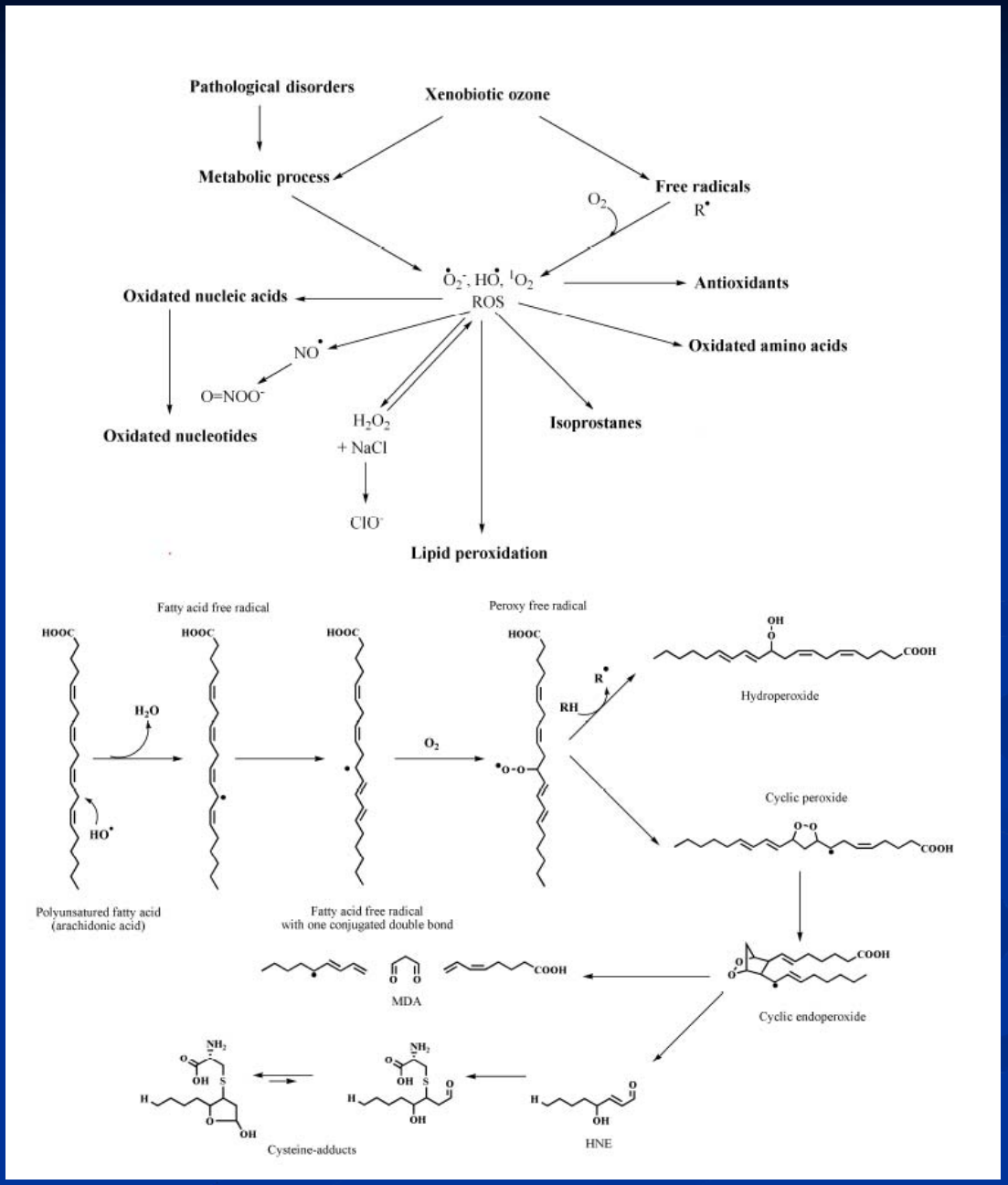
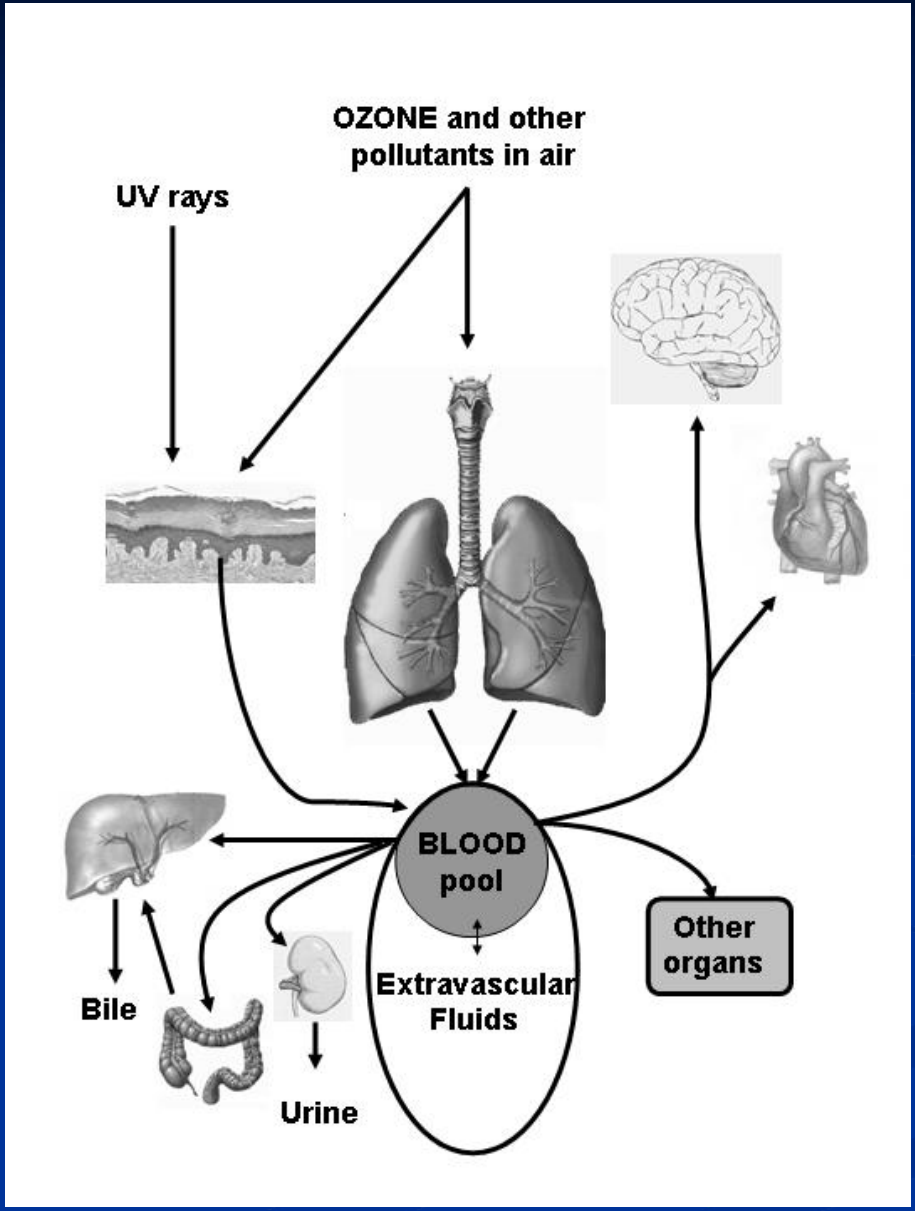
DOES OZONETHERAPY HAVE A FUTURE IN MEDICINE ?

