Expert Opinion on Drug Delivery

ISSN: 1742-5247 (Print) 1744-7593 (Online) Journal homepage: http://www.tandfonline.com/loi/iedd20

Immunomodulatory activities of alpha lipoic acid with a special focus on its efficacy in preventing miscarriage

Giovanni Monastra, Sara De Grazia, Serap Cilaker Micili, Asli Goker & Vittorio Unfer

To cite this article: Giovanni Monastra, Sara De Grazia, Serap Cilaker Micili, Asli Goker & Vittorio Unfer (2016): Immunomodulatory activities of alpha lipoic acid with a special focus on its efficacy in preventing miscarriage, Expert Opinion on Drug Delivery, DOI: 10.1080/17425247.2016.1200556

To link to this article: http://dx.doi.org/10.1080/17425247.2016.1200556

Accepted author version posted online: 13 Jun 2016.
Published online: 13 Jun 2016.

Submit your article to this journal

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=iedd20

Download by: [Giovanni Monastra] **Date:** 13 June 2016, At: 05:09

Publisher: Taylor & Francis

Journal: *Expert Opinion on Drug Delivery*

DOI: 10.1080/17425247.2016.1200556

REVIEW

Immunomodulatory activities of alpha lipoic acid with a special focus on its efficacy in preventing miscarriage

Giovanni Monastra[1], Sara De Grazia[2], Serap Cilaker Micili[3], Asli Goker[4] and Vittorio Unfer*[5]

*Author for correspondence

[1]Visiting scientist in the Systems Biology Group, Department of Experimental Medicine, University la Sapienza, Rome, Italy

[2]LO.LI. Pharma, Department of Research and Development, Rome, Italy

[3]Dokuz Eylul University Faculty of Medicine Department of Histology and Embryology, Izmir, Turkey

[4]Celal Bayar University Faculty of Medicine Department of Obstetrics and Gynecology, Manisa, Turkey

[5]UNIIPUS - Private Swiss University Institute, Via Vincenzo Vela 4, 6830 Chiasso, Switzerland

e-mail: vunfer@gmail.com

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Abstract

Introduction: Alpha lipoic acid (ALA) is an essential mitochondrial co-factor and, as a free molecule, it can exert multi-level immunomodulatory functions. Both ALA and its reduced form, dihydrolipoic acid (DHLA), are believed to be able to chelate heavy metals, to regenerate essential antioxidants and to repair important molecules damaged by oxidation. The largest part of the effects of ALA/DHLA couple can be explained by a specific stimulatory activity on Nrf2-dependent gene transcription and by the inhibition of NF-kB activity. These features have prompted its as a drug for several diseases.

Areas covered: This article surveys the main features of ALA/DHLA and its therapeutic effects. Its complex and differentiated function cannot simply be reduced to anti-inflammatory, antioxidant and detoxifying action. We highlight its capability to finely modulate several physiological pathways when unbalanced. In particular, we focus our attention on pregnancy, in relation to ALA administration by oral route and by a new formulation for vaginal delivery, in patients with threatened miscarriage.

Expert opinion: Future efforts should be devoted to explaining carefully ALA/DHLA mechanism of action to reactivate the physiological balance when modified during pregnancy. On the other hand, ALA safety in pregnant women and its pharmacokinetics by vaginal route, have to be studied in depth. Moreover, ALA efficacy has to be confirmed in a much larger sample of patients.

Keywords: Alpha-lipoic acid, antioxidant activity, drug delivery, gynecology, immunomodulation, inflammation, miscarriage, NF-kB, Nrf2, subchorionic hematoma, vaginal route.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Abbreviations:

α-SMA: α-smooth muscle actin; Akt: Protein kinase B; ALA: Alpha Lipoic Acid; ARE: Antioxidant response element; CD: Cluster of differentiation; COX2: cyclooxygenase 2; DHLA: Dihydrolipoic acid; EGF: Epidermal growth factor; HUVECs: Human umbilical vein endothelial cells; ICAM: Intracellular adhesion molecules; IFN-γ: Interferon-γ; IgE: Immunoglobulin E; IkB: Inhibitor of κB; IL: Interleukin; iNOS: inducible nitric oxide synthase; KEAP1: Kelch-like ECH-associated protein 1; MMP-9: Matrix metalloproteinase 9; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NK cells: Natural killer cells; NO: Nitric oxide; Nrf2: Nuclear factor erythroid 2-related factor 2; PDGF: Platelet-derived growth factor; PGE2: Prostaglandin E2; PI-3K: Phosphatidylinositide 3-kinase; PKA: Protein kinase A; RA: Rheumatoid arthritis; RNS: reactive nitrogen species; ROS: reactive oxygen species; TGF-β: Transforming growth factor-β; Th: T helper; TNFα: Tumor Necrosis Factor α; Treg cells: Regulatory T cells; VCAM: Vascular cell adhesion molecule; VEGF: Vascular endothelial growth factor.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Article highlights

- Alpha-lipoic acid (ALA) is an organosulfur compound playing a pivotal role as cofactor within α-keto-acid dehydrogenase complexes and glycine cleavage system.
- In living organisms, ALA is present either in its reduced, as dihydrolipoic acid (DHLA), and oxidized, disulfuric form.
- ALA is synthesized in plants and animals, but its production in humans is very low.
- ALA and DHLA play a key role in a large amount of physiological processes, exerting antioxidant, anti-inflammatory and detoxifying activities. On the whole the result is a fine immunomodulatory effect.
- ALA can be used efficaciously as drug or dietary supplement.
- ALA has been demonstrated to be safe at therapeutic doses.
- ALA and DHLA rescue altered physiological balance without negatively affecting other cascades of metabolic events not involved in pathological alterations.
- ALA treatment is effective in improving many diseases, such as cancer, sepsis, insulin resistance and diabetes, neurodegenerative disorders and neuropathy, ischemia-reperfusion injury, tissue regeneration, aging.
- Two recent clinical studies demonstrated that ALA administered by oral or vaginal route can also significantly contribute to speed up the healing process of subchorionic hematomas related to threatened miscarriage.
- ALA biological properties and the first therapeutic results obtained in miscarriage prevention suggest a promising application of this molecule also in gynecology; this research area deserves an in-deep investigation.

This box summarizes the key points contained in the article.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

1. Introduction

Alpha-lipoic acid (henceforth: ALA), an organosulfur compound (chemical formula: $C_8H_{14}O_2S_2$) with MW 206.32, derived from octanoic acid, is a safe natural molecule that exerts pleiotropic actions. ALA was initially isolated from potatoes by Snell, Strong and Peterson [1] in 1937, and called "accessory factor for lactic acid bacteria". Then it was again isolated and chemically identified by Lester J. Reed and his team [2]. Though ALA can exist in the R- and S-enantiomeric forms (Figure 1), only the R-structure is synthetized endogenously and has shown to be indispensable in living systems. In some cases, ALA stereochemistry can play a key role in determining the specific activity of this molecule [3-5]. Also dihydrolipoic acid (DHLA), the reduced counterpart of ALA (Figure 2), has the R- and S-enantiomers. DHLA is the form found within the cell, and it has been suggested that the reduction of ALA to DHLA is required to obtain at least some biological effects (for instance, neuroprotection) [6]. ALA is synthesized in plants and animals, but its production in humans is very low; tissues characterized by the presence of numerous mitochondria are the richest [7]. Potatoes, broccoli, spinach, tomatoes, Brussels sprouts, peas, brown rice contain great quantities of this compound, but red meat (especially liver, heart and kidney) is the most relevant source [8].

As shown by many researches and clinical trials, ALA and DHLA play a key role in a large amount of physiological processes, and ALA can be used efficaciously in the treatment of several pathological conditions.

2. Absorption, bioavailability, metabolism and safety of use

When absorbed from food, ALA is taken up by cells and can be intracellularly converted into DHLA by enzymes such as dihydrolipoamide dehydrogenase, glutathione reductase, or thioredoxin reductase [9-10]. In living organisms, ALA is present in its reduced and oxidized, disulfuric form. The main limitations in achieving effective doses of ALA in humans are caused by the degradation process of the molecule, the short plasma half-life, the first-pass effect and the metabolic

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

elimination [11]. The cellular transport of ALA probably occurs through several systems, such as the medium-chain fatty acid transporter, a Na^+-dependent vitamin transport system, and a H^+-linked monocarboxylate transporter for intestinal uptake [12]. The endogenous plasma levels of ALA and DHLA are respectively 1-25 and 33-145 ng/ml [13]. The absorption of ALA as dietary supplement is decreased by the concomitant food intake. Available data [13-16] show that ALA, administered as single-dose tablets (50 to 600 mg), is entirely absorbed within 30-60 minutes, and its plasma half-life (t ½) is 30 minutes. Despite the rapid and almost complete absorption after oral administration, ALA does not extensively accumulate in tissues (the richest among them are heart, kidney and liver) and is quickly metabolized. The metabolic process mainly occurs in the liver, through a mitochondrial β-oxidation. After oral administration of ALA to healthy volunteers, dimethylated products such as 4,6-bismethylmercapto-hexanoic acid and 2,4-bismethylmercapto-butanoic acid, were detected in plasma [17]. Its bioavailability is limited (about 30%), due to a significant pre-systemic metabolism in liver (first-pass effect). The oral use of ALA is hampered by its fast plasma clearance and excretion. In healthy subjects, (ALA) 600 mg/day of ALA, administered by oral route for 4 days, is recovered unchanged in urine at the percentage of: 0.19% (day 1) and 0.22% (day 4). Therefore, the central problem to deal with consists in increasing ALA bioavailability, allowing the compound to reach the proper concentration to ensure a therapeutic effect. In ongoing experiments, addressed to verify ALA distribution in uterus and cervix tissues, female rats have been administered with 50 and 500 mg/kg by vaginal route for 4 days. In control group samples, uterus, endometrium, myometrium and perimetrium have shown a normal histomorphological structure, as well as in lining simple columnar epithelium and basal membrane. Under the basal membrane the stroma with the endometrial glands and vessels has presented a normal structure in agreement with the cyclic period of the rats. In all layers of control uterus, no anti-ALA immunopositive stained cells have been found. Also in low and high dose ALA groups endometrium, myometrium and perimetrium have shown a normal histomorphologic structure. In the immunohistochemistry of endometrium the lining simple columnar epithelium, the endometrial

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

glands and the stroma were stained with intense amount of anti-ALA antibody. Between the samples of ALA low and the high dose, no significant differences have been found for immunopositivity. In the cervix tissues, control group had normal histomorphological structure, as seen for the uterus. In low and high dose ALA groups, histomorphological structures were similar to controls. The stratified squamous epithelium and the muscle tissue were stained with the same intense amount of anti-ALA antibody both in low and high dose groups. Therefore also the lowest ALA dose (50 mg/kg), administered in this experiment by vaginal route, is able to reach its pharmacokinetic targets at local level.

ALA shows no toxicity even at much higher doses than those normally used in treatments. Both acute and chronic doses have provided very reassuring results in animals. Indeed, 31.6 or 61.9 mg ALA/kg bw/day orally for 4 weeks to rats did not induce any adverse effect. Similar results were obtained with two years oral administration up to 60mg/kg/day. Furthermore, ALA by oral route in acute dose was not toxic to rats (LD50>2000mg/kg bw) [18-19]. Animal studies have proven that ALA has a protective effect on the foetus in mothers who are diabetic, alcoholic or exposed to toxic pollutants such as dioxin [20-23]. In humans, numerous clinical trials were performed to test the tolerability of this compound. ALA was administered up to 1200 mg once a day i.v. over two 5-day periods [24] or (at) 600 mg once a day i.v. for 3 weeks, followed by 600 mg three times a day orally for 6 months [25]; both treatments did not produce significant adverse effects compared to placebo. The same reassuring profile emerges from two reviews [26-27] where the authors display several data on ALA treatments during pregnancy or postpartum. We also report the results of a recent *in vitro* experiment with human lymphocytes after 24 hs incubation with four different concentrations of ALA (1 to 1000 μM). The compound confirmed its safety, and, at high doses such as 100 and 1000 μM, it has even significantly enhanced the lymphocytes viability vs the control group (more than two times at 1000 μM) [28]. Finally, in ongoing experiments for testing the safety of vaginal route, ALA, at two different doses, has been administered to healthy pregnant rats (50 and 500 mg/kg) to detect whether the treatment can modify the normal levels of Tumor Necrosis Factor α

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

(TNFα), Vascular endothelial growth factor (VEGF) and α-smooth muscle actin (α-SMA). The two groups were compared with a third one which did not receive any treatment. The experimental protocol established to determine the oestrus cycle and to apply vaginal ALA for 5 days until the second oestrus cycle. After mating, vaginal smears were taken from the female rats and sperm searched under optical microscope. If the smear was found positive, the rats, considered at the first day of pregnancy, were separated and scarified six days later. The results of semi-quantitative score of immunohistochemistry demonstrated that TNF α, VEGF and α-SMA levels were similar among the three groups (50 mg/kg ALA, 500 mg/kg ALA and controls), without any significant difference. Therefore, ALA did not induce changes in the normal values, other evidence that strengthens its safety.

