

# Are Polyunsaturated Fatty Acid Metabolites, the Protective Effect of 4-hydroxytyrosol on Human Red Blood Cell Membranes and Oxidative Damage (4-hydroxyalkenals) Compatible in Hypertriglyceridemic Patients?

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## ABSTRACT

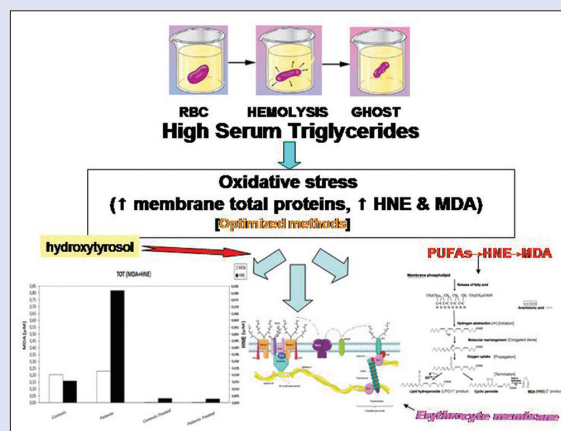
**Background:** Increased levels of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are demonstrated in plasma of uremic patients. A study showed that the comparison of erythrocytes of healthy and diseased patients (obese, hypertensive, and Type 2 diabetics) with age is associated to a disturbed oxidant/antioxidant balance when obesity is associated with hypertension. 4-hydroxytyrosol is shown to significantly protect red blood cells (RBCs) from oxidative damage (4-HNE). In literature, there are partial discussions on the role of lipids and their oxidation products. The products of degradation of membrane proteins are observed as self-consisting products without interrelations with membrane lipids. **Objective:** The aim of this study is to evaluate the role of polyunsaturated fatty acid (PUFA) metabolites on oxidative damage (4-hydroxy-alkenals) in RBCs of hypertriglyceridemic patients after membrane treatment with 4-hydroxytyrosol. **Materials and Methods:** The authors optimize the isolation of RBC ghosts and spectrophotometric method to measure free 4-hydroxyalkenals in human RBC membranes and investigated the effect on oxidative damage in human erythrocyte membranes and *in vitro* 4-hydroxytyrosol treatment to evaluate the membrane lipids reducible by this phenol. **Results:** Plasma triglyceride levels in patients are clearly higher than in controls. Moreover, total membrane proteins data are similar to previously described ones. **Conclusion:** According to experimental data, the accumulation of the alkenals in RBC membrane could be produced either by partial PUFA oxidation contained in glycerides and plasma glycerides and by glycerides into plasma membrane recycled RBC.

**Key words:** 4-hydroxyalkenals, 4-hydroxytyrosol, hypertriglyceridemia, lipid peroxidation, malondialdehyde, oxidative stress

## SUMMARY

- Hypertriglyceridemia induces oxidative stress in human red blood cell (RBC) membranes

- Oxidative stress causes increased plasma membrane total protein concentration and hydroxynonenal and malondialdehyde levels
- The authors optimize the isolation of RBC ghosts and spectrophotometric method to measure free 4-hydroxyalkenals in human RBC membranes
- After the reduction with 4-hydroxytyrosol, oxidized lipid concentration significantly decrease.



**Abbreviations used:** RBC: Red blood cell; MDA: Malondialdehyde; HNE: 4-hydroxyalkenals; LPO: Lipid peroxidation; ROS: Reactive oxygen species; ORAC: Oxygen Radical Absorbance Capacity.