V. BOCCI
Department of Physiology of the University of Siena, Italy


Pathological disorders
Xenobiotic ozone
Metabolic process
Free radicals
R•
O2
O2•-, HO•, 1O2
ROS
Antioxidants
Oxidated nucleic acids
Oxidated amino acids
NO•
O=NOO-
H2O2
+ NaCl
ClO-
Oxidated nucleotides
Isoprostanes
Lipid peroxidation
Fatty acid free radical
Peroxy free radical
HOOC
H2O
HO•
O2
RH
R•
OH
COOH
Hydroperoxide
Cyclic peroxide
Polyunsatured fatty acid
(arachidonic acid)
Fatty acid free radical
with one conjugated double bond
MDA
Cyclic endoperoxide
HNE
Cysteine-adducts


OZONE and other pollutants in air
UV rays
BLOOD pool
Other organs
Extravascular Fluids
Bile
Urine

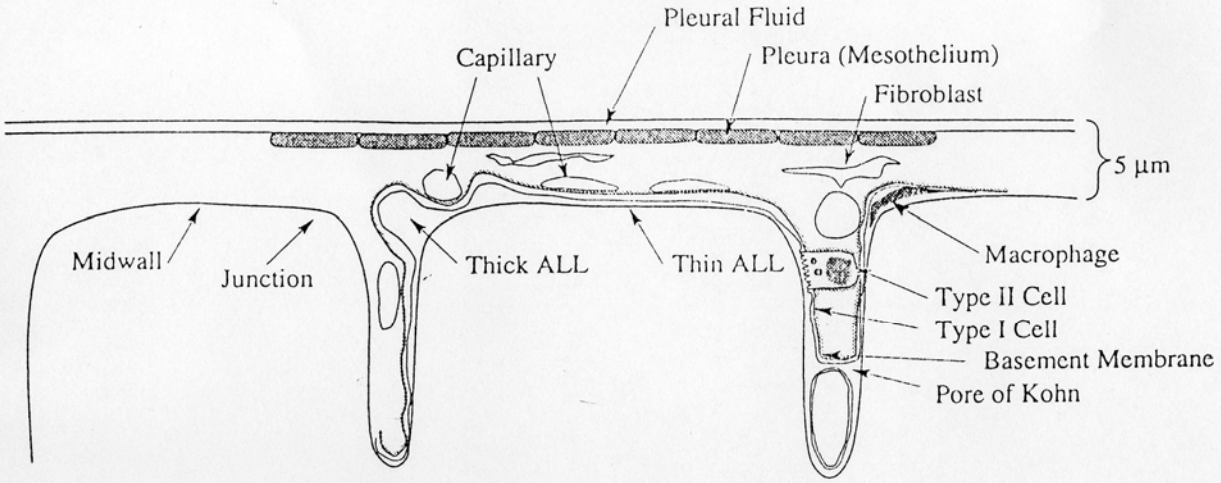


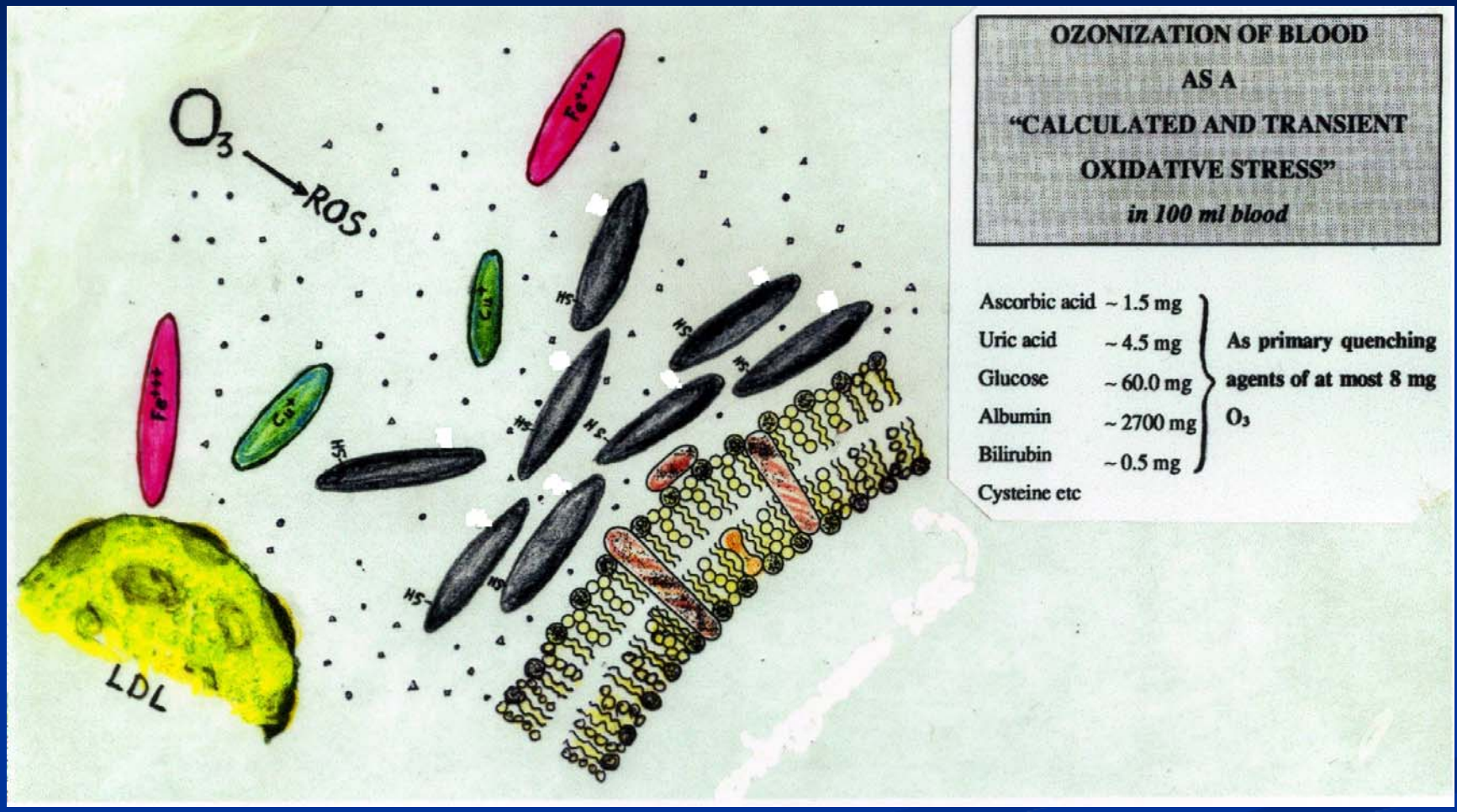
FIG. 1. Alveolar lining layer (ALL) and its relationship to pleura and alveolar wall. ALL is thin at midwall and thick and thin at alveolar junctions. Very rapid freezing could be achieved because cryoprobe in contact with pleural fluid was within a few micrometers of ALL on outermost alveolar wall. Lengths of alveolar walls are not to scale, and thickness is exaggerated for clarity.