3. ALA/DHLA as direct and indirect antioxidants

For decades ALA has been mainly known as an essential prosthetic group of various cellular enzymatic complexes, and as a molecule involved in turning glucose into energy at mitochondrial level. When ALA is endogenously produced (in R-form), great part of it is not found as free molecule; indeed, its precursor, the octanoic acid, is already bound to enzymatic systems before the introduction of sulfur atoms [29]. ALA, in protein-bound form, plays the role of cofactor in the α-keto-acid dehydrogenase complexes (KADC) and in the glycine cleavage system (GCS), which are involved in the metabolic pathways of pyruvate oxidation, the citric acid cycle, the amino acid biosynthesis and degradation [9]. The α-keto acid dehydrogenase complexes are located in the mitochondrial matrix, associated with the inner membrane, and are constituted by the pyruvate dehydrogenase complex (PDC), the α-ketoglutarate dehydrogenase complex (α-KGDC), and the branched chain α-ketoacid dehydrogenase complex (BCKADC). Their function is to catalyze the oxidative decarboxylation of several α-keto-acid substrates to the corresponding acyl-CoA forms, giving rise to NADH. The complexes have numerous copies of three enzymes: α-keto dehydrogenase (E1), dihydrolipoyl acyltransferase (E2) where ALA can be found attached with an

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

amide bond to a lysine residue (Figure 3), and dihydrolipoyl dehydrogenase (E3) [30]. Furthermore, free ALA interacts with several protein systems as substrate. It was demonstrated that the E2 and E3 components of the α-keto-acid dehydrogenase complexes use free ALA as substrate; thus E3 catalyzes the reduction of ALA to DHLA by means of NADH oxidation [9]. Free ALA plays also other relevant functions, acting as inhibitor, or effector. In this context, several authors highlighted that ALA and DHLA constitute a very powerful redox couple. When addressing the biological activity, we should consider ALA and DHLA together, since in some situations they have interchangeable effects, and also in consideration of their fast mutual transformation, which depends on the microenvironment conditions (for instance, since the cell cytoplasm is a reducing environment, ALA is transformed to DHLA after entering the cell). It is generally accepted that ALA/DHLA couple can *directly* scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), though some authors disagree [31]. Moreover, it is thought that ALA/DHLA also exert an indirect antioxidant action when this couple boosts the cellular antioxidant system, by reducing back other essential antioxidant molecules, such as Coenzyme Q_{10}, vitamin C and vitamin E [11,32-34]. Another indirect antioxidant action occurs when ALA and/or DHLA, as detoxifying agents, chelate several heavy metals (for instance, iron, lead, cadmium, mercury, copper, arsenic) that cause oxidative processes [11,35-36]. Finally, ALA/DHLA have a role in repairing biological molecules, such as proteins, lipids, DNA, damaged by oxidation. In proteins, oxidation occurs at the level of their amino acid residues, such as methionine, cysteine, histidine, tyrosine, tryptophan. For instance, the oxidation of specific methionine residues immediately leads to protein inactivation that entails an impaired or blocked activity of many enzymes, hormones and chemotactic factors. It has been shown that lipoamide, the neutral amide of ALA, is involved in repairing oxidized methionine residues [37]. Thus, ALA/DHLA perform antioxidant activities, through four different mechanisms, at least *in vitro* in some cases, namely by scavenging oxidants, regenerating endogenous antioxidants, chelating transition metals and finally contributing to repair oxidative damage. Some researchers questioned that the effects of ALA/DHLA, detected *in vitro* against ROS and RNS, also

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

occur *in vivo*, mainly owing to the impossibility that this compound, given orally, can reach a sufficient stoichiometric concentration to exert a free radical-scavenging action [31]. Clearly, ALA/DHLA activities (in particular the scavenging) may be more plausible when ALA is given as topical drug, strongly increasing the possibility to reach the therapeutic site at an effective concentration. Furthermore, as we will discuss in the "Expert Opinion" section, there are other alternative hypothesis to explain the final antioxidant result obtained by ALA/DHLA [38-39]. Together with the antioxidant activity, or even in certain cases separately from that, ALA/DHLA couple exerts anti-inflammatory and immunomodulatory effects.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

4. Modulation of Nrf2 and NF-kB

In most cases the main mechanism of action proper to ALA/DHLA seems to be associated to the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and to the inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). Nrf2 is the master of redox homeostasis, acting as regulator or inducer for the expression of several antioxidants and cytoprotective genes [40-42], though in certain cases its expression can be positively associated to aggressive cancer progression [43]. The PI-3K/Akt pathway is involved in the activation/repression of this nuclear factor. Nrf2-induced phase II antioxidant enzymes are related to the most important cellular endogenous antioxidant system to counteract oxidative stress [44]. Nrf2 is kept inactive in the cytoplasm where it is associated to Kelch-like ECH-associated protein 1 (KEAP1), and eventually degraded; instead, in presence of specific inducers or environmental situations, conformational changes occur in Keap1, thereby producing free Nrf2 that is activated by means of its transfer to the nucleus where it forms a complex with the antioxidant response element (ARE), called also electrophile-responsive element (EpRE), and other proteins, then giving rise to gene transcription [45]. ARE/Nrf2 pathway takes on great interest as potential target for pharmacological interventions on degenerative and immunological diseases, and its regulation plays a key role in the development of new therapies [46-47]. An increasing evidence has

demonstrated that ALA/DHLA can stimulate Nrf2 pathway with beneficial consequences on health. ALA i.p. injection in old rats was able to enhance nuclear Nrf2 levels and to induce Nrf2 binding to ARE, with the resulting increase of GSH levels [32]. An array of studies and researches with different experimental models confirmed that ALA is an effective chemoprotective agent and that its action is mediated by Nrf2 in many contexts [48-52].

On the other side, Nuclear Factor kB (NF-kB) plays a pivotal role in the expression of pro-inflammatory genes, including those ones for cytokines, chemokines, and adhesion molecules. Similarly to Nrf2, the PI-3K/Akt cascade is also involved in the NF-kB activation/repression. This mechanism preserves living beings from external damages and allows fundamental steps in the organism development (for instance, pregnancy), but an excessive reaction can be seriously harmful and therefore it has to be finely down-modulated, without a drastic inhibition. NF-kB normally is in a latent, inactive state bound to the inhibitor of kappa B (IkB) protein forming a complex in the cytoplasm. The expression of pro-inflammatory genes follows NF-kB activation and its translocation from cytoplasm to nucleus. This process is triggered by oxidative molecules associated to inflammation, mainly proinflammatory cytokines such as Tumor Necrosis Factor α (TNF α) and Interleukin 1β (IL-1β), but also CD45 [53-57]. The inhibition of NF-kB translocation from the cytoplasm to the nucleus assumes a pivotal role in reducing uncontrolled oxidative and inflammatory reactions. Some evidences indicate that a cross talk between Nrf2 and NF-kB exists [58]. This is an important issue according to the relationship with the cascade of events triggered by some immunomodulators.

ALA/DHLA couple has been reported to act as a down-modulator of metabolic pathways leading to inflammation therefore having an inhibitory activity on the nuclear transcription factor NF-κB [59]. This activity probably parallels the simultaneous stimulation of Nrf2. Acute treatments with ALA have been shown to prevent IκB degradation, the protein that inhibits NF-κB, and also to directly decrease NF-κB expression [60].

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

5. An overview on some specific effects of ALA

In glycinin-sensitized weaned rats, an animal model which mimics food-induced hypersensitivity and is predominantly characterized by a Th2-type immune response, it has been shown that the number of mast cells in the mucosa, the level of histamine release, and total serum IgE and glycinin-induced IgE levels were significantly decreased after feeding a low dose of ALA [61]. Furthermore, both INF-γ and IL-2 levels of animals treated with ALA were significantly higher in comparison to the group of sham-sensitized animals with purified glycinin and megadose of vitamin C. ALA treatment also decreased the ratio of $CD4^+$ to $CD8^+$ which was altered by glycinin-induced allergy [61]. This observation suggests that, at least in this rat model, a small dose of ALA has much more effect than a mega-dose of vitamin C. Moreover, other studies indicated that this compound can be active in *in vivo* and *in vitro* experimental models, as in asthma mouse model [62], or primary cultures of fibroblast-like synovial (FLS) cells from patients with long established rheumatoid arthritis (RA) [63]. Furthermore, early pediatric allergies in babies and children were treated successfully with ALA [64]. It has also been demonstrated that ALA slows down the progression of some autoimmune diseases such as collagen-induced arthritis [65]. In another study [59] human umbilical vein endothelial cells (HUVECs) treated with TNF-α, only ALA, but not the other tested antioxidants (tiron, apocynin, and tempol), was able to block NF-kB activation. Noteworthy, ALA/DHLA induced activation of the PI-3K/Akt pathway in HUVECs did not contribute to NF-kB inhibition. The authors conclude that the inhibitory effect of ALA on NF-kB activation is due to the inhibition of IKK-β, an enzyme that serves as a protein subunit of IκB kinase. As an alternative mechanism, Zang and co-workers suggested that ALA/DHLA can prevent NF-kB stimulation by activating PI3K-Akt pathway [66]. It has also been reported that ALA, but not other tested antioxidants, inhibits IkBα degradation and NF-kB dependent gene expression, strongly suggesting that this activity is independent of its antioxidant function [59]. This is consistent with previous studies showing that ALA/DHLA, but not ascorbic acid or N-acetyl cysteine (NAC), inhibited TNFα induced IKK/NF-kB signaling in human aortic endothelial cells

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

(HAEC) [67]. These data are in contrast with previous studies supporting the necessary involvement of the PI-3K/Akt pathway to inhibit NF-kB activation. Moreover, in a research carried out in rats, ALA/DHLA were shown to increase the mRNA expression of proteins involved in the redox system. Consistent with this protective activity, a dose dependent decrease of malondialdehyde concentrations was detected [68].

