

Step 2: The dissolved powders are poured into the mixer and around 100 cc of vitamin E is added slowly while mixing. Gurgle the nitrogen at medium flow rate until the organic solvent has been eliminated.

Step 3: Gradually increase the temperature to 40±2 °C while continuously stirring. Leave to cool for 15–20 minutes and slowly add another 100 cc of vitamin E, stirring all the time; increase the mixing speed slightly for about 10 minutes.

Step 4: Add 0.5 g of axerophthol palmitate in drops and continue to stir for 10 minutes; add another 100 cc of vitamin E, keeping the stirring speed low for at least 15 minutes; repeat this operation up to 1 000 g of vitamin E. Close the mixer lid and leave it to stir for at least 6 hours.

Step 5: Pour the mixture into dark glass bottles and close them immediately; store them away from light and heat (if not used immediately, introduce nitrogen flow before sealing the bottles).

Materials

- Chemical hood
- Red light.
- Mixer for high viscosity liquids with temperature controller
- 5 mc nitrogen cylinder, 200 bar, with flow system.
- 0.22 micron gas filter (Millipore)
- Stainless steel container
- Dark glass bottles.

Analyses

The solution of retinoids in tocopherol must be CRYSTAL-CLEAR, dark red, viscous, odourless, tasteless. The simplest and most direct evaluation criterion is the transparency; true solutions are, in fact, optically empty, nor is there evidence of Tyndall effect as, instead, is normal in solubilised forms. Absence of acetone.

Check of the homogeneity of dispersion of vitamin A, Beta-carotene, retinoic acid in vitamin E (titres). Check for the presence of any alteration compounds formed during preparation.

Long-term stability

Storage: The finished product should be stored in dark glass bottles at room temperature. For ideal storage, the bottles should be filled using nitrogen insufflation.

Dosage-labelling: The mixture should be taken in the morning before eating, at the dose, referring to the vitamin E content (which alone represents 997*1000), of 90 to 150 mg per Kg of body weight. It is advisable not to eat or drink acid substances (e.g. lemons) at the same time as the mixture so as not to alter its absorption and activity. Do not use a metal spoon in order to avoid oxidation. The dark glass bottles should be correctly labelled.

Using titration curves and HPLC analyses, it is necessary to check the stability of the solution, ascertain the presence of any undesired molecules such as cis retinoic acid and make sure the organic solvent has been eliminated.

RATIONALE OF THE THERAPY

The solution of retinoids in vitamin E was formulated by Prof. Luigi Di Bella on the basis of biological, biochemical, pharmacological and physiological studies, carried out over the course of many years, with a vast range of clinical indications mainly for the prevention and treatment of tumors, but also in view of their anti-degenerative, anti-infectious and trophic effects. The documented primary role of retinoids, interacting with vitamin E in vital reactions, in the trophism and functionality or organs and tissues, in anti-cancer, immuno-neuroendocrine and antidegenerative homeostasis, explains the rationality and logic of the vast range of indications for the non-toxic formulation of this solution. To understand the enormous value of retinoids in the setting of biological economy, it is sufficient to consider that they provide the large amounts of energy needed for growth and for the physiological order of growth, contributing to anti-cancer homeostasis. The growth of a living substance requires a considerable amount of energy, but the physiological order of growth requires an equally large amount of energy. Retinoids are the most potent non-hormonal activators of growth, but only of ordered, functional growth for optimum biological equilibrium, while they inhibit the aimless and disorderly neoplastic growth, directing the tumor towards apoptosis. Together with Melatonin, they are the only differential toxicity molecules that have a cytostatic effect on tumor cells but not on healthy cells. Retinoids thus have the ability to preserve and enhance the trophism, vitality and efficiency of healthy cells at the same time as diminishing the progression, vitality and marked mutagenic behaviour of the neoplastic phenotype. A tumour is a deviation from normal life, which means that it is necessary to redirect the deviated reactions to their normal status by reinforcing the means considered physiologically essential for life. Retinoids intervene in two critical aspects of tumoral biology: the uncontrolled proliferation and the sequence of mutations, common denominators of all tumors. The cytostatic properties of retinoids counter the proliferation of the tumor cells, while their differentiating properties counter the progression of mutations with which, during its evolution, the neoplastic phenotype selects and maintains a series of advantages, becoming more and more aggressive, resistant, mobile and toxic. There is now an enormous quantity of literature that confirms the multiple and determining therapeutic properties of axerophthol, retinoic acid and Betacaro-

tene (Sommer & Vyas 2012; Alizadeh *et al.* 2014; Brun *et al.* 2013; Álvarez *et al.* 2014; Angulo-Molina *et al.* 2014; Das *et al.* 2014; Israel *et al.* 1980; McLaren & Kraemer 2012; Papadimitrakopoulou *et al.* 2009; Prasad *et al.* 2003; Chen *et al.* 2014; Carratù *et al.* 2012; Alizadeh *et al.* 2014; Israel *et al.* 1980; Ling *et al.* 2012; Mettlin 1984; Nesaretnam 2008; Smith 1998; Zhang *et al.* 2012; Barroga *et al.* 2000; Black 2013; Franke *et al.* 2013).

The basic outlines of the mechanism of action of retinoids in the growth and repair of tissues were already described back in 1985 in volume 113 of the Ciba Foundation Symposia (Ciba Foundation Symposia 1985).