BASTACKY J. et al., 1995
J. Appl. Physiol. 79, 1615

A comparison between the composition of ELF and blood of a normal 70 Kg human showing the great difference in antioxidant capacity of these two fluids.

ELF	Blood
Volume: 17-20 ml	Plasma volume: ~2.7 L
	Erythrocytes: ~2.3 Kg
Total proteins:~7mg/ml	Total plasmaproteins:~75 mg/ml
(total:~130mg)	(total: ~202.5 g)
Albumin: ~3.5 mg/ml	Albumin: ~45 mg/ml
(total: ~63 mg)	(total: ~ 121.5 g)
Transferrin: ~0.3 mg/ml	2-4 mg/ml
Ceruloplasmin:~ 25µg/ml	140-400 µg/ml
Lactoferrin: ~0.5 µg/ml	?
GSH: 300-400 µM	in plasma: ~3µM
	in erythrocytes: ~2.2 mM
Vitamin E: ~2 µg/ml	10-20 µg /ml
Vitamin C: ~3.5 µg/ml	~9 µg/ml
Uric acid: ~0.05 mg/ml	0.04-0.07 mg/ml
Glucose: ~0.4 mg/ml	0.7-1.0 mg/ml
Total Bilirubin: ?	~1.0 mg/dL
Na:~82; Cl:~84; K:~29 mM	Na:~139; Cl:~103; K:~4 mM
pH: 6.9	pH: 7.4

Data reported by Schultze and Heremans, (1966); Davis and Pacht, 1997; Di Simplicio et al., 1998; Hawgood, 1997; Repine and Heffner (1997); Whitsett, 1997.

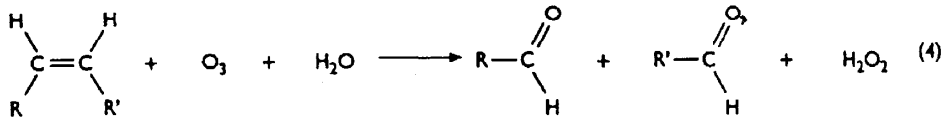


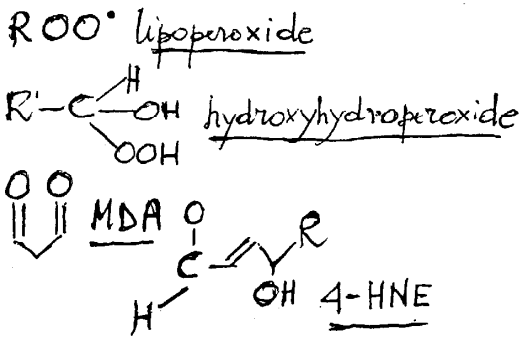
OZONIZATION OF BLOOD
AS A
"CALCULATED AND TRANSIENT
OXIDATIVE STRESS"
in 100 ml blood

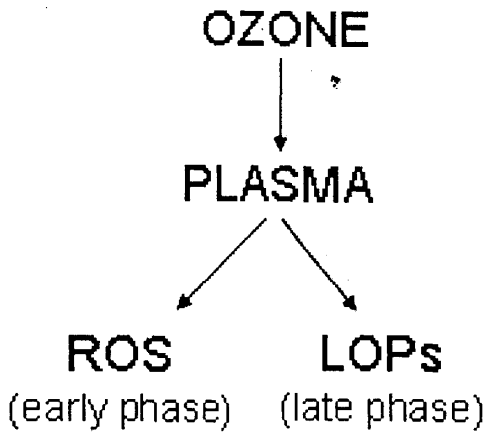
Ascorbic acid	~ 1.5 mg	As primary quenching agents of at most 8 mg O_3
Uric acid	~ 4.5 mg	
Glucose	~ 60.0 mg	
Albumin	~ 2700 mg	
Bilirubin	~ 0.5 mg	
Cysteine etc		

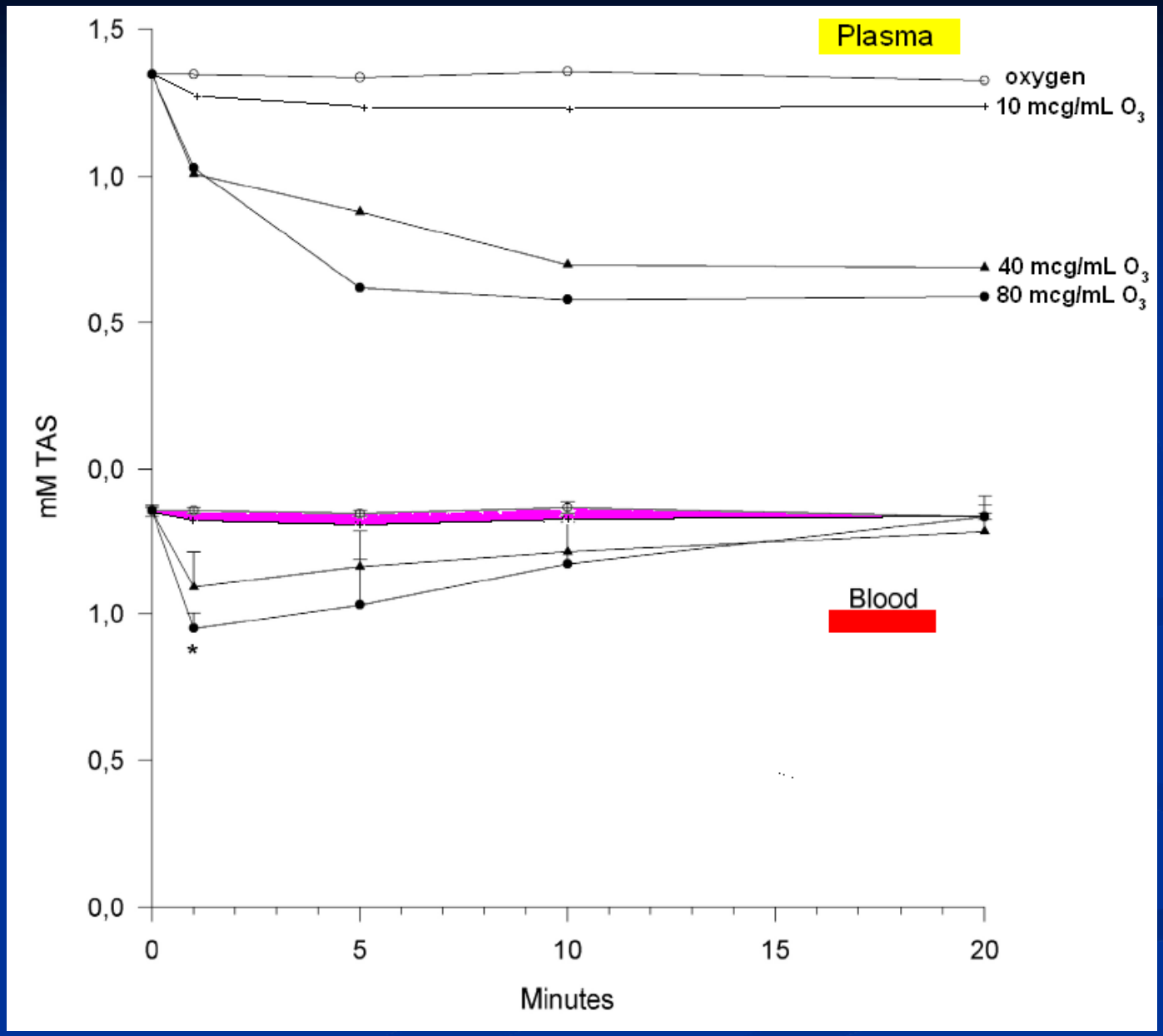
HOW DOES OZONE ACT? HOW AND WHY CAN WE AVOID OZONE TOXICITY?