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## INTRODUCTION

As described by Sommerburg *et al.*, patients with end-stage renal failure undergoing hemodialysis are exposed to oxidative stress. Increased levels of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are demonstrated in plasma of uremic patients, indicating accelerated lipid peroxidation (LPO).<sup>[1]</sup> According to Breusing *et al.*,<sup>[2]</sup> human plasma samples exposed to UVA irradiation at different doses (0, 15 J, 20 J) where analyzed for MDA, 4-HNE, and isoprostanes contents, result in a variation of free HNE by HPLC analysis in an interlaboratory validation of methods of LPO measurement. A study of Constantin *et al.* investigated the effects of aging on the carbonyl stress and glutathione antioxidant defenses in red blood cells (RBCs) of obese Type 2 mellitus

diabetic patients with/without hypertensive complications. The results showed that compared to RBCs of healthy controls, in obese Type 2

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diabetics, aging is associated with: (i) an increase in the concentration and expression of carbonylated proteins, a marker of oxidative stress; (ii) a decrease of both nonenzymatic and enzymatic endogenous glutathione defences; (iii) a severely disturbed oxidant/antioxidant balance when obesity is associated with hypertension.<sup>[3]</sup> 4-hydroxytyrosol is shown to significantly protect RBCs from oxidative damage in a dose-dependent manner (20–30  $\mu\text{M}$ ).<sup>[4]</sup> 4-hydroxytyrosol inhibits 4-HNE-induced apoptosis, characterized by increased production of reactive oxygen species (ROS).<sup>[5]</sup>

4-hydroxytyrosol shows a substantial degree of inhibition against ascorbate- $\text{Fe}^{2+}$  induced LPO in rat liver microsomes.<sup>[6]</sup> In a previous study of Gallo and Martino<sup>[7]</sup> on RBC membranes damages by oxidant agents, authors demonstrated the conformational alterations and polyphenols repairing in the same structure. Among the known risk factors of atherosclerosis-associated oxidative stress are hyperlipidemia and/or hyperglycemia.<sup>[8–10]</sup> Currently, in the literature, there are only partial discussions on the role of lipids and their oxidation products as intermediates of their membrane structural damage and/or on the protective role in the same structures.<sup>[11,12]</sup> Furthermore, the products of degradation of membrane proteins are observed as self-consisting products without interrelations with membrane lipids. In this manuscript, we examine the role of triglycerides overload on degradation of membrane lipids in the presence of other reducing agents (protective effect of 4-hydroxytyrosol).<sup>[13–15]</sup> The studied membrane model is the RBC that is damaged from ROS.<sup>[16]</sup> The RBC is an experimental model of essential cellular structure. Hypertriglyceridemia denotes high blood levels of triglycerides, the most abundant fatty molecules in organisms. Elevated levels of triglycerides are associated with atherosclerosis and predispose to cardiovascular disease.<sup>[17]</sup> Oxidative stress, i.e., an altered balance between the production of free radicals and antioxidant defenses.<sup>[18]</sup> The peroxidation of *n*-3 and *n*-6 polyunsaturated fatty acids (PUFAs) and of their metabolites is a complex process. It is initiated by free oxygen radical-induced abstraction of a hydrogen atom from the lipid molecule followed by a series of nonenzymatic reactions that ultimately generate the reactive aldehyde species 4-hydroxyalkenals (HAE). 4-HNE has been implicated in various pathophysiological interactions due to their chemical reactivity and the formation of covalent adducts with macromolecules. The progressive accumulation of adducts alters normal cell functions that lead to cell death. At low and noncytotoxic concentrations, these molecules may function as signaling molecules in cells. This paper addresses the origin and cellular functions of 4-HAE.<sup>[19]</sup> The scavenging activity is studied after the “*in vitro*” treatment with 4-hydroxytyrosol that is classified as a phytochemical compound expressing strong antioxidant properties. The Oxygen Radical Absorbance Capacity index<sup>[20]</sup> for 4-hydroxytyrosol totaled (Trolox equivalent [TE] antioxidant capacity) 40,000  $\mu\text{mol TE/g}$  that is about ten times greater than green tea at least twice compared to  $\text{CoQ}_{10}$ . One study showed that a low dose of 4-hydroxytyrosol in rats reduces the consequences of the side effects of oxidative stress induced by smoking.<sup>[21]</sup> 4-hydroxytyrosol is shown to significantly protect RBCs from oxidative damage (LPO [4-HNE]). The authors<sup>[7]</sup>