The DBM retinoid solution, together with the other components such as vitamin D^3, C, and Melatonin, acts on the biological conditions to create an environment that is not pharmacologically toxic but is biochemically unfavourable to the neoplastic biology, having a negative effect on the reactions that take place in the evolution of the tumor, at the same time activating the reactions involved in the healing process. Despite the scientific evidence, in the rare cases in which oncological protocols foresee the use of retinoic acid (the only one of the retinoids that is used, and only in promyelocytic leukemia), for its lability and easy oxidability (common to all retinoids), its efficacy is limited by the absence of the protective and antioxidizing functions of the high doses of Vitamin E and Beta carotene present in the DBM solution. Considering the number of cases treated by Prof Di Bella and by his students and the doctors who prescribe the solution, it can be estimated that since the late 1960s at least 50,000 people have benefitted from taking this solution, without any toxic effects. The literature contains numerous publications concerning a total of 754 cases of various forms of tumors, including retrospective observational studies on NSCLC, lymphoproliferative diseases, cervicofacial tumors, breast cancer, prostate cancer, etc. All these studies document the positive anti-cancer non-toxic effect of this vitamin solution, in tandem with the other components of the DBM, such as Melatonin, prolactin inhibitors, somatostatin, Vitamins D^3 and C (Todisco *et al.* 2001; Todisco 2009; Norsa & Martino 2006; Norsa & Martino 2007; Di Bella L *et al.* 1979; Di Bella 1998; Di Bella 2005; Di Bella & Gualano 2006; Di Bella 2010; Di Bella 2011; Di Bella *et al.* 2012; Di Bella & Colori 2012; Di Bella *et al.* 2013; Di Bella *et al.* 2013; Di Bella *et al.* 2013; Margheri *et al.* 2012).

The term retinoids includes vit. A or Retinol, which chemically speaking is a primary alcohol, the provitamins A (around ten), which include Carotenoids, the byproducts of vit. A, such as Retinoic Acid, the aldehyde known as Retinene, an essential component of the visual purpura of the rods, and retinal photoreceptors, essential for vision, a process that involves a recall of vitamin A from the blood to the retina, studied by Wald (Wald 1960).

Retinoids are associated by a common metabolic destiny, albeit with specific peculiarities and different levels of activity. They have some structural chemical elements in common, such as the beta ionone and the four unsaturated bonds in the side chain. They differ in their terminal chemical function which binds to the last carbon atom of the side chain. The structure formula of retinoids, which chemically speaking are hydrocarbons, makes it possible to imagine their lability and easy oxidability (Álvarez *et al.* 2014; Das *et al.* 2014; Eroglu & Harrison 2013). Thus, in order to stabilize them, avoid oxidation, enhance their activity, bioavailability and half-life, the retinoids are solubilized in the DBM vitamin solution in high doses of vitamin A which, as well as in turn having a number of documented anticancer mechanisms, maintains its pharmacological and therapeutic properties. At intestinal level, vitamin A is absorbed chemically conjugated with substances that make it more stable, such as palmitic acid; vitamin A is thus present in the DBM solution in the form of palmitic ester (Reboul 2013; Takahashi *et al.* 1997; Vilanova & Solans 2015). The absorption of retinoids is facilitated by lipids and bile acids. Through the lymph ducts, the retinoid esters collect rapidly, especially in the liver which contains up to 90%; from the liver, they are mobilized as required and/or eliminated through the bile acids. The liver has a leading role in the metabolism of vitamin A which, with the retinoids, plays a fundamental role both in preventing and treating tumors, and also tends to limit the consequences caused by the tumor and by the usual anticancer treatments. Concentrations of vitamin A of up to 100–300 mg/kg have been detected in the liver. Vitamin A is transported through the blood by protein complexes associated with a prealbumin fraction and thyroxine. These substances are vital for its transport and diffusion to the cell organelles like mitochondria, the Golgi network and the cell nucleus. The sero-protein, thyroid and hypophysis equilibrium is therefore essential for the transport and use of vitamin A. The blood concentration of vitamin A can increase through food intake or due to tissue lipolysis caused by raid weight loss, but homeostatic regulation mechanisms tend to maintain a stable concentration in the various cases. A first indication of liver disease is an alteration in the absorption and metabolism of vitamin A and retinoids, as in alcohol abuse disorders, chemotherapy and the use of oral contraceptives. In the various stages of the menstrual cycle, the rate of vitamin A; in the first stages of life it is mainly absorbed through milk where it is present in ester form. The Retinol-Binding-Protein complex is the keystone of the transformation of chemical energy at cell level, through the extremely delicate processes of vision, growth and reproduction.

From a protein point of view, the alcoholic or vitamin A (axerophthol), aldehydic (Retinene), acid (Retinoic acid) and Betacarotene byproduct varieties, with molecularly different mechanisms, influence life in its essential and delicate expressions of cell energy dynamics. It is no longer possible to deny the primary

role of retinoids in preventing and treating carcinogenesis (Hinds *et al.* 1997). A simple and easily recognizable demonstration is the limitation and subsequent suppression of the aggressive behaviour of early-stage melanomas on local application of a few drops of the DBM solution. A melanocytic mole can be gradually normalized in a few months by daily application of the solution. The true possibility of retinoid assimilation depends on the ability of the intestine to extract them; they are transferred, through the circulation and lymphatic system, to the liver where they are deposited and processed, above all in the Kupfer cells, then mobilized to satisfy organic requirements (Reboul 2013).

Small amounts of vitamin A pass through the renal tubule epithelia; the presence of vitamin A is essential even in the first few days after conception for the formation and function of the placenta, and after the first 10–20 days the embryo starts to synthesize the association of proteins with vitamin A and retinoids. Although there may be only a small amount of vitamin A in our bodies, it is deposited in the liver cells, where it is accumulated and protected against attack from the oxidating agents. The integrity of our skin, airways, glandular and urogenital systems, the ability of these tissues to react to trauma and/or infection is always certainly an expression of a sufficient presence of vitamin A, whose sphere of action is therefore immense and vital (Hinds *et al.* 1997). For any kind of damage to these tissues, vitamin A is the supreme remedy. In appropriate quantities it causes no harm; 40–50 000 units per day are tolerated without damage. By regulating the thickness of the skin, trophism and evaporation, retinoids are involved in body temperature control mechanisms. A deficit of retinoids causes thickening and dryness, and reduces heat conduction by evaporation, turning the skin into a heat insulator. A tumor patient undergoing conventional treatments, which lead to a deficiency of vitamin A, can experience an increase in body temperature which is non-febrile hyperthermia. In hyperthermia the regulation mechanisms are altered due to the absence of thermolysis and heat dispersion. The skin becomes thicker due to the increase of the horny layer, it loses elasticity and the sweat and sebaceous glands tend to atrophize, as do the hair bulbs which leads to alopecia. These regressive-degenerative phenomena also extend to the epithelium coating of the respiratory, digestive, genito-urinary and glandular systems. The skeletal system is also affected by a deficiency of Vitamin A. An international consultation group of the WHO studied vitamin A deficiency states in poor countries, and observed symptoms such as follicular hyperkeratosis, skin infections, eczema, bronchitis, cystopyelitis, etc. In areas where the *deficiency is severe, there was an increased frequency of fetal malformation, degenerative* inflammatory disorders of the generative tract and of mammary secretions. Teratogenic effects and miscarriages were also frequent, while skeletal malformations depend on the altered activity of the osteoclasts that control the metabolism of calcium The immunitary system is also impaired by a deficiency of vitamin A, affecting various mechanisms such as the synthesis of immunoglobulins and a leuko-erythro-plateletpoietic depression of the bone marrow (Álvarez *et al.* 2014; Flajollet *et al.* 2013).