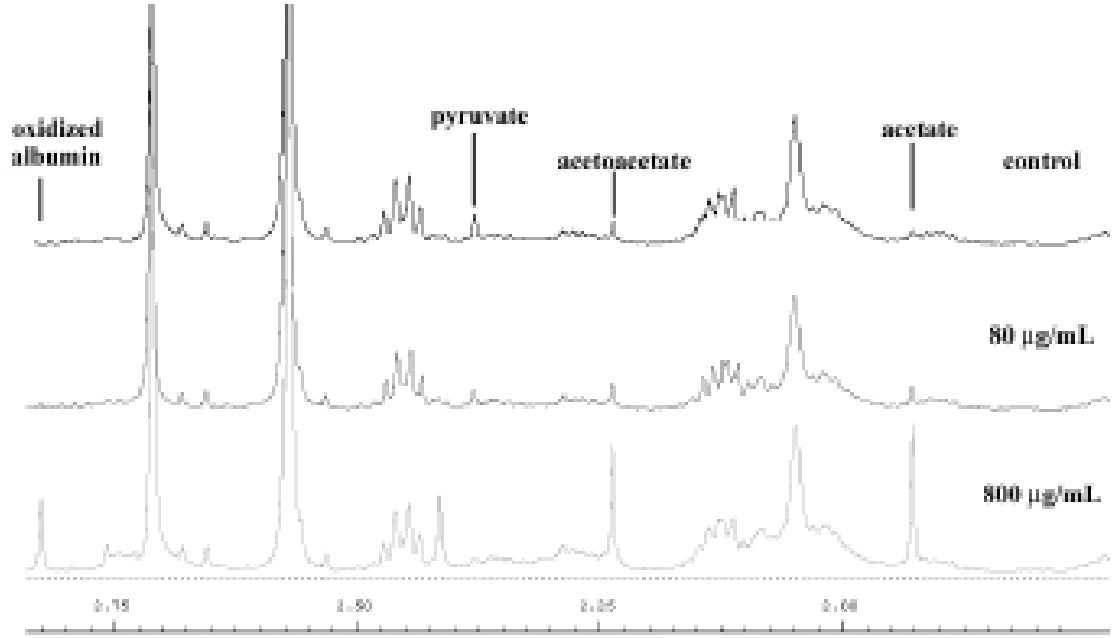
Net reaction

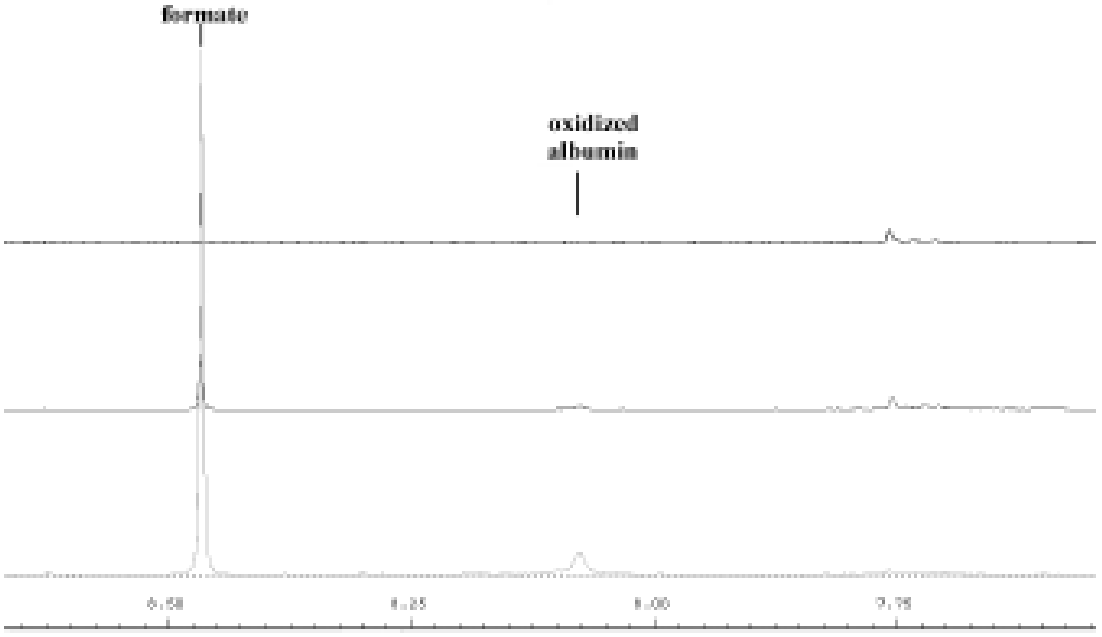




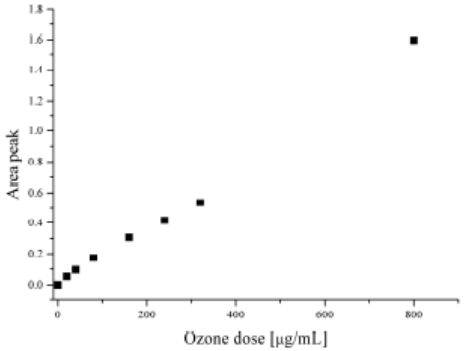



Plasma
oxygen
10 mcg/mL O3
40 mcg/mL O3
80 mcg/mL O3
Blood
mM TAS
1,5
1,0
0,5
0,0
1,0
0,5
0,0
*
0
5
10
15
20
Minutes