studied the protective effect of 4-hydroxytyrosol on human RBCs and oxidative damage so, the intent is to examine the role of triglycerides overload on degradation of membrane lipids in the presence of other reducing agents (protective effect of 4-hydroxytyrosol). For instance, in complete eukaryotic cells, the following damages can happen: 4-HAE are endogenous ligands for peroxisome proliferator-activated receptor- $\delta$ . 4-HNE affects p38 mitogen-activated protein kinases, c-Jun N-terminal kinase, protein kinase C $\beta$  and - $\delta$ , cell cycle regulators, epithelial growth factor receptor, microRNAs, and the nuclear factor- $\kappa\text{B}$  [Figure 1].<sup>[22]</sup> Cytotoxic effects furthermore result from covalent interactions of 4-HNE with aminophospholipids and distort the bilayer structure of membranes and consequently alter their barrier and transport functions [Figure 2].<sup>[23,24]</sup>

## MATERIALS AND METHODS

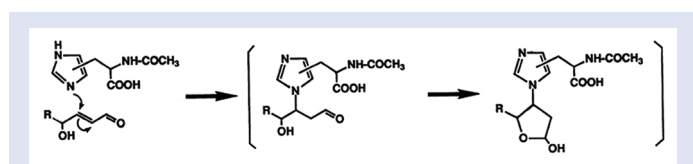
### Patients

The studied population consisted of 10 patients with endogenous hypertriglyceridemia and ten normal controls who are recruited from the Health Center of the University of Calabria, Italy. All patients followed traditional local diet. The diagnosis of endogenous hypertriglyceridemia is based on the peroxidase-coupled method for the colorimetric determination of serum triglycerides.<sup>[25]</sup>

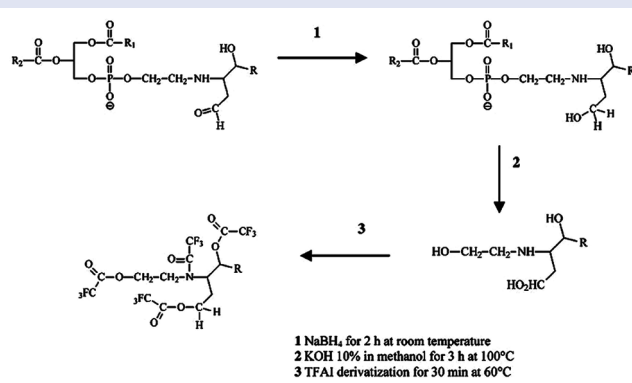
### Blood samples

After fasting, blood is sampled on ethylenediaminetetraacetic acid (EDTA)-coated tubes, spun down at 1550 g for 10 min, plasma and leukocyte layers removed, and the packed RBCs layer used for the subsequent assays.

RBCs layer is lysed in 5 mM hypotonic phosphate buffer pH 8, containing in origin 1 mM EDTA (to complex all interfering cations, authors simplified the method increasing EDTA concentration at 100 mM) and 0.03 mM phenylmethylsulphonyl fluoride, followed by 20 min centrifugation at 16,000 g, the RBCs ghost sediment are resuspended by swirling while adding back sufficient of the fresh hypotonic buffer to reconstitute the original volume. The ratio of erythrocytes to washing solution is 1/40. The ghosts are washed 3 times subsequent to the hemolysis by sedimenting in a swinging-bucket rotor (16,000 g for 30 min), till free of hemoglobin. Aliquots of this should be taken for sample replicates, as well as for protein determination. 200  $\mu\text{L}$  portions are required for use in this assay. Sample homogenates should be made as concentrated as possible. The concentration of protein in the homogenate



**Figure 1:** Proposed molecular mechanism of formation of hydroxynonenal-histidine adducts, according to Uchida and Stadtman, 1992