Vitamin A probably plays a determining role in cell proliferation, also through metabolism of the polyamines, on the regulation of reproduction and tissue growth speed. The metabolism of some amino acids such as Ornithine and Lysine, of the respective decarboxylases and the interactions with Betacarotene is still being studied. There are three forms of the yellow-orange pigment carotenoids: alpha, beta and gamma. The most common form is Beta-carotene, a terpene provitamin with a slow metabolism producing two molecules of vitamin A (Eroglu & Harrison 2013). Betacarotene consists of 8 isoprene units, cyclised at each end, and is a precursor of vitamin A which is synthesized in the liver thanks to the enzyme carotenase (Goodman *et al.* 1967; Leuenberger 2001). Like all retinoids, Betacarotene is a hydrocarbon and, as such, is a typically apolar molecule, devoid of fillers and inert. It can therefore be included among those molecules, or those apolar parts of molecules, that belong to fatty acids, i.e. to those structures that contribute to form one of the basic elements of life: the cell membrane. It is like a barrier, an obligatory gateway through which everything must pass from the cell outwards and vice versa to allow the cell to live. As a molecule ($C_{40}H_{56}$), Betacarotene has a direct preventive and therapeutic action in neoplastic diseases, as demonstrated by the considerable number of relative publications. It is also involved in the mechanism of bone growth and, in general, contributes to the correct functioning of many organs. It has a strong antioxidant effect, thus protecting cells from the damage caused by free radicals and plays a fundamental role in the immune system (Lo *et al.* 2014; Han *et al.* 2014). Onogi *et al.* (Onogi *et al.* 1998) demonstrated the direct antiproliferative effect on colon cancer cells of the carotenoids, regardless of their conversion to ATRA. It has been shown that carotenoids can exert their tumoral suppression effect even without being converted into their metabolites, retinol or retinoic acid. Beta-carotene 15, 15'-monooxygenase is an enzyme belonging to the class of oxydoreductases, which catalyzes the reaction: β-carotene + O_2 ⇄ 2 retinale, and requires bile salts and Fe. Unlike retinol, the body takes in the necessary quantity of Beta-carotene, eliminating any excess amounts. Epidemiological studies have shown a significant correlation between the onset of cancer and the intake (in high doses for several years) of betacarotene through food only in heavy smokers, confirming on the other hand the positive anticancer effect of betacarotene in all tumors in non-smokers. The cancerogens involved in smoking, such as nitrosamine, combustion products, hydrocarbons, 3–4 Benzopyrene, together with the oxidant effect of nicotine, in subjects exposed to extraor-

dinary oxidative stress due to the amplified effect of the high concentrations of oxygen in the lungs, degrade the labile and easily oxidable molecule of betacarotene by asymmetric splitting, releasing toxic metabolites (CBP). The degradation of Betacarotene, in addition to inactivating its particular and specific anticancer properties, causes a deficiency of its physiological metabolites, retinoic acid and axerophthol, fundamental molecules in the treatment of tumors for their cytostatic, differentiating, epithelioprotective, immunostimulating and trophic effect. Highly reactive products of the irregular degradation of carotenoids (CBP), including aldehydes and epoxides, form during these oxidative reactions.

The mechanisms of degradation of Betacarotene include the influence of activated neutrophils; the mitochondrial toxicity that the CBPs can cause, with effects on cell respiration, on variations in the potential of the mitochondrial membrane and relative patency and on dislocation of the nucleotide adenine was also evaluated. The inhibition of mitochondrial respiration is accompanied by a reduction in the cytosolic content of sulfureted proteins, of GSH and redox state, and a considerable accumulation of malondialdehyde. It is only recently that several authors have pointed out the need to counter the degradation of Betacarotene by means of potent biological antioxidants such as Vitamin E, thereby neutralizing the strong cancerogenic and toxic effects of the CBPs and the serious deficit of fundamental anticancer molecules (Al-Malki & Moselhy SS 2013); Khuri *et al.* 2001; Liede *et al.* 1998; Lubin *et al.* 2008).

With the solution of retinoids in Vitamin E, prof. Luigi Di Bella anticipated these therapeutic concepts by more than 30 years, identifying the biochemical, molecular and pharmacological bases for an ideal and constant therapeutic response without any toxicity. It is only the contemporary, non-sequential administration of retinoids in vitamin E, in a molecular dispersion (thus in solution, and not in suspension) which represents the correct response (available and used for decades with positive effects and no toxicity) to this problem, stabilizing the three retinoids.