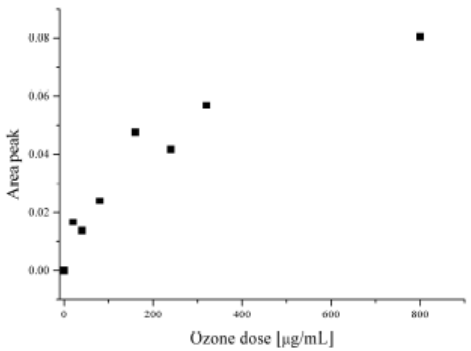

oxidized albumin
pyruvate
acetoacetate
acetate
control
80 µg/mL
800 µg/mL


formate
oxidized albumin

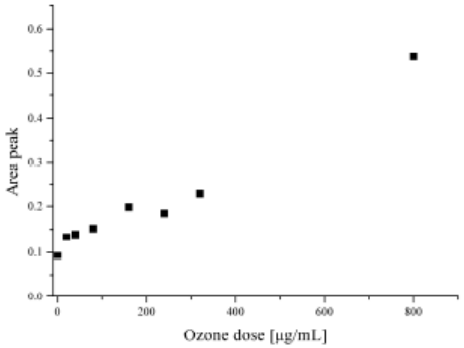
Formate



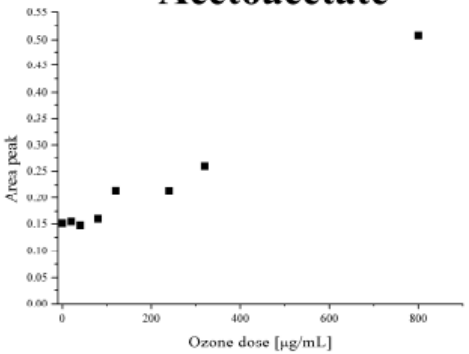
Allantoine



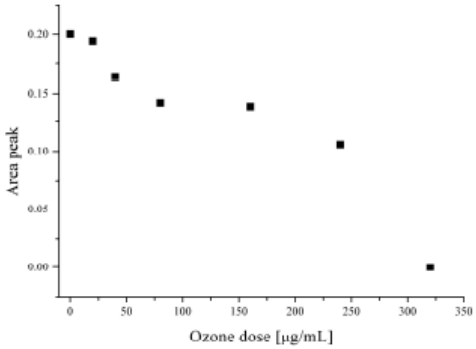
Acetate

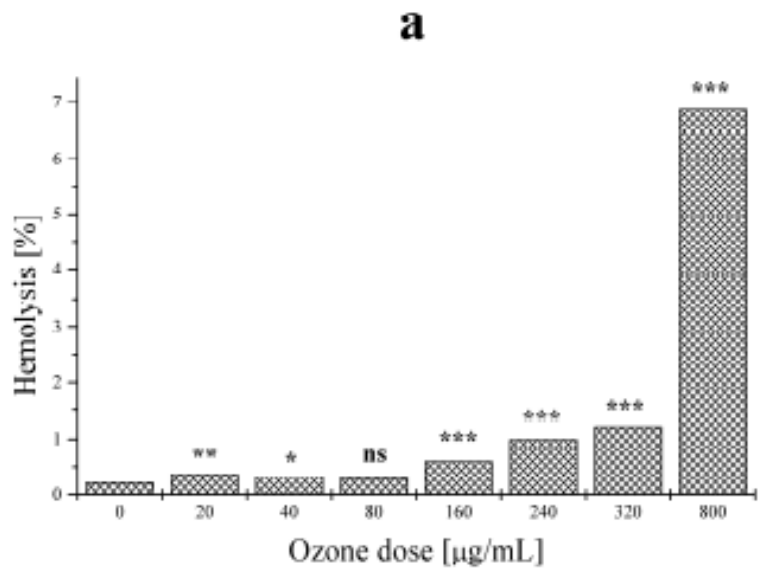


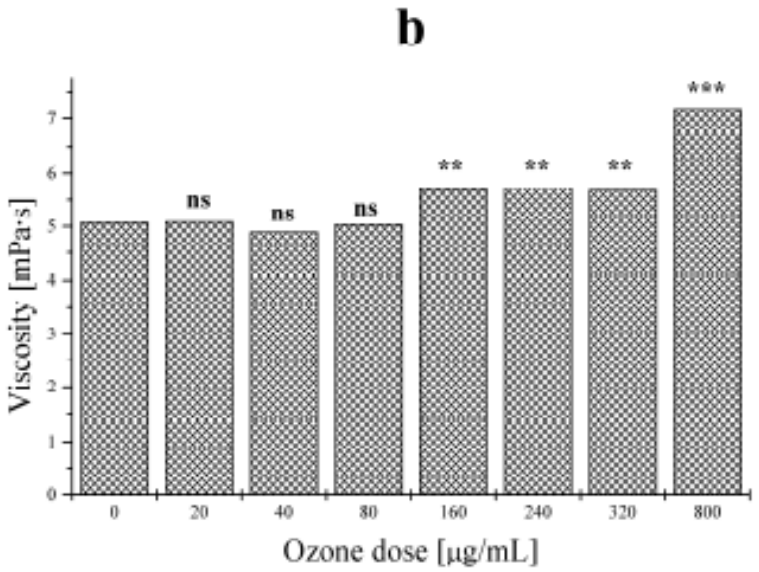
Acetoacetate



Pyruvate

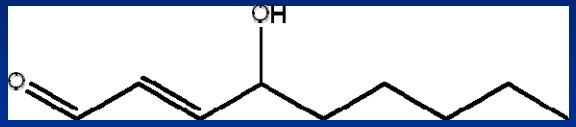



a
Hemolysis [%]
0
1
2
3
4
5
6
7
**
*
ns
***
***
***
***
0
20
40
80
160
240
320
800
Ozone dose [μg/mL]


b
Viscosity [mPa·s]
0
1
2
3
4
5
6
7
ns
ns
ns
**
**
**
***
0
20
40
80
160
240
320
800
Ozone dose [μg/mL]

HOW ALDEHYDES' TOXICITY IS AVOIDED?

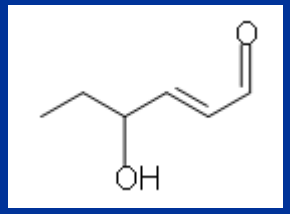
Among Lipid Oxidation Products (LOPs), the quantitatively most relevant aldehyde is *trans*-4-hydroxy-2-nonenal (HNE)



HNE is a normally detectable molecule (0,7-1,0 microM) and on its own, is very unstable and toxic.

It mainly derives from arachidonic acid (ω-6 PUFA)

From ω-3 PUFA, such as docosahexaenoic acid (DHA) derives another aldehyde: the *trans*-4-hydroxy-2-hexenal (HHE)



Which is the fate of these aldehydes?

Most of them form an adduct with either free GSH or they react with Cys 34 or His and Lys of albumin.