On the other side, the treatment with ALA/DHLA is associated with apoptotic DNA fragmentation in several cultured murine and human tumor cell lines (for instance, Jurkat and CCRF-CEM leukemic T cell lines) [69-76]. It should be highlighted that such effect was not observed in healthy cells, such as normal peripheral blood lymphocytes [75]. ALA supplementation in elderly rats was associated with a decrease of lipid peroxidation (a typical phenomenon in aging), and with an increased activity of mitochondrial enzymes, such as isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, NADPH dehydrogenase and cytochrome c oxidase [77]. Noteworthy, young rats treated with ALA did not display any significant change of enzymatic activity in mitochondria [77]. Indeed, ALA is referred as a molecule able to positively counteract several pathological conditions, without affecting the normal physiological status, as we have also shown for TNF α, VEGF and α-SMA levels in the aforementioned experiment on pregnant rats. Moreover, ALA administration *in vitro* decreased the secretion of inflammatory cytokines, such as TNF α, IL-1β and IL-6, induced by LPS stimulation in rat mesangial cells. A similar decrease was detected in the secretion levels of prostaglandin E2 (PGE2) and nitric oxide (NO), due to cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) inhibition by ALA pretreatment [78]. ALA also inhibits the expression of the inflammatory cytokine IL-8 [79], whereas it stimulates the release of the anti-inflammatory cytokine IL-10 [80]. ALA treatment significantly suppressed the number and percentage of encephalitogenic T helper 1 (Th1) and T helper 17 (Th17) cells, and increased splenic regulatory T (Treg) cells, which play an essential part in the development of immunoregulation and tolerance induction. These data suggest that ALA per se (or through its transformation in DHLA) enhances systemic Treg-cells to inhibit the

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

inflammatory response [81]. Furthermore, it blocks activation and cytotoxicity of natural killer (NK) cells and inhibits IFN-γ synthesis [82]. ALA has also been reported to downmodulate the expression of CD4 on the surface of human T lymphocytes in culture [83]. In addition, ALA inhibited the production of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecules-1 (ICAM-1), proteins involved in T cell adhesion [84]. Noteworthy, it has been demonstrated *in vivo* that cAMP levels significantly raise after ALA administration by oral route [85]. The same authors reported that in cultured T cells enriched with PBMCs, the addition of 50 mg/ml or 100 mg/ml ALA significantly reduced the secretion of IL-17, a proinflammatory cytokine, respectively by 35 and 50%. Moreover, the same study showed that ALA treatment also inhibits IL-6 production and decreases T cell proliferation and activation, while possibly maintaining IL-10 levels. In addition, ALA activated the PKA signaling pathway and this evidence prompted to state that the anti-inflammatory effects of ALA are mediated, at least in part, by cAMP/PKA signaling cascade [85].

In an animal model, 15 or 30 days of ALA administration by oral gavage to rats significantly reduced wound healing time in uterine full thickness injury, and increased the levels of VEGF and α-SMA in tissues [86]. In addition, other studies highlighted ALA/DHLA efficacy in reducing the expression of MMP-9, a metalloproteinase involved in the degradation of the extracellular matrix [60], and in countering TNF-induced and thrombin-induced weakening of human fetal membranes [87-88].

Finally, it has been demonstrated that ALA and DHLA can be useful to treat successfully severe hypertriglyceridemia and diabetic dyslipidemia [89]; furthermore, they are insulin sensitizers that modulate IR/PI3K/Akt pathway at different levels. In skeletal muscle, ALA may recruit GLUT4 from its storage site in the Golgi to the sarcolemma, therefore stimulating glucose uptake [90].

In summary, ALA/DHLA couple has been reported to be involved both in energy metabolism and in protection/recovery of normal physiological status when impaired by pathogenic agents (for a synthetic view, see figure 4). All the studies cited above highlighted very promising applications for

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

ALA in multiple therapeutic areas. In the last two decades, these evidences were transferred to the clinical practice and the use of ALA in diverse inflammatory diseases constantly increased. Noteworthy, ALA and lipoate, which is its conjugate base and the most common form of ALA in physiological conditions, are amphipathic. This versatility allows ALA to be ubiquitous in plant and animal organisms, and also gives it the ability to cross the blood-brain barrier [7]. This molecule can produce its effects both in an aqueous (cytoplasm, serum), and in a lipidic environment (cell membranes, plasma lipoproteins). This constitutes a "competitive advantage" in comparison to other antioxidants, such as Vitamin C, which is highly hydrophilic, and Vitamin E or carotenoids, which are strictly hydrophobic. This trait offers advantages when ALA is used as *autacoid* drug, namely "a drug made with a natural bioactive compound".

An array of experimental researches and clinical trials have generated a consistent knowledge dealing with the therapeutic applications of ALA in different pathological conditions. To name a few, cancer [69-75, 91], sepsis [92-94], insulin resistance and diabetes [95-99], neurodegenerative diseases and neuropathy [26-27, 100-105], ischemia-reperfusion injury [106-108], tissue regeneration [86,109], aging [77, 110-113] have been proposed as potential field of ALA utilization. Recently, in women with threatened miscarriage at the first trimester of pregnancy, ALA has shown its efficacy in accelerating the resorption of subchorionic hematoma (a gathering of blood in the subchorial area, between the membranes of the placenta and the chorion, deriving from a subchorionic haemorrhage) and in significantly decreasing abdominal pain [114-115].

6. Threatened miscarriage and its causes

Threatened miscarriage refers to an intrauterine viable clinical pregnancy with a frequent adverse pregnancy outcome [116]. It occurs during the first twenty weeks of gestation and it is a common problem in pregnancy which can be often affected by complications due to genetic abnormalities [117], inflammatory processes and immunologic disorders [118-119]. Its main sign is vaginal bleeding, with or without abdominal pain and uterine contractions. Around 20% of women during

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

early gestation are affected by threatened miscarriage, and approximately half of them have a preterm delivery [120]. The occurrence of a first-trimester subchorionic hematoma represents a useful marker for women having a risk of adverse pregnancy outcome [121], with a 46% risk of miscarriage [122]. About 18% of all cases of vaginal bleeding in the first trimester are caused by a subchorionic hematoma [123]. Its presence may indicate an increased risk of pregnancy losses or preterm delivery by immune inflammatory condition. In the last years, several studies have focused the role played in the fatal outcome of pregnancy, by molecules, such as cytokines, growth factors, chemokines, and T helper (Th) cells.

Few studies focused the role played, in the fatal outcome of pregnancy, by specific cytokines and by two subsets of $CD4^+$ lymphocytes, namely T helper 1 (Th1) and T helper 2 (Th2) cells, which are divided depending on the type and the prevalence of secreted cytokines. Overall, Th1 cells secrete exclusively or predominantly TNF-β, IFN-γ, IL-2, TNF-α, TGF-β, and others (for instance, IL-10) in lesser amount. IL-10 is a crucial cytokine, involved in the auto-regulation of Th1 activation and in the control of inflammation [124-126]. On the other hand, Th2 lymphocytes release more or exclusively IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TGF-β, but small quantities of TNF α and IL-2. All these molecules perform important functions although different, in allergic reactions [127-129], and the cytokine network is deeply involved in the positive or negative development of pregnancies [130]. Th1 cells are essential for the cellular immunity and they secrete some cytokines, involved in the stimulation of macrophages, lymphocytes, and PMNs, finely targeted to destroy bacterial pathogens [131]. Furthermore, these cytokines lead to the development of cytotoxic T cell, necessary for the cell-mediated immune response against the aggression of foreign agents such as viruses and tumor cells. This pivotal function exerted by Th1 cells in the immune system can sometimes engender an overactivation with "misdirected" attacks against particular "autologous" tissues. This feature makes Th1 cells central players in autoimmune diseases and also in particular life phases, such as pregnancy, where foreign cells develop. On the other hand, Th2 cells, involved in humoral immunity, stimulate strong antibody responses and eosinophil

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

accumulation, and inhibit various functions of phagocytic cells (phagocyte-independent inflammation) [131]. IL-1β, a pro-inflammatory cytokine released by monocytes, macrophages, and epithelial cells (but not by Th cells), is also deeply involved in pregnancy, exerting a dual effect, either negative or positive: one has an abortogenic outcome, while the other effect increases the likelihood of reaching the gestational term, thus protecting from miscarriage and pre-term delivery [132]. When a fatal pregnancy outcome occurs, the prevalence of cytokines secreted by Th1 cells [133-135] is not the only marker, because Th2-type cytokines levels, i.e., the anti-inflammatory IL-4 and the pro-inflammatory IL-6, are also elevated [136] and recurrent abortions can be observed with Th2-dominant immunity [137-138]. Serum IL-6 concentrations increase significantly in women with recurrent miscarriage, compared to controls [139]. Instead, IL-4 is an anti-inflammatory cytokine, as also IL-10, and, normally, both molecules are involved in preventing miscarriage. It was seen that the decrease of their physiological levels, during pregnancy, promote persistent inflammation and facilitate miscarriage [140]. However, it is noteworthy that IL-4-knockout mice show normal pregnancies [141]. Many other factors, such as enzymes, adhesion molecules, pro-inflammatory cytokines and other endogen mediators, are involved in favouring miscarriage too, as shown by clinical studies and animal research; activated COX2 [142] and iNOS [143], as well as high levels of ICAM-1 [144], VCAM-1 [144], IL-8 [145], IFNγ [146], PGE2 [142] and NO [143]) are detrimental, though some of them can have conflicting behaviours in different contexts.

7. A new explanatory paradigm

Saito et al. [136] proposed an inclusive and explanatory paradigm involving, Th1, Th 2, Th 17 and regulatory T (Treg) cells [147] allowing to clear out several aspects of the complex mechanisms underlying many immune diseases. In the detail, Th17 cells produce IL-17, a proinflammatory cytokine, and exert a central role in giving rise to inflammation [147-149]. It is accepted that Th 17 cells can induce pathogenetic mechanisms in autoimmunity and acute transplant rejection, also

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

affecting the development of gestation. It is worth of note that Th17 cells and the cytokine IL-17 released might exert a dual action on pregnancy. In fact, increased Th17 cells in pregnancy *decidua* might be disadvantageous for the maintenance of the physiological process [150], and conversely Th17 cells, frequency found in the deciduas, are substantially higher in comparison to their frequency in peripheral blood. Th17 cells could exert a protective immune response against microbial presence and, as well known, a bevy of microbes exist in the womb (the maternal microbiome). On the other side, Treg cells play an essential part in the development of immunoregulation and the induction of tolerance. They release the anti-inflammatory cytokines IL-10, IL-35 and TGF-beta, which, directly or indirectly, block the secretion of pro-inflammatory cytokines or their activity. As overall action, Treg cells induce immune tolerance by inhibiting the proliferation of $CD4^+$ and $CD8^+$ T cells and their synthesis of cytokines, the cytotoxic activity of natural killer (NK) cells, the immunoglobulin production by B cells, and the dendritic cells (DCs) maturation [151-152]. We have to keep in consideration that a marked increase of NK cell cytotoxicity was found indicative of subsequent miscarriage in women [153]. It is noteworthy that Treg cells can be converted to Th17 cells [154] and Th1 cells into Th17 cells [155]. Consequently, Th1/Th2/Th17 and Treg lineages are associated to each other, and, in many cases, able to convert to other lineages [134]. This plasticity excludes that a rigid separation among these cell lineages exists, and provides a favorable landscape for the intervention of fine immunomodulators. In the normal development of pregnancy, inflammation is a necessary response for a successful implantation and this evidence justifies the role of IL-1β and IL-17. However, excessive inflammation can harm embryo development and cause its resorption, a damage that can be neutralized by Treg cells, which can offset inflammation signals in the uterus during the implantation period. Of note, both excessive inflammation and immune suppression can prompt embryo resorption [136,156]. Therefore, the maintenance of a healthy gestation needs an adequate balance between Th1- and Th2-mediated immunity, more specifically an equilibrium slightly shifted toward Th2-type immunity, without any harmful overstimulation of Th1 or Th2 immunity. All these findings, along with many others, prove

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

that a good gestation cannot be identified in some single cell lineage or molecule, but consist in a complex and balanced interplay among numerous cell lineages (mainly Th1, Th2 and Treg cells) and molecules (cytokines and so on) acting in a spatiotemporal way. Furthermore, in this picture, redundancy is essential to overcome possible deficiencies. For instance, we see that various, and often overlapping, physiological activities are performed by cytokines. The most important thing is to keep an overall dynamic balance among all components, in order to compensate some deficits. This means that the best therapeutic compounds have to be able to finely modulate most of, if not all, cells and molecules that favour or prevent miscarriage.