**Figure 2:** Scheme of the procedure used to evaluate ethanolamine phospholipid-alkenal Michael adducts by gas chromatography-mass spectrometry as their ethanolamine-alkenal residue, according to Bacot *et al.*, 2007

should be determined. It is recommended that 0.2  $\mu\text{L}$  of a homogenate containing 15–60 mg/mL protein be assayed for initial studies in a previously untested biological sample. The supernatant should be kept on ice before analyze or, if not analyzed immediately after preparation, frozen at  $-70^{\circ}\text{C}$  for longer storage.<sup>[3]</sup>

## ASSAY OF TOTAL PROTEINS IN RED BLOOD CELLS GHOSTS

The determination of protein concentration is performed by the Biuret method.<sup>[26]</sup> The principle on which is based on the reaction of the peptide bond of proteins with  $\text{Cu}^{2+}$  ions. In alkaline solution, the formation of an indigo colored complex: The intensity of the color is directly proportional to the protein concentration. The standard used is bovine serum albumin from Sigma. Procedure: Samples prepared by plugging the tubes are shaken, and after 45 min it reads the absorbance of each sample at 540 nm on Shimadzu UV 2100 spectrophotometer.

## Spectrophotometric analysis of free hydroxynonenal

### *Assay procedure for malondialdehyde ([concentrated hydrochloric acid] and malondialdehyde + hydroxyalkenals [concentrated methane sulfonic acid procedure])*

All reagents are from Enzo Life Science. The standards (HNE standard: (E)-Hydroxynonenal I $\kappa$ B inhibitor) are prepared in clean glass test tubes or polypropylene microcentrifuge tubes. Preferably, standards should be run in triplicate. Add 200  $\mu\text{L}$  of sample to a clean glass test tube or polypropylene microcentrifuge tube. Unknowns should be run in triplicate. Add 650  $\mu\text{L}$  of diluted N-methyl-2-phenylindole reagent. Mix gently by vortexing the sample. Add 150  $\mu\text{L}$  concentrated (12 N) HCl or methanesulfonic acid reagent. Mix well and stopper the tube. Incubate at  $45^{\circ}\text{C}$  for 60 min. Centrifuge turbid samples (e.g., 15,000  $\times$  g for 10 min) to obtain a clear supernatant. Transfer the clear supernatant to a cuvette. Measure the absorbance at 586 nm.<sup>[27]</sup>

## In vitro 4-hydroxytyrosol treatment

The concentration of reducing agent is chosen according to literature data.<sup>[4]</sup> Reducing agent is provided from Sigma Chemical Co. (St. Louis, MO, USA) and used at the final concentration of 80  $\mu\text{M}$  for 3 h at  $37^{\circ}\text{C}$ .

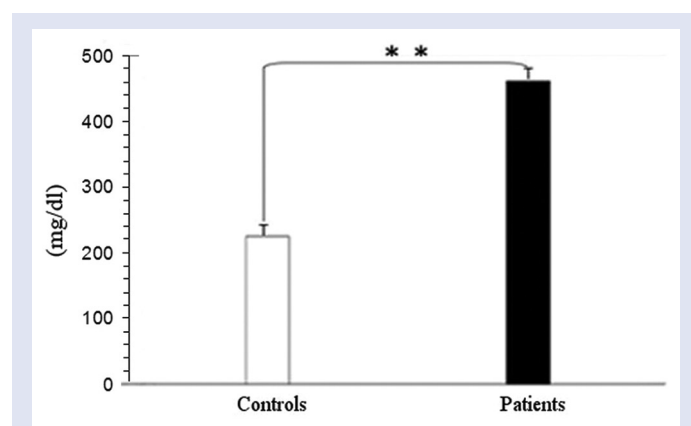
## Statistical analysis

For statistical analysis, one-way ANOVA and Bonferroni *post hoc* test are used. Results are given as mean  $\pm$  standard error of the mean on at least ten independent determinations.