The constant availability of a sufficient quantity of Betacarotene obtained as described above is fundamental for biological equilibrium, homeostasis, preservation and recovery of cell and mitochondrial membrane functionality, receptorial physiology, ion channels, membrane potential, intercellular communication and cell adhesion. The DBM solution forms a reserve of Betacarotene that is always readily available when due to oncological, degenerative or infectious diseases an increase in the requirements Betacarotene or its metabolites – Axerophthol and Retinoic acid – is required. Axerophthol and Retinoic acid are therefore reinforced by Betacarotene in the DBM solution; and to obtain maximum efficacy and effect, the ratio of Betacarotene to Axerophthol (or vit. A) and Retinoic acid must be four to one. The continued administration for many years of the DBM solution has never caused an accumulation or toxicity, or cases of carotenemia whose presence is instead due to a deficiency of carotenase, hepatic dysfunction or an excessive dietary intake of carotenoids. Carotenemia is characterized by the jaundice-like colour of the skin except for the sclerae, a fundamental element in the differential diagnosis with respect to liver disease. Free radicals, produced in oxidative reactions, are notoriously highly reactive and instable fragments of molecules, classified as ROS, reactive oxygen species, and RNS, reactive nitrogen species. Damage caused by free radicals includes disruption of the structure and functionality of cell membranes, in infinitesimal fractions of a second the rupture of molecules, the formation of new bonds, the oxidation of membrane phospholipids with alteration of membrane fluidity and permeability. The degradation of lipids by free radicals is shown by the presence of end products of advanced lipoxylation such as malondialdehyde. Free radicals can also act on mitochondria, modify amino acids, proteolysis of cytosolic proteins, damage enzymes, create cross bonds and aggregation between proteins, degrade nucleic acids with rupture of single and double filaments forming alternative nitrogen bases with alteration of the genetic information and of the physiological mechanisms of transcription, translation and replication. The extent and severity of the damage caused by free radicals are considerably limited and countered by the antioxidant properties of the DBM solution, in synergy with Melatonin (Igielska-Kalwat *et al.* 2015). The anticancer mechanisms of action of Betacarotene include maintaining the physiological levels of Glutathione, which rapidly decreases in the presence of tumors, and the protective-antitoxic effect by countering the lipid peroxidation increase due to the progress of the tumor (Basu *et al.* 2000).

Vitamins are physiological catalyzers between energy and matter. Any change in living matter must be accompanied by an adjustment in energy. Only slight quantitative variations in the production and absorption, i.e. processing, of the biological terrain and its energy equivalent are compatible with life, and reactions must therefore take place in gradual stages with minimal amounts of matter-energy, mutually compensated over time. These reactions lead very gradually to the production and absorption of energy and matter. This continuous process must, for the exceptional purposes, be gradually modulated and finely adjusted, and this would be impossible without vitamins, whose purpose is to condition and regulate the matter/energy equilibrium on which life is balanced. A complete knowledge of vitamins means knowledge of the finest equilibria, energy/matter relationships and all the effects on vital activities. Knowing the chemical composition, the formation, the localization inside the cell, the time of their intervention, and the regulation and extent of their activity makes it possible to understand the essence of physiological life and to correct patho-

logical deviations. Thus, from the original biochemical-vital role, vitaminology is raised in the DBM solution to a rational and essential role in the prevention and cure of various diseases. The study and in-depth knowledge of the regulatory mechanisms of normal physiological life thus permits the realization of effective countermeasures to prevent and/or contrast degenerative and/or neoplastic deviations. In tumor-predisposing situations and in neoplastic diseases, especially during chemo-radiotherapy, the structure and potential of cell membranes, and consequently receptorial expression and functionality, can be disrupted, with aggravation of oxidative processes and a consequent peak in the production of free radicals (Odeleye *et al.* 1992; Elangovan *et al.* 2008; Launoy *et al.* 1998; Shimizu *et al.* 2004; Di Bella, 2005; Neuzil *et al.* 2002; Frei & Lawson 2008). The DBM solution supplies apolar components such as Betacarotene and vit. E to the phospholipids of the cell membranes, stabilizing them and preserving them from oxidative damage and from free radicals (Shklar & Schwarts 1996; Israel *et al.* 2000; Kini *et al.* 2001; Di Bella, 2005; Dong *et al.* 2008; Lubin *et al.* 2008; Nesaretnam 2008; Watters *et al.* 2009).

FUNCTIONS OF BETACAROTENE:

- It has a protective effect on cell membranes (Di Bella 1998);
- It decreases lipid peroxidation and increases Glutathione (Basu *et al.* 2000);
- It has a direct antiproliferative effect (regardless of the conversion to ATRA) on tumor cells, it significantly suppresses both the mobility measured by means of tetrazolium MTT) and the synthesis of DNA (controlled by capitation of 3H-thymidine) and cell proliferation (measured by means of cell count) (Onogi *et al.* 1998).

FUNCTIONS OF RETINOIC ACID (ALL TRANS RETINOIC ACID –A.T.R.A.):

- It acts by redifferentiating blast and tumor cells (Hassan *et al.* 1990);
- It triggers the synthesis of leukotriene C4 (Abe *et al.* 2003);
- It suppresses the gene transcription of oncogenic factors and promotes the antiproliferative effect (Arnold *et al.* 1994);
- It has an anti-angiogenetic action (Majewski *et al.* 1994);
- It reduces the microvascular density of the bone marrow in leukemia and of hot point density. It interrupts the production of VEGF by NB4 cells, suppressing angiogenesis (Kini *et al.* 2001);
- It stops the cell development associated with the increase in levels of interferon 1 [IRF-1] with activation of p21WAF1 (Arany *et al.* 2003);
- It activates apoptosis, with the contribution of IRF-1 and STAT1, by means of caspase 1 (Arany *et al.* 2003);
- It stops the progression of the cell cycle (Wu *et al.* 2009);
- It stops the cell cycle in G0/G1 (Wu *et al.* 2009);
- It triggers the expression of p21 WAF1/CIP 1, by means of p 53-dependent and independent pathways (Wu *et al.* 2009);
- In tumor cells, it inhibits the activation of AP-1 by means of its RAR-alpha receptor and suppresses the expression of cJun and cFos (Wu *et al.* 2009);
- It synergizes the effect of Bcl-2, both on growth arrest and expression of the p21 gene (Chou *et al.* 2000);
- It prevents the invasion of colon cancer cells and decreases the expression of matrilysin (Adachi *et al.* 2001);
- In tumor cells, it causes morphological and biochemical changes, such as membrane shrinkage, chromatin condensation and DNA splitting, typical characteristics of cells during apoptosis (Lee *et al.* 2008);
- By means of RAR-beta it leads to a net increase of c-myc and Bax proteins, with greater susceptibility to apoptosis (Lee *et al.* 2008);
- It decreases the potential of neoplastic proliferation and has an important role in differentiation, apoptosis and cell adhesion (Voigt *et al.* 2000);
- It makes tumor cells particularly sensitive to chemotherapy, also causing an increase in inter-cell communication in the gap junctions (Carystinos *et al.* 2001);
- It reduces the level of glial fibrillary protein and the synthesis of DNA, and induces apoptosis, demonstrating considerable synergism and reinforcement of the efficacy with TNF-alpha by increasing the receptors of p55 TNF (Chambaut-Guérin 2000);
- It induces a gene, autotaxin [ATX], which decodes a stimulation factor of tumor motility (Dufner-Beattie *et al.* 2001);
- It induces neurotic differentiation with extensive neurite growth, and a decrease of the oncoprotein n-Myc and of the mRNA of Gap-43. It exerts an antiproliferative effect by increasing the kinase A of the type II/RII beta protein and kinase A of the W protein (Kim *et al.* 2010);
- It differentiates tumor cells through its effect on A2, Ca^{2+}-dependent phospholipases (Antony *et al.* 2001);
- It reduces the expression of VnR, correlated with the organisation of fibronectin and cell adhesion and expansion (Baroni *et al.* 2003);
- It reduces the inhibition chemically induced by RAR Beta, blocking the cell cycle in the G1 phase (Song *et al.* 2001);
- It reduces tumor invasiveness, by inhibiting matrix metalloproteinase (MMP). (Pham *et al.* 2013);
- It increases the activity of P 53 (Lu *et al.* 2000);