Moreover, with reference to the subcorionic haemorrhage and the resulting hematoma, a large spectrum of different bio-active molecules, such as growth factors, cytokines and chemokines, are involved in acute wound and wound healing where an increase of TNF-α, IL-1β and IL-6 has been observed together with VEGF and also with Epidermal growth factor (EGF), Fibroblast growth factor 2 (FGF-2), Transforming growth factor beta (TGF-β), Platelet-derived growth factor (PDGF) [157]. Many of these factors induce inflammation in the acute wound. Others, like VEGF and α-SMA, help reepithelization, taking part in vasculogenesis and angiogenesis. In particular, α-SMA stimulates fibrogenesis and myofibroblast generation [158]. Finally, it is important to mention the role of Matrix metalloproteases (MMPs), which belong to the group of neutral proteases. Inflammation, extracellular matrix (ECM) degradation and new ECM remodeling are positively affected by MMPs, though a selective control of its activity is necessary to avoid the wound worsening [60]. Therefore, all these molecules, in different ways, are involved in hematoma resorption. Moreover, it has been shown that premature or aberrant activation of NF-kB impairs pregnancy [159], and this fact can be explained by the effect of NF-kB on cytokine network and other molecules. Last, but not least, recent evidences highlighted on Nrf2 pivotal role in pregnancy in protecting the fetus in presence of oxidative stress in utero. This role has been confirmed in Nrf2 knockout mice that suffer of placental oxidative stress and show decreased fetal growth. Furthermore, a deficit in Nrf2 activity may cause pre-term delivery, and the knockdown of Nrf2 in

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

amnion cells increases pro-inflammatory cytokines secretion that, in turn, contributes to fetal membrane rupture [160]. Obviously we have to remember the TNF-induced and thrombin-induced weakening of human fetal membranes [161-162].

8. ALA/DHLA putative mechanism of action and mediators in preventing miscarriage

The recent use of ALA in patients with threatened miscarriage to improve the subchorionic hematoma resorption is a promising area of researches and studies. On the sidelines of the general topic of pregnancy, we remind that ALA deficiency has been proposed to be responsible for deficits in foetus development [20-21,23], suggesting that a linkage between this phenomenon and Nrf2-Nf-kB interplay exists. The use of ALA in threatened miscarriage derives from its promising activity profile, outlined before. In the miscarriage prevention the downstream effects might concern the modulation and regulation of cell lineages and/or the synthesis/release of several molecules without any fixed unidirectional effect, within the physiological framework of the first trimester pregnancy

We can herein summarize the most important effects, pertinent with this therapeutic area. ALA suppresses the number and percentage of encephalitogenic Th1 and Th17 cells and the NK cell cytotoxicity [81-82], whereas it increases splenic Treg-cells [82], involved in fighting excessive inflammation. Moreover, ALA reduces proinflammatory cytokine levels, such as TNFα, IL-1β, IL-6, IL-8, IL-17 and INFγ [78, 79, 82, 85], while it induces anti-inflammatory IL-10 release [78]. ALA also decreases VCAM-1 and ICAM-1 [84] and MMP-9 production [60], and increases VEGF and alpha-SMA [86]. A reduction in prostaglandin E2 (PGE2) and nitric oxide (NO) levels was detected also, due to cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) inhibition by ALA [78]. Moreover, this molecule impedes TNF-induced and thrombin-induced weakening of human fetal membranes [87-88]. All these activities can strongly contribute to counteract many alterations, as above, involved in inducing miscarriage (Table 1).

9. ALA by oral route in threatened miscarriage

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Porcaro et al. [114] carried out a randomized controlled clinical trial in pregnant women with threatened miscarriage. The study was aimed to test the contribution of ALA supplementation (600 mg by oral route) in improving the standard treatment with progesterone vaginal suppositories, in healing subchorionic hematomas and also in reducing subjective and objective signs such as vaginal bleeding, abdominal pain, and uterine contractions. Controls received only vaginal suppositories containing progesterone. This compound is routinely administered to ward off threatened miscarriage in pregnancy, according to its ability in reducing pregnancy loss in this kind of patients, but the real extent of its efficacy is strongly debated [163-166]. All treatments were administered until complete resolution of the clinical picture. Both sets of patients improved, but those treated with progesterone plus ALA had a better and faster evolution during the first twenty weeks of gestation. The monitoring of the main signs of threatened abortion (chorioamniotic separation and uterine hematoma, vaginal bleeding, abdominal pain, and uterine contractions) clearly indicated that all symptoms decreased or disappeared in the group treated with ALA plus progesterone, faster than in the group treated with progesterone alone. The clinical evolution of uterine wound healing and hematoma resorption clearly indicated that patients treated with ALA had a better improvement compared to the progesterone group.

Although preliminary, these results can provide the background for a promising therapeutic activity of ALA in preventing miscarriage. In fact, as mentioned above, pregnancy is characterized by a cross talk among different immune cells, communicating with each other by means of strictly related messenger molecules in a balanced but fragile network. ALA is likely to act as an accurate and selective regulator in this network, able to re-establish the lost equilibrium, modulating the levels of various molecules involved in the biochemical mechanisms, underlying such pathological states of pregnant women. Therefore, in a situation such as threatened abortion, ALA might play a pivotal role in significantly improving the health conditions of both mother and foetus.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

10. Vaginal administration of ALA and hematoma resorption

The results shown in the previous study prompted to develop the use of ALA in the same therapeutic area, testing the vaginal route so far not treated with ALA in a new formulation. This kind of deliver of active molecules has the advantage to bypass some problems related to the systemic absorption of ALA. A recent Randomized Controlled Trial pilot study [115], carried out at the Women's Health Center, Azienda USL Ferrara (Italy), compared the therapeutic efficacy of ALA vs progesterone, by vaginal route, on subchorionic hematoma resorption in women at the first trimester with threatened miscarriage, also evaluating the effects of the treatment on pelvic pain and vaginal bleeding. The patients suffered of pelvic pain with or without moderate vaginal bleeding. In all subjects the presence of subchorionic hematoma was diagnosed. One group of patients received 400 mg of vaginal Progesterone, and a second one (case study) vaginal ALA. A third group was formed by twenty-two women receiving no treatment according to their personal choice. All treatments were administered until the total resolution of the clinical picture. The resorption of subchorionic hematoma related to threatened miscarriage was chosen as primary outcome; the secondary outcomes were reduction/disappearance of vaginal bleeding and pelvic pain symptoms. Both groups of patients were assessed for the incidence of miscarriage. The therapy was considered effective when pregnancy went over 20 weeks. In this study, the subchorionic hematoma had a statistically significant quicker resorption in women treated with ALA, than in those ones treated with progesterone or in controls. These findings were in agreement with the study mentioned above based on the oral administration of ALA plus progesterone by vaginal route [114]. The other parameters (namely pelvic pain and vaginal bleeding) were not significantly affected by specific treatments. A smaller number of miscarriages, although not significant, was found in ALA-treated patients, compared to progesterone group. ALA administration did not exert adverse effects on foetus until the final check-up of the study, sixty days from the beginning of the treatment. Sporadic episodes of mild vaginal burning were reported by the patients but there was no need to discontinue the therapy. This first example of ALA use by vaginal route in clinical practice provides the first

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

proof supporting the efficacy and tolerability of ALA, administered by vaginal route, in hematoma resorption in patients with threatened miscarriage. We may speculate that, compared with the unidirectional immunosuppressive action of progesterone, ALA is able to finely modulate the complex of cells and molecules involved in threatened miscarriage, therefore offering new avenues to substantially reduce the complications linked to threatened abortion, and ameliorate pregnancy outcome .

11. Conclusion

ALA has been considered an expedient for a therapeutic strategy in several pathologies and its role in the field of pharmacology and dietary supplements has already gained an important space. ALA and its reduced form, DHLA, have been demonstrated to act as immunomodulatory molecules in different inflammatory diseases where a selective and fine regulation of entangled pathways needs to be set. It is noteworthy the capability of ALA/DHLA to rescue an altered physiological balance without negatively affecting other cascades of metabolic events not involved in the pathological alterations. We have shown several activities of ALA/DHLA couple tested in experimental research and clinical practice, taking into consideration many data, with particular concern to the gynecology area. ALA/DHLA couple has been reported to be able to act as a “bona fide” free radical scavenger, to chelate transition metals, to regenerate essential antioxidants and to contribute to the repair of important molecules damaged by oxidation. More recently, it has been proposed that the real protective mechanism of ALA/DHLA is mediated by the ability to trigger the activation of Nrf2 and quench NF-kB. The picture drawn in the previous pages provides a promising complex of biological properties in support of the therapeutic value of this molecule, also in gynecology, with a specific use in miscarriage prevention.

12. Expert opinion

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Data available so far indicate that the key finding of ALA/DHLA is the immunoregulatory effect exerted in several therapeutic areas. Its potential role in gynecology to ward off miscarriage in the first trimester of pregnancy also took advantage from new vaginal formulations. An obvious weakness, in the studies so far carried out, is in the small number of patients.

We have to clarify that ALA by vaginal route is a *potential* treatment for miscarriage that deserves to be investigated and evaluated carefully in the future from several point of view. A preliminary discussion has to evaluate the current framework of therapies, mainly by vaginal route, available for preventing miscarriage. For this therapeutic aim, the only drug still administered is progesterone. Other treatments, such as vitamins [167], were found completely ineffective in preventing miscarriage, even if administered prior to pregnancy. The treatment with progesterone to prevent miscarriage is approved by therapeutic protocols, but its real efficacy was strongly questioned [163-166]. It is administered orally, vaginally or intramuscularly, and it was shown that the intramuscular route is the only one able to exert a real effect. The results of a recent multicenter, double-blind, placebo-controlled, randomized trial [168] did not support some earlier studies that suggested a benefit of progesterone therapy, by vaginal route, in the first trimester among women with recurrent miscarriages. The outcomes are very clear and persuasive. The progesterone inefficacy was confirmed by another multicenter, double-blind, placebo-controlled randomised trial [169] focused on the pre-term birth prevention. After progesterone failure in reducing the miscarriage rate and in absence of other drugs, there is a "therapeutic vacuum" which might be filled by Alpha-Lipoic Acid. Many data prompt us to investigate this possibility.

Overall, numerous other steps in research are necessary, concerning not only the gynecological area, but also some intriguing points of ALA/DHLA activity.

The first challenge is to better understand how ALA/DHLA mechanism of action works to reactivate the physiological balance. In fact, we need a clear explanation of some varying effects due to different doses/concentrations of ALA, also to define the best therapeutic regimen in the most accurate way. These crucial parameters should be carefully evaluated since, at least in a very

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

small number of experimental models, high amounts of ALA have been observed to reverse the expected anti-oxidant effect to pro-oxidant. According to the prevailing thought in the sector, this phenomenon means a change from anti-inflammatory to pro-inflammatory effect. For instance, it has been observed that in the heart muscle tissue of aged rats, ALA (100 mg/kg body wt/day), administered i.p. for 14 days, raised protein oxidation parameters such as protein carbonyl (PCO), nitrotyrosine (NT), advanced oxidation protein products (AOPP), and protein thiol (P-SH) levels, whereas oxidative stress parameters such as total thiol (T-SH) and non-protein thiol (Np-SH) levels were not modified. In the same study only lipid hydroperoxide (LHP) levels were reduced [170]. The increasing concentrations of protein oxidation markers such as PCO, NT, and AOPP in ALA-supplemented rats compared to controls prompted the authors to suggest that ALA stimulates *in vivo* an oxidative protein damage. Therefore, it seems that in this specific situation ALA exerts a pro-oxidant effect. It can be assumed that ALA may exert both pro- and antioxidant effects, depending on the underlying physiological and metabolic state. This is surely an intriguing issue which deserves an in-depth investigation, by testing different doses in different experimental models. In relation to this matter a review [38] and an experimental research [39] have given new supports to outline a framework which can connect a slight pro-oxidant effect, initially exerted by ALA, with its final overall effect, which remains antioxidant. In their review, Linnane and Eastwood [38] speculated that antioxidants, such as ALA, could give rise to a "hormetic" phenomenon, with different effects at the dose variation, where Nrf2 and NF-κB pathways are probably involved [171]. On the other hand, Ursini and Forman suggested that the effect of antioxidants is due to a para-hormetic mechanism [39, 172]. This interesting hypothesis looks more realistic than the one made by Linnane and Eastwood, and would deserve to be tested also for ALA/DHLA. There are differences between hormesis and para-hormesis. Both mechanisms start with a slight pro-oxidant effect, but with some dissimilarities mainly regarding the inducer molecule. Hormesis constitutes an adaptive mechanism in response to minimal exposure to agents producing a well-defined damage at higher exposure. In this case the permanent activation of

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

different mechanisms, including repair, are involved and a new homeostatic condition, which means a new phenotype, arises and becomes stable. Therefore, in hormesis a dangerous agent induces the change. It reminds us of the behaviour of Mithridates VI, the king of Pontus and the ideas of Paracelsus. The first one was suspicious to be poisoned to death, and, trying to save himself, he regularly ingested small doses of venom to become resistant to its lethal effects. Paraclesus, wrote that "All things are poison and nothing is without poison, only the dose makes something not a poison". Only at the correct dose, it can be also a precious and safe remedy. Instead, para-hormesis is the mechanism by which exogenous agents (nutritional antioxidants) help maintain redox homeostasis, essential for a physiological steady state. Antioxidants, by activating Nrf2, mimic the effect of endogenously produced "oxidants", exerting a mild stimulation which protects from a second more powerful challenge. The final effect is "antioxidant". Unlike hormesis, in para-hormesis the inducer molecule is not harmful at a wide range of doses, and obviously this is the case of ALA. We are in front of a new paradigm that clearly has to be verified in-depth. Therefore new research and studies on ALA are needed to provide a robust conceptual background and to draw proper consequences for its therapeutic application, with potential implications in gynecology, and in particular as a preventive treatment for miscarriage.