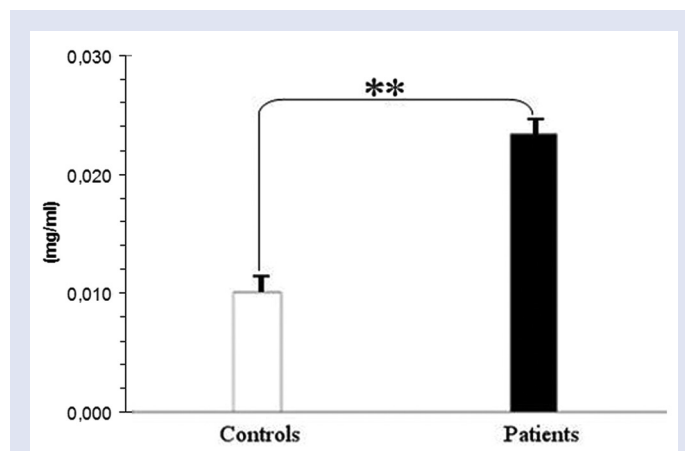
## RESULTS

Authors studied the effect of oxidative stress of RBC ghosts by the method of Gérard-Monnier *et al.*<sup>[27]</sup> The diagnostic criteria for endogenous hypertriglyceridemia are as follows: Total plasma triglyceride level  $> 200$  mg/dL [Figure 3]. So that triglyceride plasma levels in patients are neatly higher than in controls. The mean values of triglyceride plasma levels in our casuistry are described in Figure 3. It is evident that data of the two groups are significantly different ( $P < 0.01$ ).

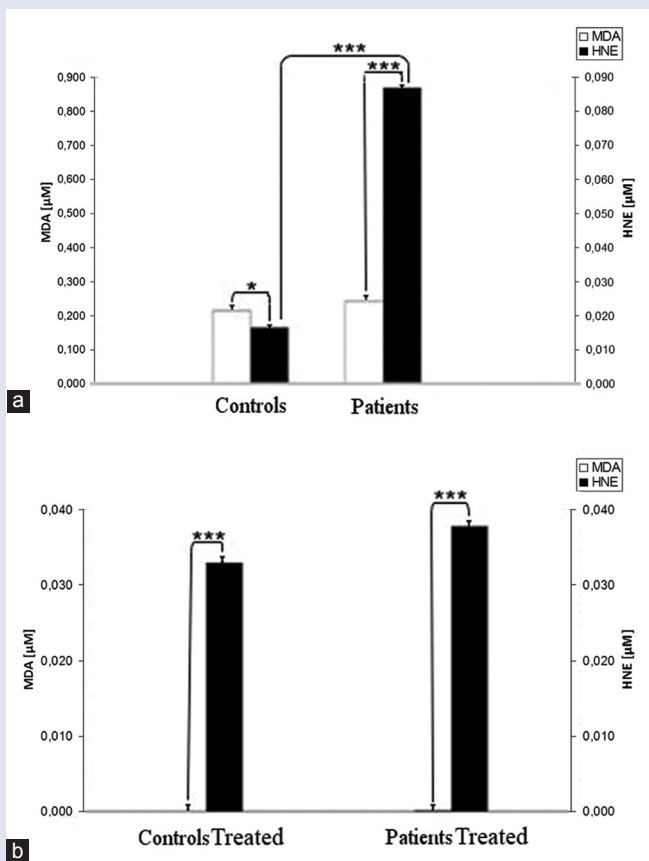
As described in Figure 4 total membrane proteins from samples of our casuistry are significantly different after extensive differential centrifugation of RBC ghosts. The mean values of total membrane protein levels in our casuistry are described in Figure 4. It is evident that the two grouped data are significantly different ( $P < 0.01$ ). The proteins of pathological samples are about two times those of controls, showing that hypertriglyceridemic patients possess RBC ghosts that are richer in protein than reference ones. As described in Figure 5a and b the level of oxidation products of membrane lipids are referred as  $\mu\text{M}$  of MDA or HNE. MDA levels are always ten times those of HNE as referred in right-hand scale. In Figure 5a and b the scales are quite different (for instance: 0.00/0.85 in A and 0.000/0.033 in B for MDA). We can also observe that MDA levels of pathological samples are slightly higher than control ones but HNE levels of pathological samples are four times those of control ones. After the 4-hydroxytyrosol action both lipid metabolites substantially decrease. The mean values of total membrane protein levels in our casuistry are described in Figure 5. It is evident that for the two dosed substances each group of data is significantly different ( $P < 0.001$ ) from the other ones. In Figure 6 are reported the total levels of MDA + HNE per milligram of total membrane proteins of RBC ghosts. As described the total oxidized lipid levels in RBC ghost membranes almost doubled in pathological samples in comparison to control ones. This shows that even if total membrane proteins dramatically increase in hypertriglyceridemic patients, also total oxidized lipids sufficiently increase in such samples so that ratio between patients RBC oxidized lipids to membrane proteins is always doubled. Such partial compensation of lipids in comparison