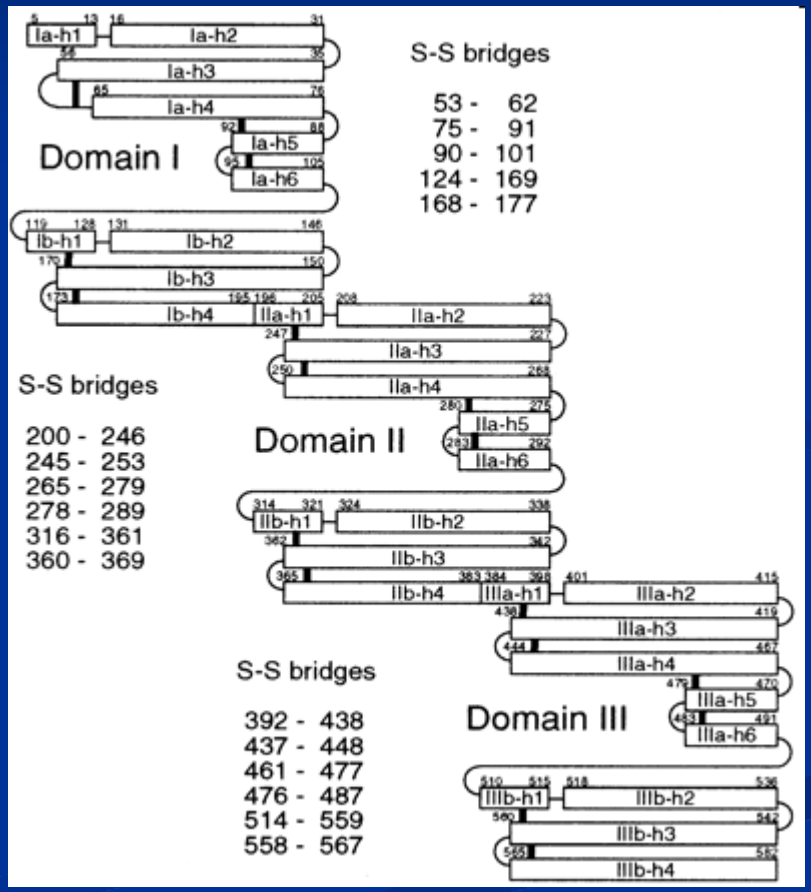
HUMAN SERUM ALBUMIN

It has a molecular mass of 67kDa constituted by 585 amino acids

It contains seventeen pairs of disulfide briges (S-S linkages), one free Cys(34) and 11 accessible nucleophilic residues, two of which constituted by Lys(199) and His(146). During ozonation, within 40 sec, it can bind up to eleven 4-HNE molecules. The rate constant of HNE albumin-trapping with the formation of adducts is far higher than GSH!

Besides the crucial functions of regulating the pH and oncotic pressure, albumin acts as an endogenous detoxifying agent of circulating reactive carbonyl species.

Within three homologous domains there are three hydrophobic pockets, transporting PUFAs, unconjugated bilirubin , hormones, and drugs and bound HNE

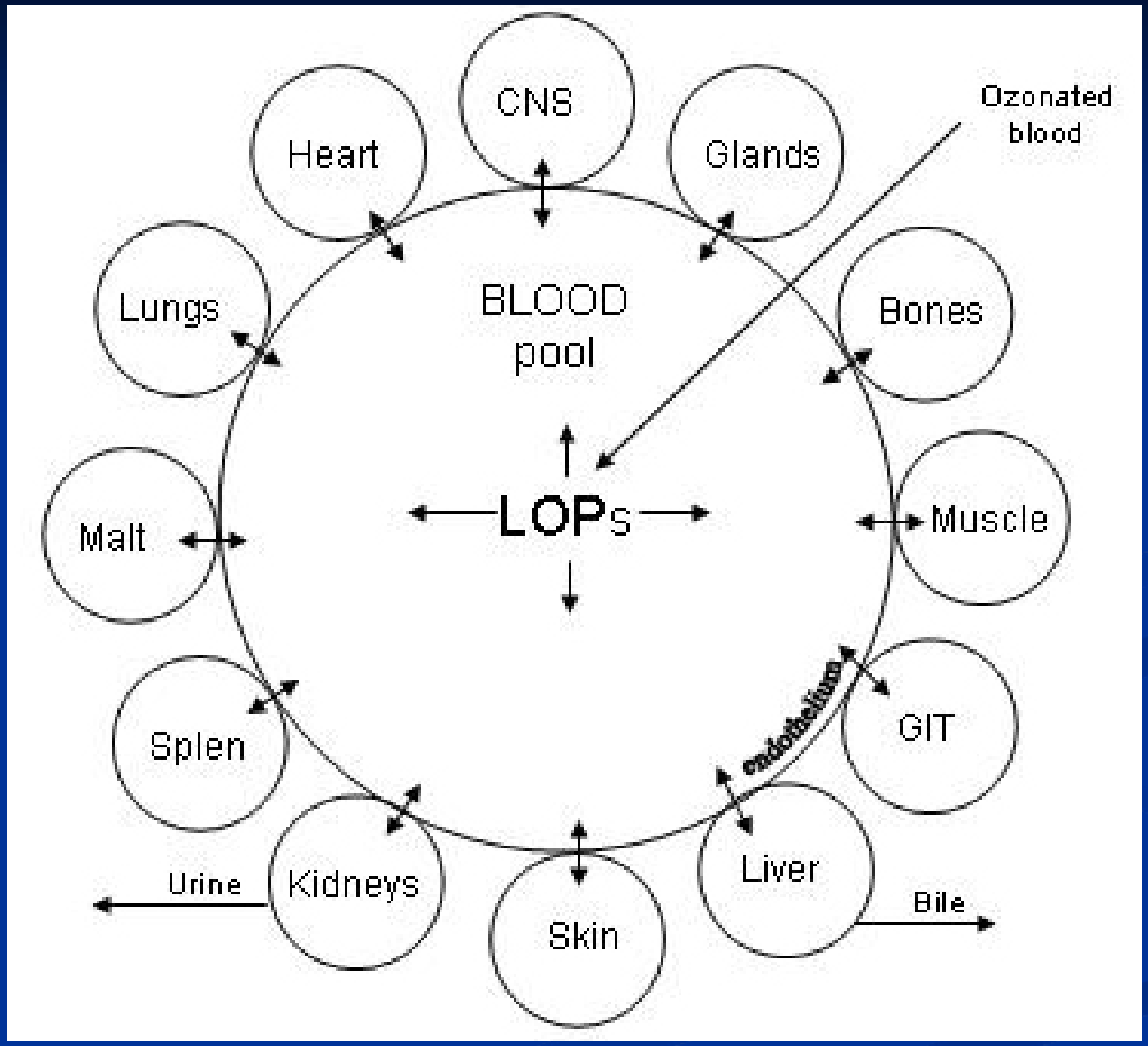


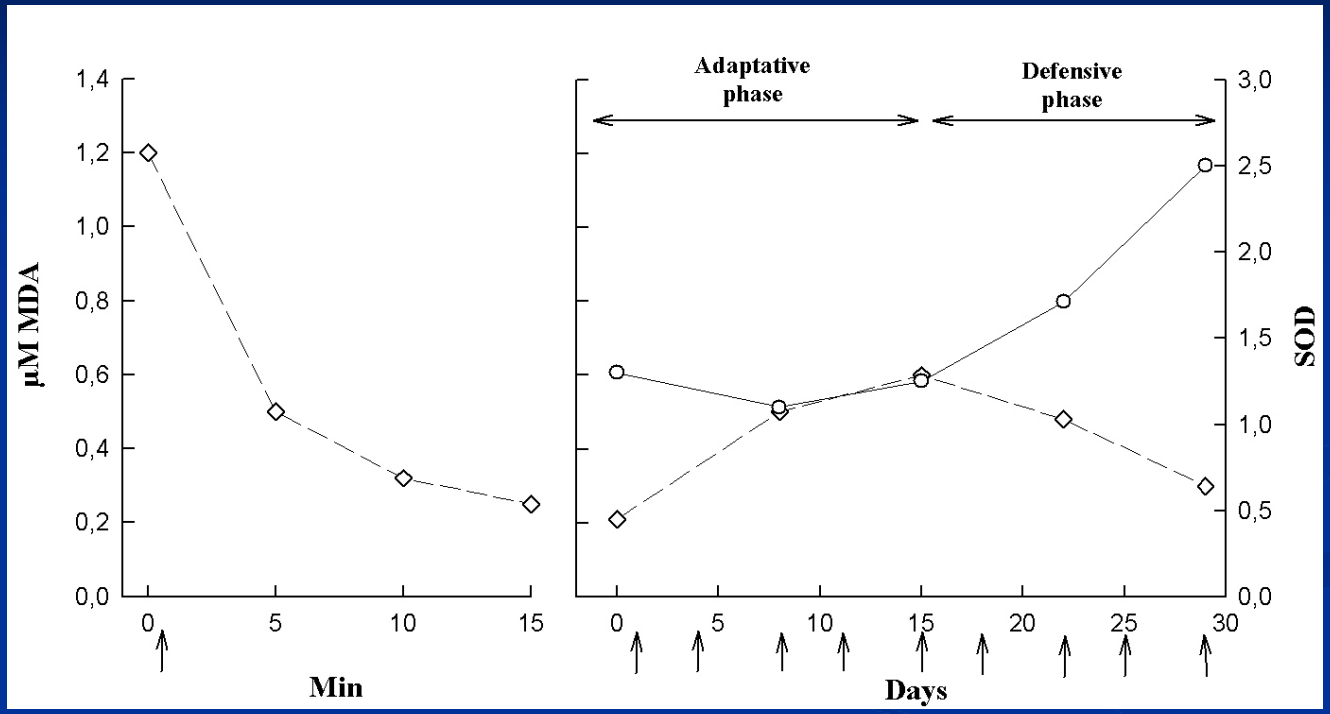
S. Sugio, A. Kashima, S. Mochizuki, M. Noda, and K. Kobayashi
Crystal structure of human serum albumin at 2.5 Å resolution
Protein Eng. 1999 12: 439-446; doi:10.1093/protein/12.6.439