- Together with Vit D, it promotes apoptosis (Sha *et al.* 2013);
- It counters the hepatotoxic effect of chemotherapy (Ewees *et al.* 2015);
- It inhibits the inactivation of caspases (Piedrafita & Pfahl 1997; Takada *et al.* 2001; Jiang 2008);
- It inhibits the expression of BCOM1 associated with an increase colon cancer cell mobility and invasivity and inhibits the expression of MMP7 and MMP28, with an antiproliferative and antimetastatic effect (Pham *et al.* 2013) .

FUNCTIONS OF VITAMIN A

The use of vitamin A in the prevention and treatment of tumors, started more than 30 years ago by prof. Di Bella, is also well documented in the international scientific literature. Piedrafita (Piedrafita & Pfahl 1997) reported the induction of apoptosis caused by vitamin A and retinoids, through activation of proteolytic cell enzymes, the caspases. The degradation of the general transcription factor Sp-1 causes tumor cell death by apoptosis. There are numerous studies on the anticancer prevention activity of vit. A (Hennekens 1986; Kelloff et al.1996; Lippman & Meyskens 1988; Redlich *et al.* 1995; Thiberville *et al.* 1996). A detailed review of the anticancer effects of vitamin A can also be found in the publications by Israel *et al.* 1980, and Pozzi *et al.* 1985. Samet *et al.* (Samet *et al.* 1985) carried out an epidemiological study, showing that a deficiency of vitamin A favours the development of lung cancer (Mettlin 1984; Barthet *et al.* 1989; Moon *et al.* 1994).

FUNCTIONS OF VITAMIN E

It inhibits the growth of various tumor cells, such as:

- Prostate cancer cells (Israel *et al.* 2000; Yu *et al.* 2002; Zhang *et al.* 2002) ;
- Breast cancer cells (Yu *et al.* 1999)
- Lung cancer cells (Neuzil *et al.* 2001);
- Parotic cancer cells (Prasad *et al.* 1996) ;
- Stomach cancer cells (Rose % McFadden 2001; Wu *et al.* 2002);
- Colon cancer cells (Neuzil *et al.* 2001);
- Pancreatic cancer cells (Heisler *et al.* 2000);
- Oral squamous cell cancer (Elattar & Virji 1999);
- Melanoma cells (Prasad *et al.* 1990);
- Neuroblastoma cells (Prasad *et al.* 2003);
- Glioma cells (Prasad *et al.* 2003);
- Leukemia cells (Yamamoto *et al.* 2000);
- Lymphoma cells (Turley *et al.* 1995; Yu *et al.* 1997; Dalen & Neuzil 2003);
- At low doses it induces differentiation and inhibition of tumor proliferation; at higher concentrations it induces apoptosis (Prasad *et al.* 2003);
- Suppression of tumor growth (Prasad 2003);
- Apoptotic and/or cytostatic activity of breast cancer cells (Malafa & Neitzel 2000);
- Colon cancer cells (Prasad *et al.* 2003);
- Melanoma cells (Malafa & Neitzel 2002);
- Neuroblastoma cells (Prasad *et al.* 2003);
- Lymphoma cells (Sarna *et al.* 2000);
- It reinforces the anticancer action of various chemotherapy agents such as adriamicin, cisplatin and tamoxifen (Ripoll *et al.* 1986; Prasad *et al.* 1994);
- It protects bone marrow cells from the lethal effects of doxorubicin (Fariss *et al.* 1994);
- It reinforces the anticancer action of various chemotherapy agents, protecting healthy cells from toxic effects (Prasad *et al.* 2003)
- Antiangiogenetic activity (Shklar & Schwartz 1996; Tang & Meydani 2001; Neuzil *et al.* 2002; Inokuchi *et al.* 2003; Miyazawa *et al.* 2004).