**Figure 3:** Plasma biochemical data of controls and hypertriglyceridemic patients (mean  $\pm$  standard deviation). The mean values of triglyceride plasma levels. It is evident that data of the two groups are significantly different for  $P < 0.01$  by one-way ANOVA and Bonferroni *post hoc* test. \* $P < 0.05$ ; \*\* $P < 0.01$  and \*\*\* $P < 0.001$



**Figure 4:** The quantitative assay of total proteins per blood ml from red blood cell ghosts. Mean values of proteins in red blood cell purified membranes. It is evident that data of the two groups are significantly different for  $P < 0.01$  by one-way ANOVA and Bonferroni *post hoc* test. \* $P < 0.05$ ; \*\* $P < 0.01$  and \*\*\* $P < 0.001$

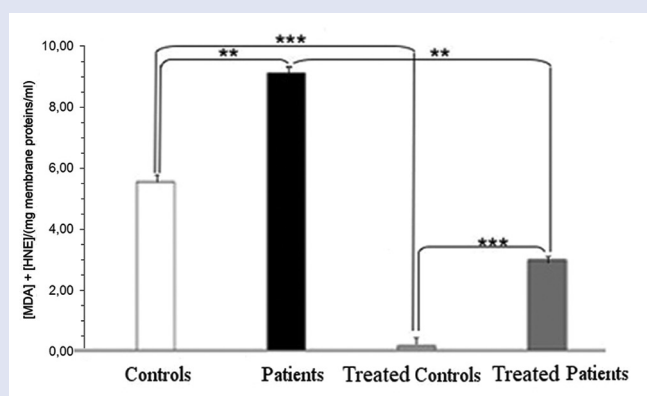


**Figure 5:** Human red blood cell membrane concentration of malondialdehyde and hydroxynonenal (μM absolute concentration in membrane samples) from controls and hypertriglyceridemic patients (a) and the same samples treated with 4-hydroxytyrosol (80 μM) (b). It is evident that for the two dosed substances each grouped data are significantly different for  $P < 0.001$  by two ways ANOVA and Bonferroni *post hoc* test. \* $P < 0.05$ ; \*\* $P < 0.01$ , and \*\*\* $P < 0.001$