THE PHARMACOKINETIC AND FATE OF FREE ALDEHYDES AND ADDUCTS

1. **FORMATION OF ALBUMIN-HNE ADDUCTS**
   Assuming to ozonate 200 ml of blood with an ozone dose of 8 mg, the presence of about 4-5 g of albumin can easily bind all the formed HNE.
2. **DILUTION IN THE PLASMATIC AND EXTRAVASCULAR ALBUMIN POOL**
   During the infusion of the ozonated blood into the patient, the albumin-HNE adducts will firstly dilute within the intravascular albumin pool represented by about 130 g albumin and then with the extravascular pool containing about 170 g albumin. Thus, in a total body pool of about 300 g albumin, the ozonated aliquot is less than 1.1%
3. **DETOXIFICATION**
   This is operated very rapidly inside billions of cells by available GSH and at least three enzymes such as aldehyde dehydrogenase, aldose reductase and glutathione S-transferase.
4. **EXCRETION**
   HNE metabolized as mercapturic acid had been detected in urine and in bile after hepatic detoxification.

THESE FOUR PROCESSES INDICATE THAT HNE PRODUCED DURING BLOOD OZONATION WILL EVENTUALLY INFORM CELLS AT SUBMICROMOLAR LEVELS, INDICATING ITS IMPORTANT ROLE AS LONG-ACTING MESSENGER ABLE TO INDUCE UPREGULATION OF ANTIOXIDANT ENZYMES AND HEME-OXYGENASE-1 OR, IN OTHER WORDS, TO COUNTERACT THE CHRONIC OXIDATIVE STRESS INDUCED BY DIABETES, ATHEROSCLEROSIS ETC


CNS
Heart
Glands
Ozonated blood
Lungs
BLOOD pool
Bones
LOPs
Malt
Muscle
Splen
endothelium
GIT
Urine
Kidneys
Liver
Skin
Bile



An AMRD patient's response to a single (left side) or intermittent (right side) infusion of O_3-AHT (300 g blood treated with an ozone dose of 21 mg per session). MDA, malonyldialdehyde (◊) and Mn-SOD (U/ml plasma, O) are reported on the ordinate. Arrows indicate the time of blood reinfusion

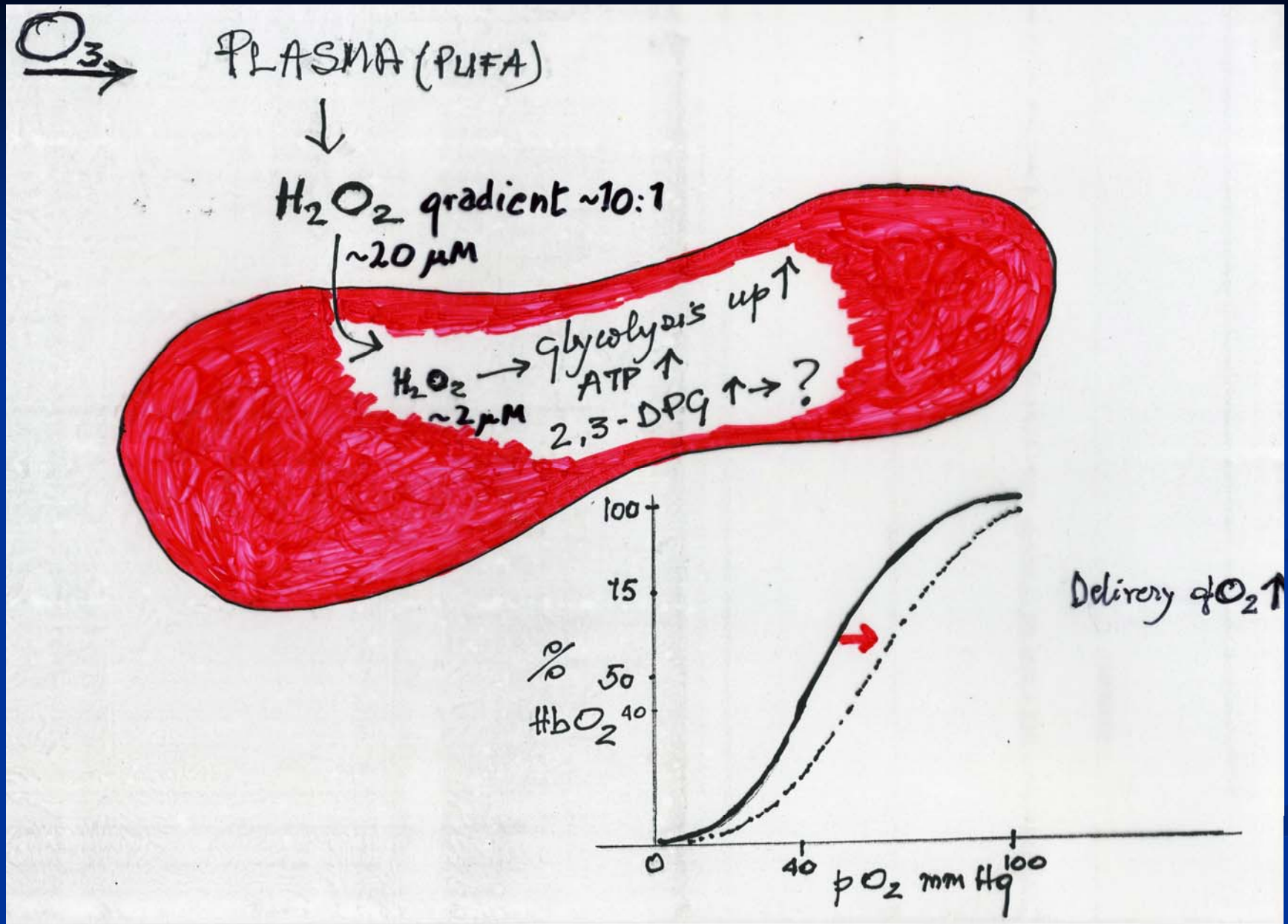
Table 2
Determination of plasma levels (mean ± S.D.) of the following parameters on whole blood samples immediately after exposure to argon, oxygen and progressively increasing ozone concentrations