Retinoids are molecules with a hydrophobic structure, able to cross the biological membranes and to directly reach the nucleus where, interacting with specific nuclear receptors, they can exert their biological activity; retinoids are also often bound to proteins both inside cells and in the extracellular compartment, such proteins being known as RBPs: Retinoid Binding Proteins. The All Trans Retinoic Acid (ATRA) enters the cells by simple diffusion or by means of conversion from retinol (vitamin A) which has been absorbed in the gastrointestinal tract. Inside the cell, ATRA binds to specific proteins, the CRABPs (Cellular Retinoic Acid Binding Proteins), only two types (I and II) of which are known so far. The function of these proteins is still not clear although they could act as a system for the accumulation and transport of retinoids in particular intracellular compartments (e.g. the endoplasmatic reticulum for oxidation or the nucleus for interaction with specific receptors). The encoding gene for the CRABP-II protein presents two elements sensitive to retinoic acid and it appears that this gene can for this reason be induced by ATRA. In fact, subjects treated with ATRA present an increased expression of CRABP-II. The cells which produce this protein after administration of ATRA have not yet been identified. The ubiquitary receptorial expression of retinoids, RAR alfa beta gamma, and RXR alfa beta gamma, has been shown both at cell membrane and cell nucleus level, where RXR and RAR dimerize with vitamin D receptors (VDR) and RZR and ROR of Melatonin (components of the DBM), amplifying a dynamic differentiating and antiproliferative gene expression.

RETINOID RECEPTORS

Two families of retinoid receptors have been identified so far:

- **RARs** (Retinoic Acid Receptors), of which we know three types (a, b and g) and various subtypes (a1, a2, b1-4, g1 and g2); these receptors bind ATRA and 9-cis retinoic acid.
- **RXRs** (Retinoid X Receptors) of which we know three types (a, b and g) which bind only 9-cis retinoic acid.

These receptors belong to a large superfamily of inducible nuclear receptors (which are also transcription factors) like steroid receptors, thyroid hormone receptors, vitamin D^3 receptors, Drosophila ecdysteroid receptors, and a number of receptors whose ligands have not yet been identified ("orphan" receptors). This superfamily can in turn be divided into two main families of nuclear receptors:

- steroid receptor family;
- non-steroid receptor family (thyroid hormones/retinoids/vitamin D).

The characteristic common to all these receptors is the ability to interact with regulatory regions of DNA called target sequences Hormone Response Elements (HREs) and to induce the transcription of specific genes. The structure of these receptors is extremely complex in relation to the functions they perform: several functional domains have in fact been identified in the context of these molecules. In the retinoid receptors, a total of 6 functional domains (A–F) have been identified in the RARs and only 5 (A–E) in the family of RXRs. A and B domains contain a transactivating region (activating gene transcription) defined AF-1, whose action inducing gene transcription is independent of the binding of the receptor with the ligand (retinoid). In the retinoid receptors there is also a second transactivating region (AF-2) present inside the domain which binds the ligand (domain E). In fact, RAR-a, -b and -g missing from the AF-2 region are unable to induce gene transcription. In an intact receptor, the function of binding with the ligand (domain E) and the transactivating function (region AF-2, also domain E) are clearly independent. Domain C functions as region for binding to DNA; it contains, in fact, two zinc-finger sequences capable of interacting with nucleotide sequences. The domains D and E contain a region implicated in the formation of dimers and a region implicated in the nuclear localization of the receptor.

RARs

The RAR class consists of three types of receptors: RAR-a, RAR-b, and RAR-g. The members of this receptor family exhibit a distinct tissue and cell expression; they share a high degree of homology in the DNA binding domains (domain C) and ligand binding domains (domain E); they bind ATR and 9-cis retinoic acid with high affinity and 13-cis retinoic acid with low affinity. Each type of RAR (a, b or g) can in turn present different subtypes (a1, a2, b1-4, g1 and g2). The synthesis of different subtypes depends on two main mechanisms:

- **alternative transcription** due to the presence of two promotors in the framework of the same encoding gene: these transcripts generate receptor subtypes with different A domains and with different nucleotide sequence binding abilities;
- **alternative splicing**.

The RARs present 6 domains (A–F) from the N-terminal to the C-terminal Until 1988 it was thought that RARs, like thyroid hormone receptors (TRs), functioned exclusively as homodimers (RAR/RAR and TR/TR). It was subsequently shown that these receptors, like the vitamin D receptor (VDR), interact with other factors present in nuclear extracts to bind specific gene sequences with high affinity. After the discovery of RXRs, a series of laboratory experiments showed that these factors were in fact the RXRs, able to form heterodimers with the RARs, VDRs and TRs. The RAR-a is mainly expressed in the cerebellum, adrenal gland, testicles and leukemic cells of the myeloid line.