It is also necessary to carefully investigate on the pharmacokinetic of ALA given by vaginal route, in consideration of its distribution in some tissues, detected by histological analysis, and to develop drug safety studies during pregnancy as well as the dose related effect. Pregnancy follow-up studies should be carried out for novel products used in pregnant women, in order to understand the side effects, if any, in embryos and fetus. Moreover, the promising results found when ALA is given by vaginal route, have to be confirmed in a much larger sample of patients by means of Randomized Controlled Trials.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Declaration of interest

V Unfer and S De Grazie are employees at LO.LI.Pharma, Rome, Italy. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Bibliography

Papers of special note have been highlighted as either of interest (●) or of considerable interest (●●) to readers.

1. Snell EE, Strong FM, Peterson WH. Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. Biochem J 1937;31(10):1789-99.
2. Reed LJ, DeBusk BG, Gunsalus, IC, Hornberger CS Jr. Crystalline alpha-lipoic acid: a catalytic agent associated with pyruvate dehydrogenase. Science 1951;114(2952):93-4.
3. Kilic F, Handelman GJ, Traber K, et al. Modelling cortical cataractogenesis XX. *In vitro* effect of alpha-lipoic acid on glutathione concentrations in lens in model diabetic cataractogenesis. Biochem Mol Biol Int 1998;46(3):585-95
4. Hagen TM, Vinarsky V, Wehr CM, Ames BN. (R)-alpha-lipoic acid reverses the age-associated increase in susceptibility of hepatocytes to tert-butylhydroperoxide both *in vitro* and in vivo. Antioxid Redox Signal 2000;2(3):473–83.
5. Streeper RS, Henriksen EJ, Jacob S, et al. Differential effects of lipoic acid stereoisomers on glucose metabolism in insulin-resistant skeletal muscle. Am J Physiol 1997;273(1 Pt 1):E185-91.
6. Wolz P, Krieglstein J. Neuroprotective effects of alpha-lipoic acid and its enantiomers demonstrated in rodent models of focal cerebral ischemia. Neuropharmacology 1996;35(3):369-75.
7. Wada H, Shintani D, Ohlrogge J. Why do mitochondria synthesize fatty acids? Evidence for involvement in lipoic acid production Proc Natl Acad Sci U S A 1997;94(4):1591-6.
8. Lodge JK, Packer L. Natural Sources of Lipoic Acid in Plant and Animal Tissues. in: Packer, Hiramatsu, Yoshikawa eds. Antioxidant Food Supplements in Human Health. Academic Press 1999

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

9. Bustamante J, Lodge JK, Marcocci L, et al. Alpha-lipoic acid in liver metabolism and disease. Free Radic Biol Med 1998;24(6):1023-39.
10. Mayr JA, Feichtinger RG, Tort F, et al. Lipoic acid biosynthesis defects. J Inherit Metab Dis 2014;37:553-63.
11. Biewenga G, Haenen G, Bast A. The pharmacology of the antioxidant lipoic acid. Gen Pharmacol 1997;29:315–31.
12. Packer L, Cadenas E. Lipoic acid: energy metabolism and redox regulation of transcription and cell signaling. J Clin Biochem Nutr 2011;48(1):26-32.
13. Teichert J. Preiss R. HPLC-methods for determination of lipoic acid and its reduced form in human plasma. Int J Clin Pharmacol Ther Toxicol. 1992;30(11):511-2.
14. Goraca A, Huk-Kolega H, Piechota A, et al. Lipoic acid – biological activity and therapeutic potential. Pharmacol Rep. 2011;63(4):849-58.
15. Teichert J, Hermann R, Ruus P, Preiss R. Plasma kinetics, metabolism, and urinary excretion of alpha-lipoic acid following oral administration in healthy volunteers. J Clin Pharmacol 2003; 43(11):1257-67.
16. Teichert J, Tuemmers T, Achenbach H, et al. Pharmacokinetics of alpha-lipoic acid in subjects with severe kidney damage and end-stage renal disease. J Clin Pharmacol 2005;45(3):313-28.
17. Locher M, Busker E, Borbe HO. Metabolism of alpha-lipoic acid in human volunteers. Naunyn-Schmiedeberg's Arch Pharmacol 1995;351:R52.
18. Cremer DR, Rabeler R, Roberts A, Lynch B. Long-term safety of alpha-lipoic acid (ALA) consumption: A 2-year study. Regul Toxicol Pharmacol 2006;46(3):193-201.
19. Cremer DR, Rabeler R, Roberts A, Lynch B. Safety evaluation of alpha-lipoic acid (ALA). Regul Toxicol Pharmacol 2006;46(1):29-41.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

20. Al Ghafli MH, Padmanabhan R, Kataya HH, Berg B. Effects of alpha-lipoic acid supplementation on maternal diabetes-induced growth retardation and congenital anomalies in rat fetuses. Mol Cell Biochem 2004; 261(1-2):123-35.
21. Padmanabhan R, Mohamed S, Singh S., Beneficial effect of supplemental lipoic acid on diabetes-induced pregnancy loss in the mouse. Ann N Y Acad Sci 2006;1084:118-31.
22. Koga T, Ishida T, Takeda T, et al. Restoration of dioxin-induced damage to fetal steroidogenesis and gonadotropin formation by maternal co-treatment with α-lipoic acid. PLoS One 2012; 7(7):e40322.
23. Antonio AM, Gillespie RA, Druse-Manteuffel MJ. Effects of lipoic acid on antiapoptotic genes in control and ethanol-treated fetal rhombencephalic neurons. Brain Res 2011;1383:13-21.
24. Ziegler D, Hanefeld M, Ruhnau KJ, et al. Treatment of symptomatic diabetic peripheral neuropathy with the anti-oxidant alpha-lipoic acid. A 3-week multicentre randomized controlled trial (ALADIN Study). Diabetologia 1995;38(12):1425-33.
25. Ziegler D, Hanefeld M, Ruhnau KJ, et al. Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: a 7-month multicenter randomized controlled trial (ALADIN III study). Diabetes Care 1999;22(8):1296-301.
26. Costantino M, Guaraldi C, Costantino D, De Grazia S, et al. Peripheral neuropathy in obstetrics: efficacy and safety of α-lipoic acid supplementation. Eur Rev Med Pharmacol Sci 2014;18(18):2766-71.
27. Costantino D, Guaraldi C, Costantino M, Bounous VE. Use of alpha-lipoic acid and omega-3 in postpartum pain treatment. Minerva Ginecol 2015;67(5):465-73.
28. Rahimifard M, Navaei-Nigjeh M, Baeeri M, et al. Multiple protective mechanisms of alpha-lipoic acid in oxidation, apoptosis and inflammation against hydrogen peroxide induced toxicity in human lymphocytes. Mol Cell Biochem 2015;403(1-2):179-86.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

29. Moura FA, de Andrade KQ, dos Santos JC, Goulart MO. Lipoic Acid: its antioxidant and anti-inflammatory role and clinical applications. Curr Top Med Chem 2015;15(5):458-83.

30. Patel MS, Roche TE; Harris RA. Alpha-keto acid dehydrogenase complexes. In: Azzi, Packer eds. Molecular and cell biology updates. Berlin: Birkhauser Verlag 1996.

31. Shay KP, Moreau RF, Smith EJ, et al. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. Biochim Biophys Acta 2009;1790(10):1149-60.

32. Suh JH, Shenvi SV, Dixon BM. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci USA 2004;101(10):3381-6.

33. Bast A, Haenen GR. Lipoic acid: a multifunctional antioxidant. Biofactors 2003;17(1-4):207-13.

34. Kozlov AV, Gille L, Staniek K, Nohl H. Dihydrolipoic acid maintains ubiquinone in the antioxidant active form by two-electron reduction of ubiquinone and one-electron reduction of ubisemiquinone. Arch Biochem Biophys 1999;363(1):148-54.

35. Ou P, Tritschler HJ, Wolf SP. Thioctic (lipoic) acid: a therapeutic metal-chelating antioxidant? Biochem Pharmacol 1995;50(1):123-6.

36. Suh JH, Moreau R, Heath SH, Hagen TM. Dietary supplementation with (R)-alpha-lipoic acid reverses the age-related accumulation of iron and depletion of antioxidants in the rat cerebral cortex. Redox Rep 2005;10(1):52-60.

37. Spector A, Huang R-R C, Yah G-Z, Wang R-R. Thioredoxin fragment 31-36 is reduced by dihydrolipoamide and reduces oxidized protein. Biochem. Biophys. Res. Commun 1988;150(1):156-62

38. Linnane AW, Eastwood H. Cellular redox regulation and prooxidant signaling systems: a new perspective on the free radical theory of aging. Ann N Y Acad Sci 2006;1067:47-55.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

39. Forman HJ, Davies KJ, Ursini F. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. Free Radic Biol Med 2014;66: 24–35.

● **It provides an alternative view on the real mechanism of action used by antioxidants**.

40. Atia A, Abdullah A. Review of NRF2-regulated genes induced in response to antioxidants. Int J Med Res Health Sci 2014;3:428-35.

41. Ma Q. Role of Nrf2 in Oxidative Stress and Toxicity. Annu Rev Pharmacol Toxicol 2013;53:401-26.

42. Johnson JA, Johnson DA, Kraft AD, et al. The Nrf2-ARE Pathway: An Indicator and Modulator of Oxidative Stress in Neurodegeneration. Ann N Y Acad Sci 2008;1147:61–9.

43. Kawasaki Y, Ishigami S, Arigami T, et al. Clinicopathological significance of nuclear factor (erythroid-2)-related factor 2 (Nrf2) expression in gastric cancer. BMC Cancer 2015;15:5.

44. Lee JM, Li J, Johnson DA, et al. Nrf2, a multi-organ protector? Faseb J 2005;19(9):1061–66.

45. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 2007;47:89–116.

46. Hybertson BM, Gao B, Bose SK, McCord JM. Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. Mol Aspects Med 2011;32(4-6):234-46.

47. Gao B, Doan A, Hybertson BM. The clinical potential of influencing Nrf2 signaling in degenerative and immunological disorders. Clin Pharmacol 2014:6:19–34.

48. Lii CK, Liu KL, Cheng YP, et al. Sulforaphane and α-lipoic acid upregulate the expression of the π class of glutathione S-transferase through c-jun and Nrf2 activation. J Nutr 2010;140:885-92.

49. Kim YS, Podder B, Song HY. Cytoprotective effect of alpha-lipoic acid on paraquat-exposed human bronchial epithelial cells via activation of nuclear factor erythroid related factor-2 pathway. Biol Pharm Bull 2013;36(5):802-11.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

50. Deng C, Sun Z, Tong G, et al. α-Lipoic acid reduces infarct size and preserves cardiac function in rat myocardial ischemia/reperfusion injury through activation of PI3K/Akt/Nrf2 pathway PLoS One. 2013;8(3):e58371. doi: 10.1371/journal.pone.0058371. Epub 2013 Mar 7.