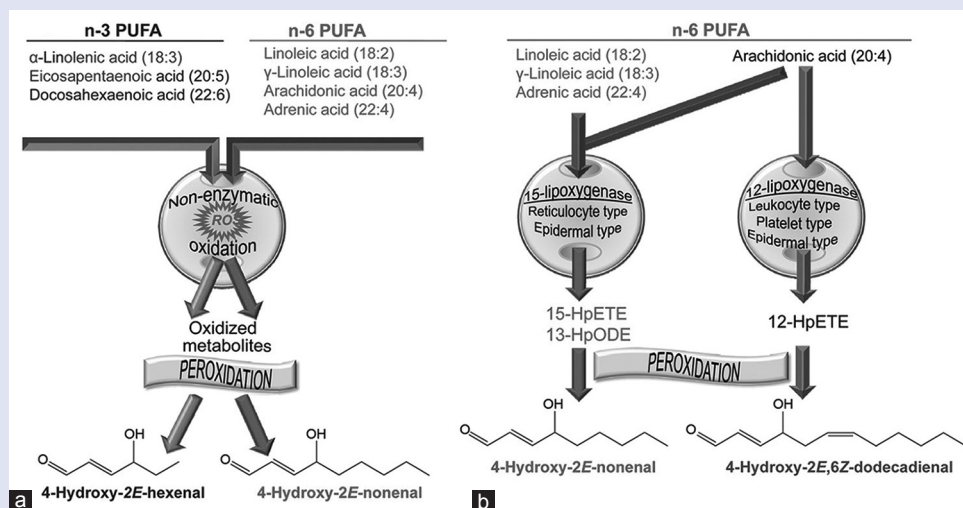
to proteins from RBC membrane could be explained as an attempt of RBC metabolism to partially compensate ghost structure damages by oxidative stress. The ratio of total oxidized lipids to membrane proteins before and after 4-hydroxytyrosol treatment are referred in Figure 6. After 4-hydroxytyrosol treatment, the ratio of oxidized lipids to total membrane proteins decreases even more, but the values in our controls are always lower than in hypertriglyceridemic patients. The mean values of ratio of oxidized lipids (MDA + HNE) and membrane proteins in our casuistry are described in Figure 6. It is evident that the two groups of data are significantly different ( $P < 0.001$ ). Neither Vitamin E nor Vitamin C are used because their ultraviolet-visible (UV-Vis) spectra superimpose to HNE determination method interfering with their evaluation.

## DISCUSSION

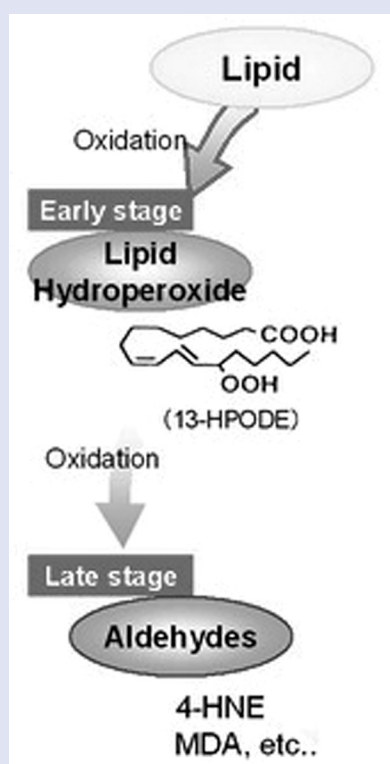
According to our data in RBC, ghosts are present both HNE and MDA products [Figure 7].<sup>[19]</sup> As demonstrated by higher levels of HNE in



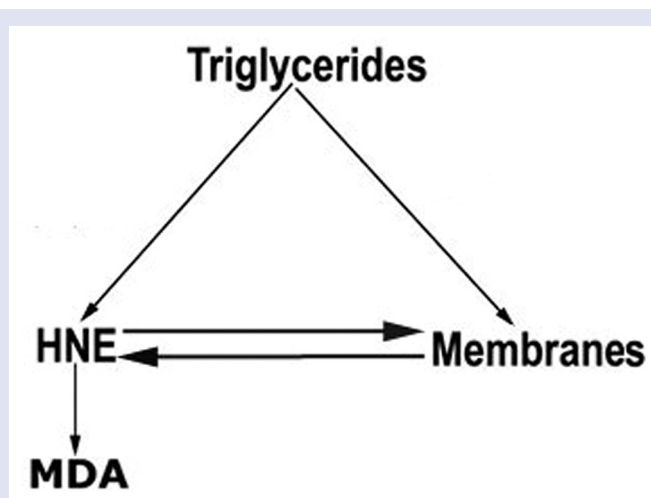
**Figure 6:** Ratio of oxidized lipids (malondialdehyde + hydroxynonenal) and membrane proteins. It is evident that all comparisons for the two dosed substances, each group of data is significantly different for  $P < 0.001$  by two-ways ANOVA and Bonferroni *post hoc* test. \* $P < 0.05$ ; \*\* $P < 0.01$  and \*\*\* $P < 0.001$  from the others