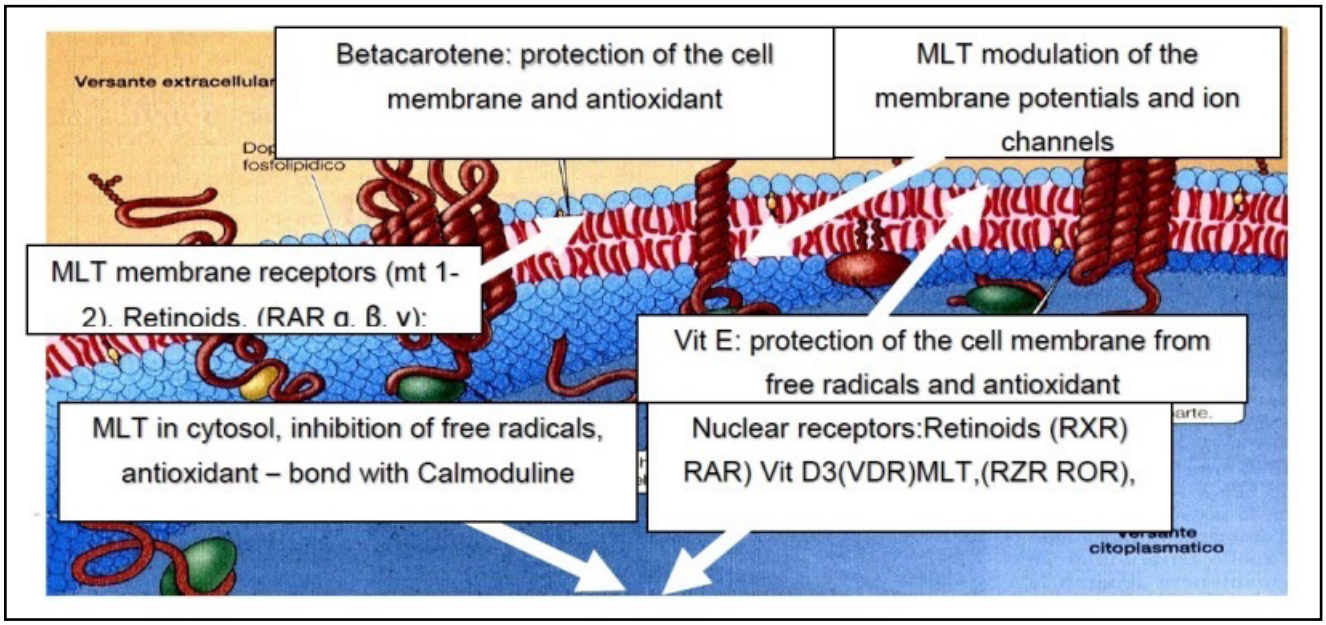
RXRs

This family of receptors is only distantly correlated with the RAR family as regards the peptide sequence and seems unable to bind ATRA with high affinity. The ligand of RXRs is 9-cis retinoic acid, an isomer of ATRA that can interact with RXRs and RARs. RXR-a/RAR-a heterodimers bind to specific sequences of DNA known as Retinoic Acid Response Elements (RARE) which are two repeated sequences: Direct Repeats (DR) of the 5'-PuG(G/T)TCA (Pu: purine) type separated by 1–5 pairs of bases (DR1-5) and usually localized in the promotor region of a target gene. In addition to the DR sequences, there are other nucleotide sequences that the nuclear receptors bind to, like the IR sequences (Inverted Repeats), which are activated by TRs, RARs and RXRs, and the ER sequences (Everted Repeats). The binding of the ligand to the dimer complex RAR/RXR involves its interaction with DR sequences, which leads to control of the transcription (activation or suppression) of the gene downstream of the interaction site. Several studies suggest that the differentiation of myeloid lines induced by ATRA is in fact mediated by RAR-a/ RXR-a heterodimer complexes and not through the activity of RAR-a (RAR-a/RAR-a) homodimer complexes.

In support of this, experiments carried out on HL60 cell lines show that RXRs are functional but not RARs as the latter are incapable of differentiating in response to ATRA. HL-60 cells resistant to the action of ATRA contain a non-sense mutation inside the region encoding for RAR-a; when transfected with a cDNA vector encoding for RAR-a, the cells can easily differentiate in response to ATRA. Dawson and Xia showed that, by using synthetic ligands for the pairs of RAR/RXR and RXR/RXR, the differentiation of the myeloid line depends almost exclusively on the action of the heterodimer RARs/RXRs (Dawson & Xia 2012; Di Masi *et al.* 2015; Eroglu & Harrison 2012; Long *et al.* 2015; Urvalek *et al.* 2014; Zhong *et al.* 2013).

After binding to RAR/RXZ, the retinoids modulate the expression of genes involved in programmed cell death (apoptosis) through inhibition of Bcl2 and consequent caspase activation.

They also have an antiproliferative effect with activation of P21/P27, and a consequent halt in the cell cycle in the G0/G1 stages, and a protective action at the level



The cell membrane (in pale blue) containing the phospholipid layer (in red) is a defence, a vital filter through which everything passes, from inside the cell towards the exterior, where the stimuli and the conditioning from the exterior towards the interior and vice versa are assimilated, in which communication takes place, where impulses and signals are emitted and received. Optimizing it, making it efficient, means making the cell able to defend itself in ideal conditions, reinforcing it: Vit. E and Betacarotene protect and stabilize the membrane, MLT physiologically modulates its potentials, regulating the ion channels and all the dynamic ands and receptorial expression.

of the cell membrane, in total synergy with ascorbic acid, tocopherol and melatonin.

Vitamin E induces neoplastic apoptosic also through externalization of phosphatydilserine from the membrane of tumor cells and relative chemotaxis of cell immunity.

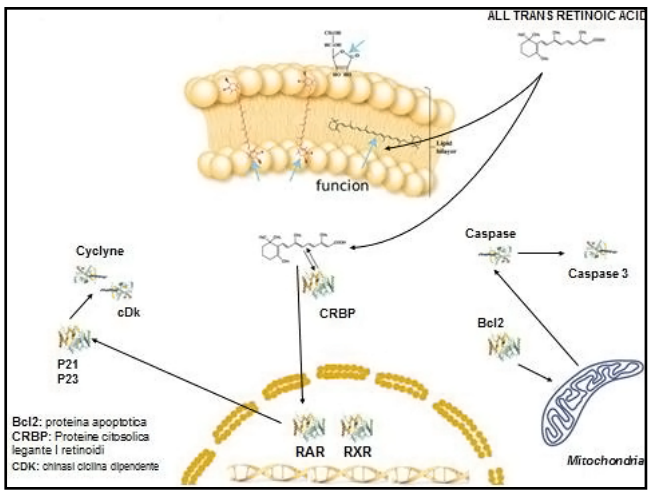
CONCLUSIONS

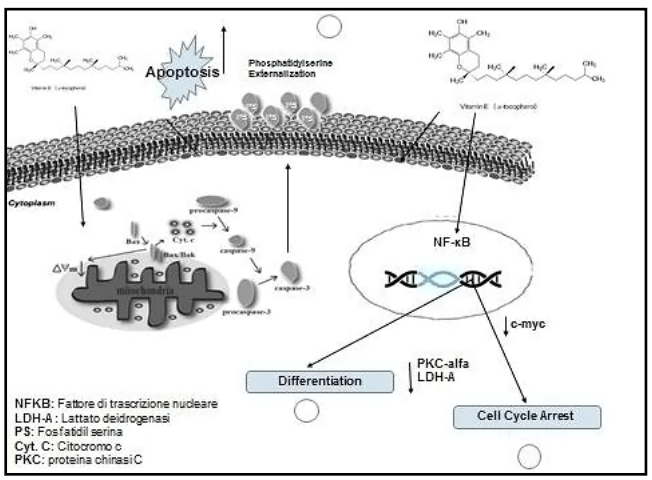
THE LITERATURE HAS EXTENSIVELY DOCUMENTED AND CONFIRMED THE DETERMINING ROLE OF RETINOIDS IN THE PREVENTION AND THERAPY OF TUMORS, AS SUMMARIZED BELOW