51. Koriyama Y, Nakayama Y, Matsugo S, Kato S. Protective effect of lipoic acid against oxidative stress is mediated by Keap1/Nrf2-dependent heme oxygenase-1 induction in the RGC-5 cell line. Brain Res 2013;1499:145-57.

52. Huerta-Olvera SG, Macías-Barragán J, Ramos-Márquez ME, et al. Alpha-lipoic acid regulates heme oxygenase gene expression and nuclear Nrf2 activation as a mechanism of protection against arsenic exposure in HepG2 cells. Environ Toxicol Pharmacol 2010;29(2):144-9.

53. Malinin NL, Boldin MP, Kovalenko AV, Wallach D. MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature 1997;385(6616):540-4.

54. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol 2009;1(6):a001651. doi: 10.1101/cshperspect.a001651.

55. Hayden MS, Ghosh S. NF-kappaB in immunobiology. Cell Res 2011;21(2):223-44.

56. Hayden MS, Ghosh S. Regulation of NF-κB by TNF family cytokines. Semin Immunol 2014;26(3):253-66.

57. Gilmore TD, Wolenski FS. NF-kappaB: where did it come from and why? Immunol Rev 2012;246(1):14-35.

58. Tusi SK, Ansari N, Amini M, et al. Attenuation of NF-kappaB and activation of Nrf2 signaling by 1,2,4-triazine derivatives, protects neuron-like PC12 cells against apoptosis. Apoptosis 2010;15(6):738-51.

59. Ying Z, Kampfrath T, Sun Q, et al. Evidence that α-lipoic acid inhibits NF-κB activation independent of its antioxidant function. Inflamm Res 2011;60(3):219-25.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

60. Kim HS, Kim HJ, Park KG, et al. Alpha-Lipoic acid inhibits matrix metalloproteinase-9 expression by inhibiting NF-kB transcriptional activity. Exp Mol Med 2007;39(1):106-13.

61. Ma X, He P, Sun P, Han P. Lipoic acid: an immunomodulator that attenuates glycinin-induced anaphylactic reactions in a rat model. J Agric Food Chem 2010;58(8):5086-92.

62. Cho YS, Lee J, Lee TH, et al. Alpha-Lipoic acid inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. J Allergy Clin Immunol 2004;114(2): 429-35.

63. Lee CK, Lee EY, Kim YG, et al. Alpha-lipoic acid inhibits TNF-alpha induced NF-kappa B activation through blocking of MEKK1-MKK4-IKK signaling cascades. Int Immunopharmacol 2008;8(2):362-70.

64. Lam PKL. Alpha-Lipoic acid: A novel therapeutic agent for intervention of early pediatric allergies. J. Allergy Clin. Immunol 2005;115(2) Suppl. S162.

65. Hah YS, Sung MJ, Lim HS, et al. Dietary alpha lipoic acid supplementation prevents synovial inflammation and bone destruction in collagen-induced arthritic mice. Rheumatol Int. 2011;31(12):1583-90.

66. Zhang WJ, Wei H, Hagen T, Frei B. Alpha-lipoic acid attenuates LPS-induced inflammatory responses by activating the phosphoinositide 3-kinase/Akt signaling pathway. Proc Natl Acad Sci USA 2007;104(10):4077–82.

67. Zhang WJ, Frei B. Alpha-lipoic acid inhibits TNF-alpha-induced NF-kappaB activation and adhesion molecule expression in human aortic endothelial cells. FASEB J 2001;15(13):2423–32.

68. Ide T, Effect of dietary a-lipoic acid on the mRNA expression of genes involved in drug metabolism and antioxidation system in rat liver. British Journal of Nutrition. 2014; 112: 295–308.

69. Choi SY, Yu JH, Kim H. Mechanism of alpha-lipoic acid-induced apoptosis of lung cancer cells. Ann N Y Acad Sci. 2009 Aug;1171:149-55.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

70. Shi DY, Liu HL, Stern JS, Yu PZ, Liu SL. Alpha-lipoic acid induces apoptosis in hepatoma cells via PTEN/Akt pathway. Febs lett. 2008; 582(12):1667-71.

71. Kafara P, Icard P, Guillamin M, Schwartz L, Lincet H. Lipoic Acid decreases Mcl-1, Bcl-xL and up regulates Bim on ovarian carcinoma cells leading to cell death. J Ov Res. J Ovarian Res. 2015;8:36. doi: 10.1186/s13048-015-0165-z.

72. Van de Mark K, Chen JS, Steliou K, et al. Alpha-lipoic acid induces p27Kip-dependent cell cycle arrest in non-transformed cell lines and apoptosis in tumor cell lines. J Cell Physiol 2003;194(3):325-40.

73. Selvakumar E, Hsieh TC. Regulation of cell cycle transition and induction of apoptosis in HL-60 leukemia cells by lipoic acid: role in cancer prevention and therapy. J Hematol Oncol. 2008;1:4. doi: 10.1186/1756-8722-1-4.

74. . Dozio E, Ruscica M, Passafaro L, et al. The natural antioxidant alpha-lipoic acid induces p27Kip1 –dependent cell cycle arrest and apoptosis in MCF-7 human breast cancer cells. Eur J Pharmacol 2010;641(1):29-34.

75. Pack RA, Hardy K, Madigan MC, Hunt NH. Differential effects of the antioxidant alpha-lipoic acid on the proliferation of mitogen-stimulated peripheral blood lymphocytes and leukaemic T cells. Mol Immunol 2002;38(10):733-45.

76. Wenzel U, Nickel A, Daniel H. Alpha-Lipoic acid induces apoptosis in human colon cancer cells by increasing mitochondrial respiration with a concomitant O^{2-*-}generation. Apoptosis 2005;10(2):359-68.

77. Arivazhagan P, Ramanathan K, Panneerselvam C. Effect of DL-alpha-lipoic acid on mitochondrial enzymes in aged rats. Chem Biol Interact 2001;138(2):189-98.

78. Li G, Fu J, Zhao Y, et al. Alpha-Lipoic Acid Exerts Anti-Inflammatory Effects on Lipopolysaccharide-Stimulated Rat Mesangial Cells via Inhibition of Nuclear Factor Kappa B (NF-κB) Signaling Pathway. Inflammation 2015;38(2):510-9.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

79. Choi JH, Cho SO, Kim H. α-Lipoic Acid Inhibits Expression of IL-8 by Suppressing Activation of MAPK, Jak/Stat, and NF-κB in H. pylori-Infected Gastric Epithelial AGS Cells. Yonsei Med J. 2016 Jan;57(1):260-4.

80. Tanaka Y, Kaibori M, Miki H, et al. Alpha-lipoic acid exerts a liver-protective effect in acute liver injury rats, J Surg Res 2015;193(2):675-83.

81. Wang KC, Tsai CP, Lee CL, et al. Alpha-Lipoic acid enhances endogenous peroxisome-proliferator-activated receptor-gamma to ameliorate experimental autoimmune encephalomyelitis in mice, Clin Sci (Lond) 2013;125(7):329-40.

82. Salinthone S, Schillace RV, Marracci GH, et al. Lipoic acid stimulates cAMP production via the EP2 and EP4 prostanoid receptors and inhibits IFN gamma synthesis and cellular cytotoxicity in NK cells. J Neuroimmunol 2008;199(1-2):46-55.

83. Marracci GH, Marquardt WE, Strehlow A, et al. Lipoic acid downmodulates CD4 from human T lymphocytes by dissociation of p56(Lck). Biochem Biophys Res Commun 2006;344(3):963-71.

84. Chaudhary P, Marracci GH, Bourdette DN. Lipoic acid inhibits expression of ICAM-1 and VCAM-1 by CNS endothelial cells and T cell migration into the spinal cord in experimental autoimmune encephalomyelitis. J Neuroimmunol 2006; 175(1-2):87-96.

85. Salinthone S, Yadav V, Schillace RV, et al. Lipoic Acid Attenuates Inflammation via cAMP and Protein Kinase A Signaling. PLoS One 2010; 5(9), e13058.

86. Micili SC, Goker A, Sayin O, et al. The effect of lipoic acid on wound healing in a full thickness uterine injury model in rats, J Mol Histol 2013;44(3):339-45.

87. Moore RM, Novak JB, Kumar D, et al. Alpha-lipoic acid inhibits tumor necrosis factor-induced remodeling and weakening of human fetal membranes. Biol Reprod 2009;80(4):781-7.

88. Moore RM, Schatz F, Kumar D, et al. Alpha-lipoic acid inhibits thrombin-induced fetal membrane weakening *in vitro*. Placenta 2010;31(10):886-92.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

89. Pashaj A, Xia M, Moreau R. α-Lipoic acid as a triglyceride-lowering nutraceutical. Can J Physiol Pharmacol. 2015;93(12):1029-41.

90. Estrada DE, Ewart HS, Tsakiridis T,et al. Stimulation of glucose uptake by the natural coenzyme alpha-lipoic acid/thioctic acid: participation of elements of the insulin signaling pathway. Diabetes 1996;45(12):1798-804.

91. Novotny L, Rauko P, Cojocel C. Alpha-Lipoic acid: the potential for use in cancer therapy. Neoplasma 2008;55(2):81-6.

92. Mérida S, Sancho-Tello M, Muriach M, et al. Lipoic acid lessens Th1-mediated inflammation in lipopolysaccharide-induced uveitis reducing selectively Th1 lymphocytes-related cytokines release. Free Radic Res 2013;47(8):593-601.

93. Petronilho F, Florentino D, Danielski LG, et al. Alpha-Lipoic Acid Attenuates Oxidative Damage in Organs After Sepsis. Inflammation. 2016;39(1):357-65.

94. Shen HH, Lam KK, Cheng PY, et al. Alpha-lipoic acid prevents endotoxic shock and multiple organ dysfunction syndrome induced by endotoxemia in rats. Shock 2015;43(4):405-11.

95. Pessler-Cohen D, Pekala PH, Kovsan J, et al. GLUT4 repression in response to oxidative stress is associated with reciprocal alterations in C/EBP alpha and delta isoforms in 3T3-L1 adipocytes. Arch Physiol Biochem 2006;112(1):3-12.

96. Eason RC, Archer HE, Akhtar S, Bailey CJ. Lipoic acid increases glucose uptake by skeletal muscles of obese-diabetic ob/ob mice. Diabetes Obes Metab 2002;4(1):29-35.

97. Ghibu S, Richard C, Vergely C, et al. Antioxidant properties of an endogenous thiol: Alpha-lipoic acid, useful in the prevention of cardiovascular diseases. J Cardiovasc Pharmacol 2009;54(5):391-8.

98. Bitar MS, Ayed AK, Abdel-Halim SM, et al. Inflammation and apoptosis in aortic tissues of aged type II diabetes: amelioration with alpha-lipoic acid through phosphatidylinositol 3-kinase/Akt- dependent mechanism. Life Sci 2010;86(23-24):844-53.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

99. Diesel B, Kulhanek-Heinze S, Höltje M, et al. Alpha-Lipoic Acid as a Directly Binding Activator of the Insulin Receptor: Protection from Hepatocyte Apoptosis, Biochemistry 2007;46(8):2146-55.

100. Marracci GH, Jones RE, McKeon GP, Bourdette DN. Alpha lipoic acid inhibits T cell migration into the spinal cord and suppresses and treats experimental autoimmune encephalomyelitis. J Neuroimmunol 2002;131(1-2):104-14.

101. Yadav V, Marracci G, Lovera J, et al. Lipoic acid in multiple sclerosis: a pilot study. Mult Scler 2005;11(2):159-65.

102. Tan EC, Bahrami S, Kozlov AV, et al. The oxidative response in the chronic constriction injury model of neuropathic pain. J Surg Res 2009;152(1):84-8.

103. Ziegler D, Ametov A, Barinov A, et al. Oral treatment with alpha lipoic acid improves symptomatic diabetic polyneuropathy: the SYDNEY 2 trial. Diabetes Care 2006;29(11):2365-70.

104. Di Geronimo G, Caccese AF, Caruso L, et al. Treatment of carpal tunnel syndrome with alpha-lipoic acid. Eur Rev Med Pharmacol Sci 2009;13(2):133-9.