**Figure 7:** Mechanisms of 4-hydroxyalkenal production. Reactive oxygen species, reactive oxygen species. (a) Nonenzymatic generation of 4-hydroxy-2E-hexenal and 4-hydroxy-2E-nonenal (4-hydroxynonenal) from n-3 and n-6 polyunsaturated fatty acids, respectively. (b) lipoxigenase-initiated peroxidation of n-6 polyunsaturated fatty acids and generation of 4-hydroxynonenal and 4-hydroxy-2E,6Z-dodecadienal

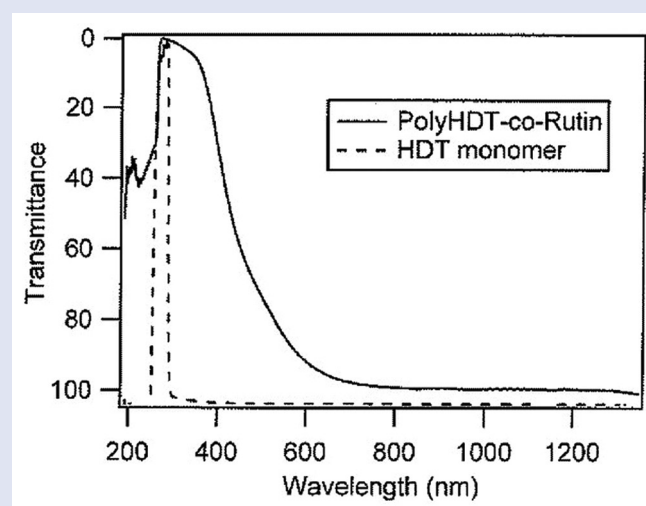


**Figure 8:** Stages of lipid peroxidation (modified by authors scheme of Abd El-Aal HAHM. Lipid Peroxidation End-products as a Key of Oxidative Stress: Effect of Antioxidant on Their Production and Transfer of Free Radicals. In: Catala A, editor. Biochemistry, Genetics and Molecular Biology "Lipid Peroxidation". Winchester (GB): InTech; 2012. p. 63-88)



**Figure 10:** Graphical resume of main metabolic steps of fatty acids in cell membrane

comparison to MDA, according to Sommerburg *et al.*<sup>[1]</sup> The RBC membrane preparations are obtained increasing the efficiency of Fe<sup>2+</sup> removal with an higher concentration of EDTA (100 mM). Spectrophotometric method (25) to measures free 4-HAE in human RBCs is optimized for the determination of alkenals [Figure 8] in membranes. Other kinds of reducing agents, such as Vitamin E, are not tested because their UV-Vis spectra superimpose to N-methyl-2-phenylindole product spectra.



**Figure 9:** 4-hydroxytyrosol ultraviolet-visible absorbance spectrum peak at 280 nm (Klen TJ. Olive fruit phenols in olive oil processing: the fate and antioxidant potential. Thesis, University of Nova Gorica, Nova Gorica, Slovenia; 2014)

In membranes of hypertriglyceridemic patients, the levels (triglycerides, total membrane proteins, total aldehydes and their ratio) are higher than in control samples. All the data decrease relevantly ( $P < 0.001$ ) after the 4-hydroxytyrosol treatment, especially those of alkenals [Figure 9].

## CONCLUSION

According to experimental data, the accumulation of the alkenals in RBC membrane could be produced either by partial PUFA oxidation contained in glycerides and plasma glycerides and by glycerides into recycled plasma membrane in RBC neogenesis [Figure 10]. According to these hypotheses, the increased charge of triglycerides in plasma forces its metabolism toward either incorporation in cell membrane of degradative oxidation. This last pathway induces increase of oxidative product such as alkenals and MDA. Furthermore, free alkenals can be dissolved in lipid membrane bilayer degrading their structures. This last process favors increased level of macromolecular assemblies in circulation that enhances microcirculation damage. Such last data could be studied in following works.

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## Conflicts of interest

There are no conflicts of interest.

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