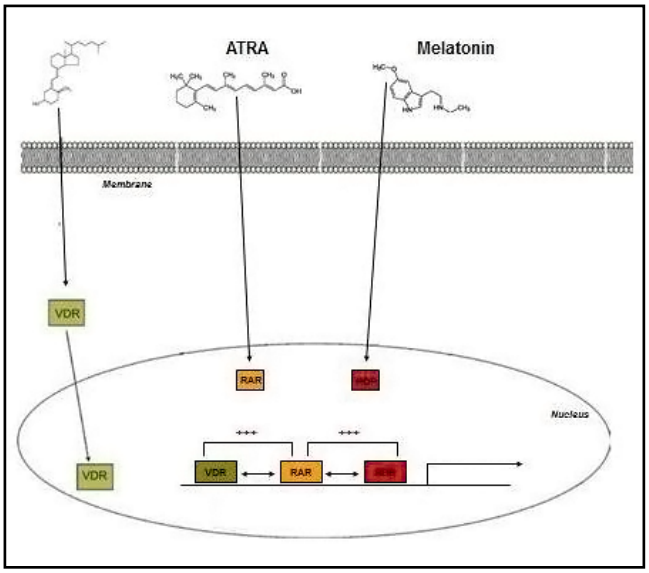
Interaction and factorial synergism of retinoid with vitamin E and other components of the DBM (Barroga *et al.* 2000; Black 2013; Suarez *et al.* 2014; Watters *et al.* 2009; Gilbert *et al.* 2012; Hu *et al.* 2014).

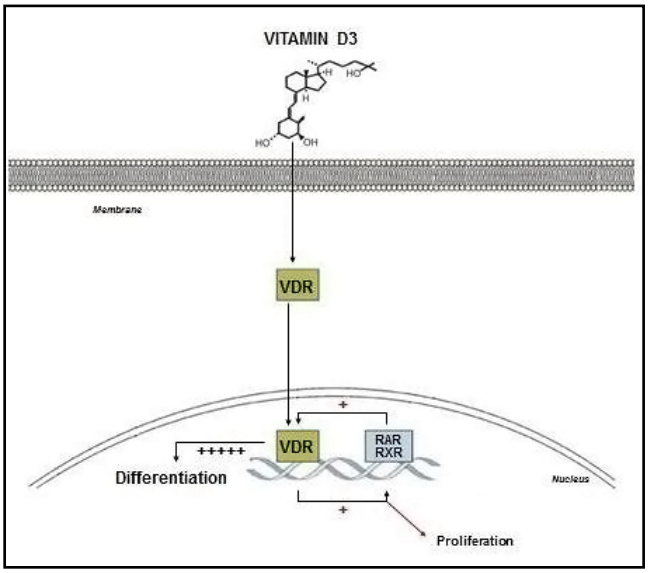
Antioxidant, antiradical and antitoxic activity preventing tumor development (Buring *et al.* 1994; Clamon 1980; Connolly *et al.* 2013; Den Hollander *et al.* 2013; Doldo *et al.* 2015; Helm *et al.* 2013; Ianhez *et al.* 2013; Jeon *et al.* 2011; Kabat *et al.* 2012; Miyanishi *et al.* 2015; Jiang 2014; Mondul *et al.* 2013; Siems *et al.* 2009; Tanaka *et al.* 2012; Virtamo *et al.* 2014; Wang *et al.* 2014; Igielska-Kalwat *et al.* 2015; Li *et al.* 2012).

Apoptotic, cytostatic, and antiproliferative properties: Retinoids inhibit mutagenesis through a pro-differentiating action, keeping the healthy cells "differentiated", they favour the reconversion to normality and redifferentiate cells that tend to become









or are already "indifferentiated" and neoplastic. The alteration of the GF-TRK ligand-receptor system and the altered response to the differentiating stimulus are also effectively countered by the retinoids. (Arany *et al.* 2003; Basu *et al.* 2000; Chang *et al.* 2013; Constantinou *et al.* 2012; Cui *et al.* 2007; Dalen & Neuzil 2003; Dong *et al.* 2008; Ginestier *et al.* 2009; Gudas 2013;, Lim *et al.* 2014; Lu *et al.* 2013; Neuzil *et al.* 2001; Onogi *et al.* 1998; Piedrafita & Pfahl 1997; Voigt *et al.* 2000; Yang *et al.* 2012; Yin *et al.* 2009; Wu *et al.* 2009).

Antiangiogenic properties: Retinoids inhibit angiogenesis in tumor tissues in synergy and interaction with other components of the DBM, such as Vitamins D and C, GH inhibitors and GH-dependent growth factors, D2 receptor agonists (Malafa *et al.* 2002; Inokuchi *et al.* 2003; Majewski *et al.* 1994; Miyazawa *et al.* 2004; Siveen *et al.* 2014; Tang & Meydani 2001).

Antimetastatic properties, by activating intercellular adhesion and inhibition of cell passage through the natural containment barriers of metastatic invasion such as the EMC, preventing lysis (Adachi *et al.* 2001; Lee *et al.* 2014; Lim *et al.* 2013; Lotan et al.1991; Pham *et al.* 2013; Siddikuzzaman, Grace, 2012; Siddikuzzaman *et al.* 2012; Walder *et al.* 1997).

Immunostimulating properties of retinoids in the natural immunity and response of NK cells, improvement of the functionality of organs and tissues with increase of cell trophism, particularly evident at epithelial level (Carratù *et al.* 2012; Ding *et al.* 2013; Han *et al.* 2014; Lo *et al.* 2014; Pekmezci 2011; Prabhala *et al.* 1991).

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"There is not and never will be a cytotoxic chemotherapy treatment that can cure a solid tumor, but only a method, a rational and biological multitherapy, a complex of synergic and factorially interactive substances, individually having atoxic anticancer activity, which sequentially or simultaneously act centripetally on the myriad of biological reactions of tumor life, gradually restoring the vital reactions deviated by the tumor to normality."

Luigi Di Bella.

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