105. Bertolotto F, Massone A. Combination of alpha lipoic acid and superoxide dismutase leads to physiological and symptomatic improvements in diabetic neuropathy. Drugs R D 2012;12(1):29-34.

106. Cao X, Chen A, Yang P, et al. Alpha-lipoic acid protects cardiomyocytes against hypoxia/reoxygenation injury by inhibiting autophagy. Biochem Biophys Res Commun 2013;441:935-40

107. Ambrosi N, Arrosagaray V, Guerrieri D, et al. α-Lipoic Acid Protects Against Ischemia-Reperfusion Injury in Simultaneous Kidney-Pancreas Transplantation. Transplantation. 2015 Oct 23. [Epub ahead of print]

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

108. Dong Y, Wang H, Chen Z. Alpha-Lipoic Acid Attenuates Cerebral Ischemia and Reperfusion Injury via Insulin Receptor and PI3K/Akt-Dependent Inhibition of NADPH Oxidase. Int J Endocrinol 2015;2015:903186. doi: 10.1155/2015/903186. Epub 2015 Jul 29.

109. Ekinci M, Cagatay HH, Ceylan E, et al. Reduction of conjunctival fibrosis after trabeculectomy using topical α-lipoic acid in rabbit eyes. J Glaucoma 2014;23(6):372-9.

110. Arivazhagan P, Panneerselvam SR, Panneerselvam C. Effect of DL-alpha-lipoic acid on the status of lipid peroxidation and lipids in aged rats. J Gerontol A Biol Sci Med Sci 2003;58(9):B788-91.

111. Liu J. The Effects and Mechanisms of Mitochondrial Nutrient alpha-Lipoic Acid on Improving Age-Associated Mitochondrial and Cognitive Dysfunction: An Overview. Neurochem Res 2008;33(1):194-203.

112. Head E, Nukala VN, Fenoglio KA, et al. Effects of age, dietary and behavioral enrichment on brain mitochondria in a canine model of human aging. Exp Neurol 2009;220(1):171-6.

113. Zhao L, Liu Z, Jia H, et al. Lipoamide Acts as an Indirect Antioxidant by Simultaneously Stimulating Mitochondrial Biogenesis and Phase II Antioxidant Enzyme Systems in ARPE-19 Cells. PLoS ONE 2015;10(6): e0128502. doi:10.1371.

114. Porcaro G, Brillo E, Giardina I, Di Iorio R. Alpha Lipoic Acid (ALA) effects on subchorionic hematoma: preliminary clinical results. Eur Rev Med Pharmacol Sci 2015;19:3426-32.

● **This trial provides the first demonstration that ALA oral supplementation can significantly contributes to speed up the process of healing in cases of threatened miscarriage**.

115. Costantino M, Guaraldi C, Costantino D. Resolution of subchorionic hematoma and symptoms of threatened miscarriage using vaginal Alpha Lipoic Acid or Progesterone: clinical evidences. Eur Rev Med Pharmacol Sci 2016;.20(8):1656-63.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

● **This trial provides the first demonstration that ALA administered by vaginal route can significantly contributes to speed up the process of healing in cases of threatened miscarriage.**

116. Paradisi R, Maldini-Casadei M, Boni P, et al. T-helper 2-cytokine levels in women with threatened abortion. Eur J Obstet Gynecol Reprod Biol 2003;111(1):43-9.

117. Kliman HJ, Milano KM. The majority of miscarriages are caused by genetic abnormalities. Fertil Steril 2013;100:S306.

118. Kwak-Kim J, Yang KM, Gilman-Sachs A. Recurrent pregnancy loss: a disease of inflammation and coagulation. J Obstet Gynaecol Res 2009;35:609-22.

119. Christiansen OB, Nielsen HS, Kolte AM. Inflammation and miscarriage. Semin Fetal Neonatal Med 2006;11(5):302-8.

120. Yassaee F, Shekarriz-Foumani R, Afsari S, Fallahian M. The Effect of Progesterone Suppositories on Threatened Abortion: A Randomized Clinical Trial. J Reprod Infertil 2014;15(3):147-51.

121. Nagy S, Bush M, Stone J, et al. Clinical significance of subchorionic and retroplacental hematomas detected in the first trimester of pregnancy. Obstet Gynecol 2003;102(1):94-100.

122. Leite J, Ross P, Rossi AC, Jeanty P. Prognosis of very large first-trimester hematomas. J Ultrasound Med 2006; 25(11):1441-5.

123. Sauebrei EE. Early pregnancy: pre-embrionic and embryonic periods. in: Sauebrei, Nguyen, Nolan eds. A practical guide to ultrasound in obstetrics and gynecology. Philadelphia (PA): Lippincott-Raven Publishers 1998.

124. Del Prete G, De Carli M, Almerigogna F, et al. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol 1993;150(2):353-60.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

125. Heo YJ, Joo YB, Oh HJ, et al. IL-10 suppresses Th17 cells and promotes regulatory T cells in the CD4+T cell population of rheumatoid arthritis patients. Immunol Lett 2010;127(2):150-6.

126. Kulcsar KA, Baxter VK, Greene IP, Griffin DE. Interleukin 10 modulation of pathogenic Th17 cells during fatal alphavirus encephalomyelitis. Proc Natl Acad Sci U S A. 2014;111(45):16053-8.

127. Carter LL., Dutton RW. Type 1 and Type 2: A fundamental dichotomy for all T-cell subsets. Curr Opin Immunol. 1996;8(3):336-42.

128. Desmedt M, Rottiers P, Dooms H, et al. Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. J Immunol 1998;160(11):5300-8.

129. Prescott VE, Hogan SP. Genetically modified plants and food hypersensitivity diseases: Usage and implication of experimental models for risk assessment. Pharmacol Ther 2006;111(2):374-83.

130. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 1993;14(7):353-6.

131. Romagnani S, T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol. 2000; 85(1):9-18; quiz 18, 21.

132. Vitoratos N, Papadias C, Economou E, et al. Elevated Circulating IL-1β and TNF-Alpha, and Unaltered IL-6 in First-Trimester Pregnancies Complicated by Threatened Abortion With an Adverse Outcome, Mediators Inflamm 2006; 2006(4):30485.

133. Hill JA, Polgar K, Andersomn DJ. T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. JAMA 1995;273(24):1933-6.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

134. Raghupathy R, Makhseed M, Azizieh F, et al. Maternal Th1-and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. Cellular Immunology 1999;196(2):122–130.

135. Makhseed M, Raghupathy R, El-Shazly S, et al. Pro-inflammatory maternal cytokine profile in preterm delivery. Am J Reprod Immunol. 2003;49(5):308-18.

136. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy. Am J Reprod Immunol 2010;63(6):601-10.

●● This review provides an innovative explanatory framework of pathophysiological mechanisms that can occur during pregnancy.

137. Linjawi S, Tin-Chiu L, Laird S, Blakemore A. Interleukin-1 receptor antagonist and interleukin-1 beta polymorphisms in women with recurrent miscarriage. Fertil Steril. 2005;83(5):1549-52.

138. Fortunato SJ, Menon R. IL-1 β is a better inducer of apoptosis in human fetal membranes than IL-6. Placenta 2003;24(10):922–8.

139. Prins JR, Gomez-Lopez N, Robertson SA. Interleukin-6 in pregnancy and gestational disorders. J Reprod Immunol. 2012 Sep;95(1-2):1-14.

140. Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the Anti-Inflammatory Cytokines Interleukin-4 and Interleukin-10 during Pregnancy. Front Immunol. 2014;5:253. doi: 10.3389/fimmu.2014.00253. eCollection 2014.

141. Svensson L, Arvola M, Sällström MA, Holmdahl R, Mattsson R. The Th2 cytokines IL-4 and IL-10 are not crucial for the completion of allogeneic pregnancy in mice. J Reprod Immunol. 2001 Jul;51(1):3-7.

142. Banerjee P, Jana SK, Pasricha P, Ghosh S, Chakravarty B, Chaudhury K. Proinflammatory cytokines induced altered expression of cyclooxygenase-2 gene results in unreceptive endometrium in women with idiopathic recurrent spontaneous miscarriage. Fertil Steril. 2013 Jan;99(1):179-87.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

143. Raffaelli F, Nanetti L, Vignini A, Mazzanti L, Giannubilo SR, Curzi CM, Turi A, Vitali P, Tranquilli AL. Nitric oxide platelet production in spontaneous miscarriage in the first trimester. Fertil Steril. 2010 Apr;93(6):1976-82.

144. Yurdakan G, Ekem TE, Bahadir B, Gun BD, Kuzey GM, Ozdamar SO. Expression of adhesion molecules in first trimester spontaneous abortions and their role in abortion pathogenesis. Acta Obstet Gynecol Scand. 2008;87(7):775-82.

145. Hattori Y, Nakanishi T, Ozaki Y, Nozawa K, Sato T, Sugiura-Ogasawara M. Uterine cervical inflammatory cytokines, interleukin-6 and -8, as predictors of miscarriage in recurrent cases. Am J Reprod Immunol. 2007 Oct;58(4):350-7.

146. Liu HY, Liu ZK, Chao H, Li Z, Song Z, Yang Y, Peng JP. High-dose interferon-γ promotes abortion in mice by suppressing Treg and Th17 polarization. J Interferon Cytokine Res. 2014 May;34(5):394-403.

147. Peck A, Mellins ED. Plasticity of T-cell phenotype and function: the T helper type 17 example. Immunology 2010;129(2):147-53.

148. Crome SQ, Wang AY, Levings MK. Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. Clin Exp Immunol 2010;159(2):109-19.

149. Romagnani S, Maggi E, Liotta F, et al. Properties and origin of human Th17 cells, Mol Immunol 2009;47(1):3-7.

150. Santner-Nanan B, Peek MJ, Khanam R, et al. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J Immunol 2009;183(11):7023-30.

151. Sakaguchi S. Naturally arising Foxp3-expressing CD25 + CD4 + regulatory T cells in immunological tolerance to self and non-self. Nat Immunol 2005;6(4):345-52.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

152. Akbar AN, Vukmanovic-Stejic M, Taams LS, Macallan DC. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. Nat Rev Immunol. 2007;7(3):231-7.

153. Emmer PM, Nelen WL, Steegers EA, Hendriks JC, Veerhoek M, Joosten I. Peripheral natural killer cytotoxicity and CD56(pos)CD16(pos) cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. Hum Reprod. 2000 May;15(5):1163-9.

154. Osorio F, Leibund Gut-Landmann S, Lochner M, et al. DC activated via dectin-1 convert Treg into IL-17 producers. Eur J Immunol 2008;38(12):3274-81.

155. Liu HP, Cao AT, Feng T, et al. TGF-β converts Th1 cells into Th17 cells through stimulation of Runx1 expression. Eur J Immunol 2015;45:1010-8.

156. Mor G, Cardenas I, Abrahams V, Guller S: Inflammation and pregnancy: the role of the immune system at the implantation site. Ann N Y Acad Sci 2011;1221(1): 80–7.

157. Barrientos S, Stojadinovic O, Golinko MS, et al. Growth factors and cytokines in wound healing, Wound Repair Regen 2008;16(5):585-601.

158. Cherng S, Young J, Ma H. Alpha-smooth muscle actin (α-SMA). J Am Sci. 2008;4(4):7-9.

159. Lindström TM, Bennett PR. The role of nuclear factor kappa B in human labour. Reproduction. 2005;130(5):569-81.

160. Chapple SJ, Puszyk WM, Mann GE. Keap1-Nrf2 regulated redox signaling in utero: Priming of disease susceptibility in offspring. Free Radic Biol Med 2015;88(Pt B):212-20.

●● It highlights the key role exerted by Nrf2 in protecting the fetus in presence of oxidative stress in utero.

161. Kumar D, Fung W, Moore RM, et al. Proinflammatory cytokines found in amniotic fluid induce collagen remodeling, apoptosis, and biophysical weakening of cultured human fetal membranes. Biol Reprod. 2006;74(1):29-34.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

162. Puthiyachirakkal M, Lemerand K, Kumar D, et al. Thrombin weakens the amnion extracellular matrix (ECM) directly rather than through protease activated receptors. Placenta. 2013;34(10):924-31.

163. Qureshi NS. Treatment options for threatened miscarriage. Maturitas. 2009;65 Suppl 1:S35-41.

164. Daya S. Luteal support: progestogens for pregnancy protection. Maturitas 2009;65 Suppl 1:S29-34.

165. Duan L, Yan D, Zeng W, et al. Effect of progesterone treatment due to threatened abortion in early pregnancy for obstetric and perinatal outcomes. Early Hum Dev 2010;86(1):41-3.

166. Di Renzo GC, Giardina I, Clerici G, Mattei A, Alajmi AH, Gerli S. The role of progesterone in maternal and fetal medicine. Gynecol Endocrinol. 2012;28(11):925-32.

167. Balogun OO, da Silva Lopes K, Ota E, Takemoto Y, Rumbold A, Takegata M, Mori R. Vitamin supplementation for preventing miscarriage. Cochrane Database Syst Rev. 2016, 6;5:CD004073.

168. Coomarasamy A, Williams H, Truchanowicz E, Seed PT, Small R, Quenby S, Gupta P, Dawood F, Koot YE, Bender Atik R, Bloemenkamp KW, Brady R, Briley AL, Cavallaro R, Cheong YC, Chu JJ, Eapen A, Ewies A, Hoek A, Kaaijk EM, Koks CA, Li TC, MacLean M, Mol BW, Moore J, Ross JA, Sharpe L, Stewart J, Vaithilingam N, Farquharson RG, Kilby MD, Khalaf Y, Goddijn M, Regan L, Rai R. A Randomized Trial of Progesterone in Women with Recurrent Miscarriages. N Engl J Med. 2015;373(22):2141-8.

169. Norman JE, Marlow N, Messow CM, et al. Vaginal progesterone prophylaxis for preterm birth (the OPPTIMUM study): a multicentre, randomised, double-blind trial. Lancet 2016 Feb 23. pii: S0140-6736(16)00350-0. doi: 10.1016/S0140-6736(16)00350-0. [Epub ahead of print].

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

170. Cakatay U, Kayali R, Sivas A, Tekeli F. Prooxidant activities of alpha-lipoic acid on oxidative protein damage in the aging rat heart muscle. Arch Gerontol Geriatr 2005;40(3):231-40.

171. Silvestri S, Orlando P, Armeni T, et al. Coenzyme Q10 and α-lipoic acid: antioxidant and pro-oxidant effects in plasma and peripheral blood lymphocytes of supplemented subjects. J. Clin. Biochem. Nutr 2015;57(1):21-6.

● **This study provides interesting new data on some seemingly contradictory effects of ALA**.

172. Ursini F, Maiorino M, Forman HJ. Redox homeostasis: The Golden Mean of healthy living. Redox Biol. 2016;8:205-215.

● **It provides an alternative view on the real mechanism of action used by antioxidants**.

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Table 1. ALA/DHLA putative mechanism of action in preventing miscarriage

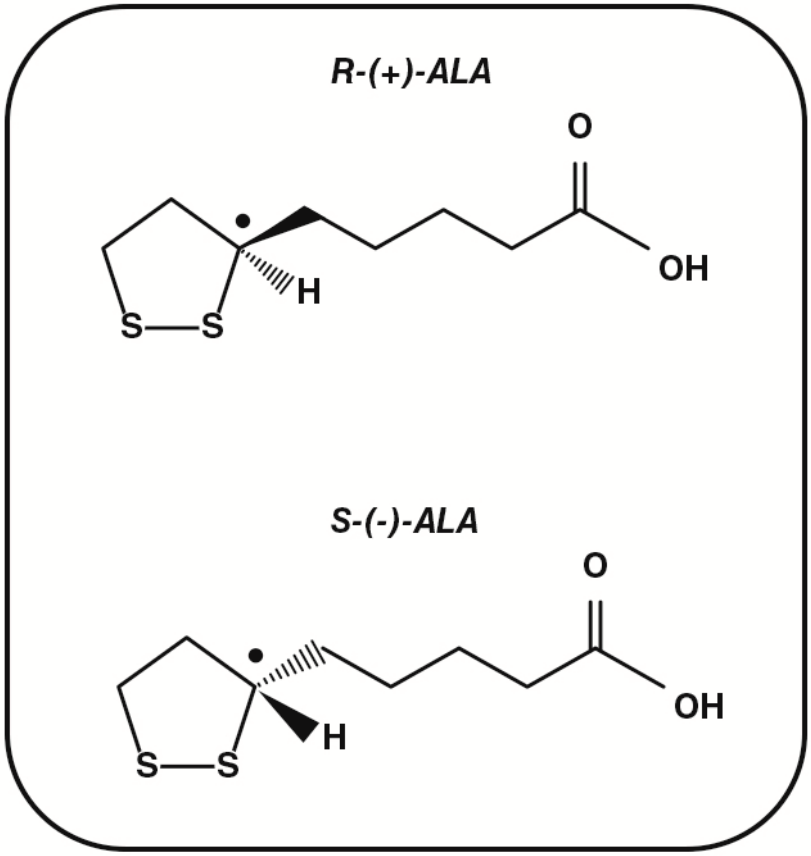
rebalancing (moderate stimulation) of pathways which can protect from miscarriage	**rebalancing (moderate inhibition)** of pathways which can induce miscarriage
Treg cells	Th1, Th17 and NK cells
IL-10	TNF-α, IL-1β, IL-6, IL-8, IL-17, IFN-γ
VEGF, α-SMA	COX 2, iNOS, PGE2, NO, ICAM-1, VCAM-1, MMP-9, Thrombin

In miscarriage, prevention of the downstream effects of ALA might concern the modulation and regulation of cell lineages and/or the synthesis/release of several molecules, in this way reactivating the physiologic framework of the first trimester pregnancy:

Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

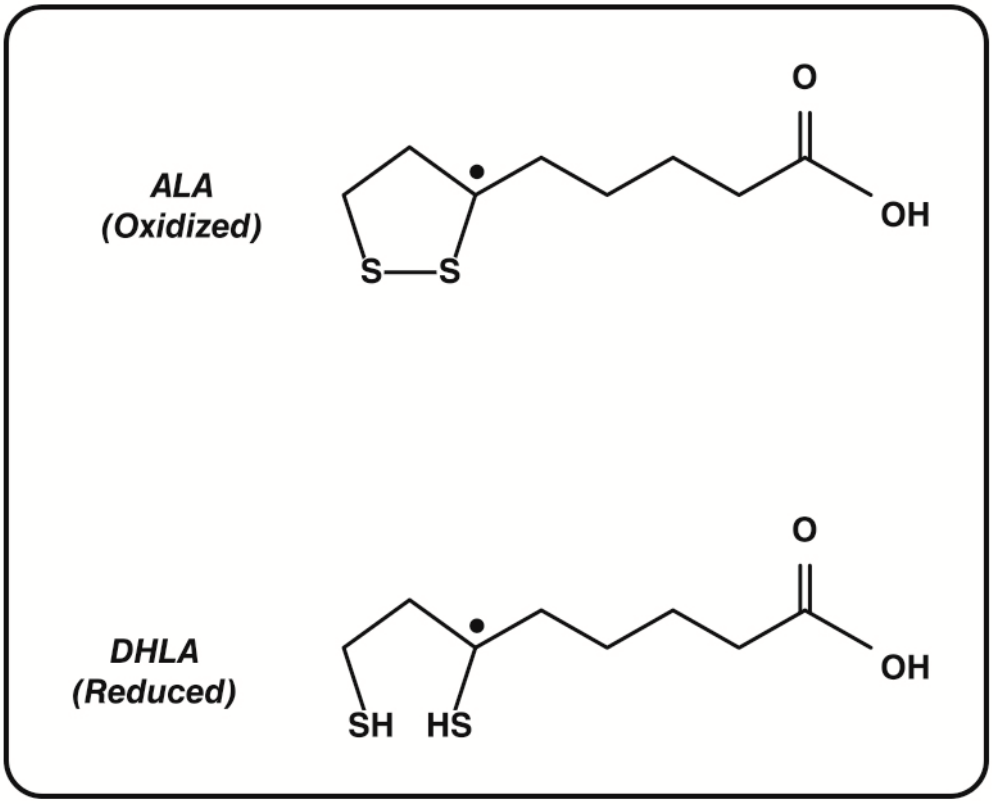
Figure 1. R- and S-enantiomeric forms of Alpha Lipoic Acid (ALA). Only the R-structure is synthetized endogenously and has shown to be indispensable in living systems.



Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Figure 2. ALA and its reduced form, dihydrolipoic acid (DHLA). Both the reduced and the oxidized forms are found in living organisms.



Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT

Figure 3. Pyruvate dehydrogenase complex (PDC). TPP (thiamine pyrophosphate): prosthetic group of E1; ALA: prosthetic group of E2; FAD (Flavin Adenine Dinucleotide): prosthetic group of E3.

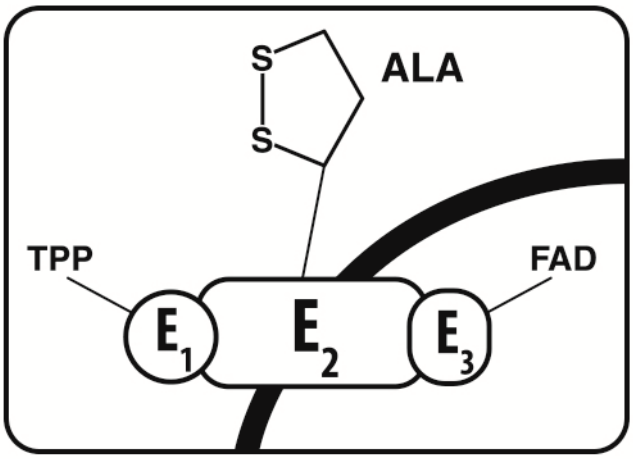
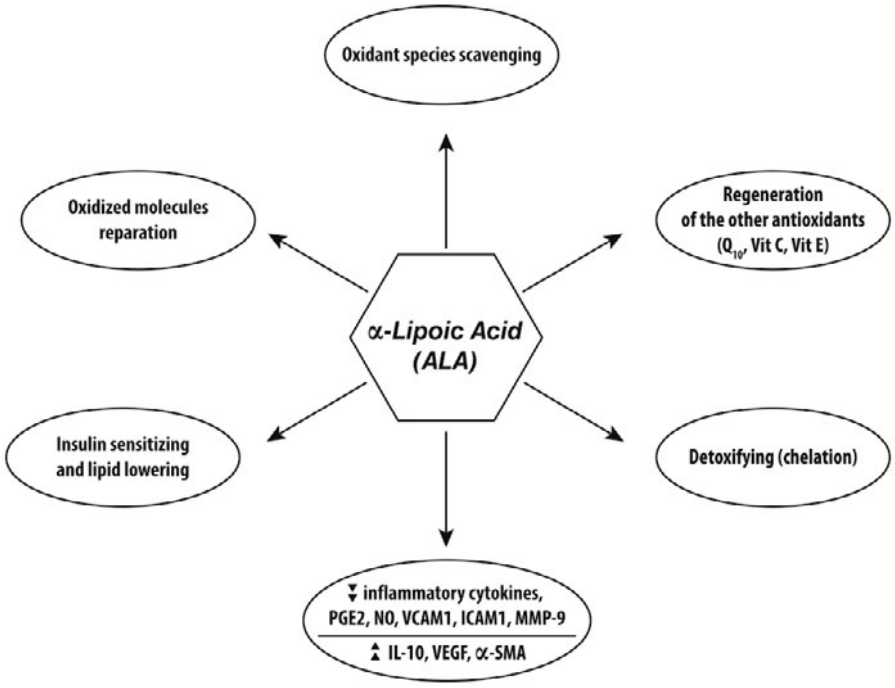


Figure 4. ALA/DHLA exert a multifaceted activity. The main biological effects are synthetized here. Many are mediated by Nrf2 and NF-kB pathways, since ALA/DHLA downregulate NF-kB expression and upregulate Nrf2 expression.



Downloaded by [Giovanni Monastra] at 05:09 13 June 2016

ACCEPTED MANUSCRIPT