Gas treatment	Fibrinogen (mg/dL)		Cholesterol (mg/dL)		Triglycerides (mg/dL)		HDL (mg/dL)		LDL (mg/dL)	
	Heparin	Citrate	Heparin	Citrate	Heparin	Citrate	Heparin	Citrate	Heparin	Citrate
Ar	272 ± 29.5	260 ± 11.2	156 ± 8.5	150 ± 10.7	61 ± 19.3	44 ± 6.8	63 ± 5.7	61 ± 8.5	75 ± 10.1	72 ± 3.1
O_2	260 ± 28.1	263 ± 3.5	156 ± 9.4	150 ± 11.4	62 ± 18.8	43 ± 4.4	64 ± 5.7	62 ± 7.7	76 ± 9.4	70 ± 6.3
20	255 ± 31.8	254 ± 7.0	156 ± 7.1	156 ± 7.5	62 ± 19.1	45 ± 4.4	64 ± 4.9	63 ± 8.2	76 ± 7.4	73 ± 11.2
40	250 ± 24.3	264 ± 10.4	157 ± 8.8	158 ± 9.2	64 ± 19.3	48 ± 5.1	62 ± 6.5	63 ± 9.3	76 ± 6.6	76 ± 6.6
80	248 ± 27.5	268 ± 5.7	158 ± 11.0	157 ± 2.5	65 ± 19.0	48 ± 6.4	61 ± 5.0	65 ± 7.9	77 ± 8.8	78 ± 6.8
160	262 ± 7.0	262 ± 10.1	156 ± 8.1	159 ± 5.7	61 ± 19.6	49 ± 6.6	63 ± 4.5	64 ± 7.6	75 ± 8.0	79 ± 6.4

Table 3
Enzymatic levels (U/g Hb) in human blood after exposing blood samples to either O_2 or O_3

Treatment	Enzymatic levels (U/g Hb)			
	SOD	GSH-Px	GSH-Rd	G6PDH
Ar	830.2 ± 249.6	24.4 ± 5.8	2.1 ± 0.5	5.2 ± 2.4
O_2	836.9 ± 293.6	19.1 ± 5.8	2.0 ± 0.5	5.3 ± 2.4
20	726.8 ± 197.5	25.2 ± 6.4	2.0 ± 0.6	5.7 ± 2.6
40	694.7 ± 140.9	25.8 ± 6.1	2.1 ± 0.5	5.2 ± 2.7
80	670.8 ± 185.5	27.2 ± 6.1	2.1 ± 0.4	5.6 ± 3.0

Values represent mean ± S.D. ($n = 6$).


O3
PLASMA (PUFA)
H2O2 gradient ~10:1
~20 μM
H2O2 → glycolysis up ↑
~2 μM
ATP ↑
2,3-DPG ↑ → ?
100
75
%
50
HbO2
40
0
40
100
pO2 mm Hg
Delivery of O2 ↑

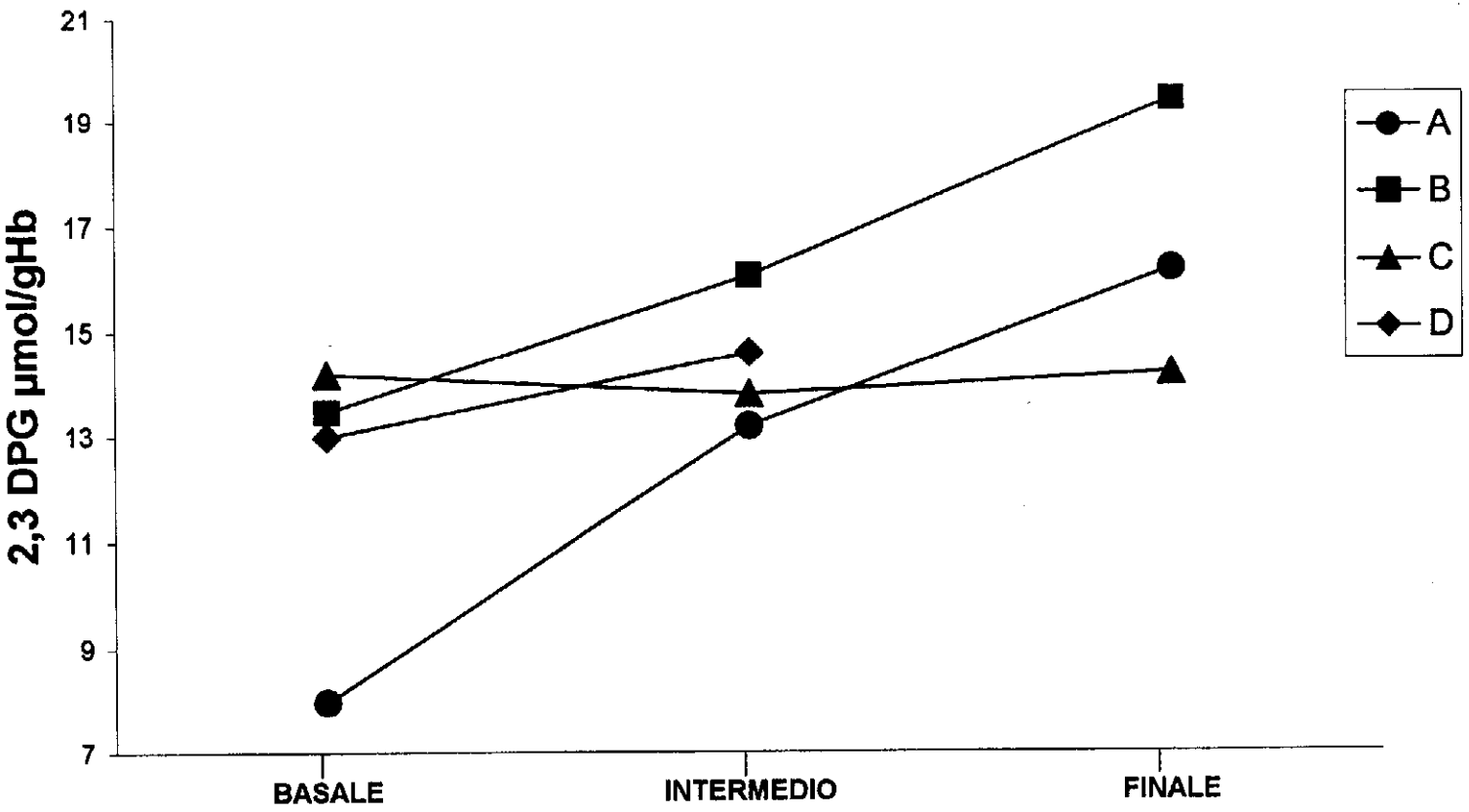
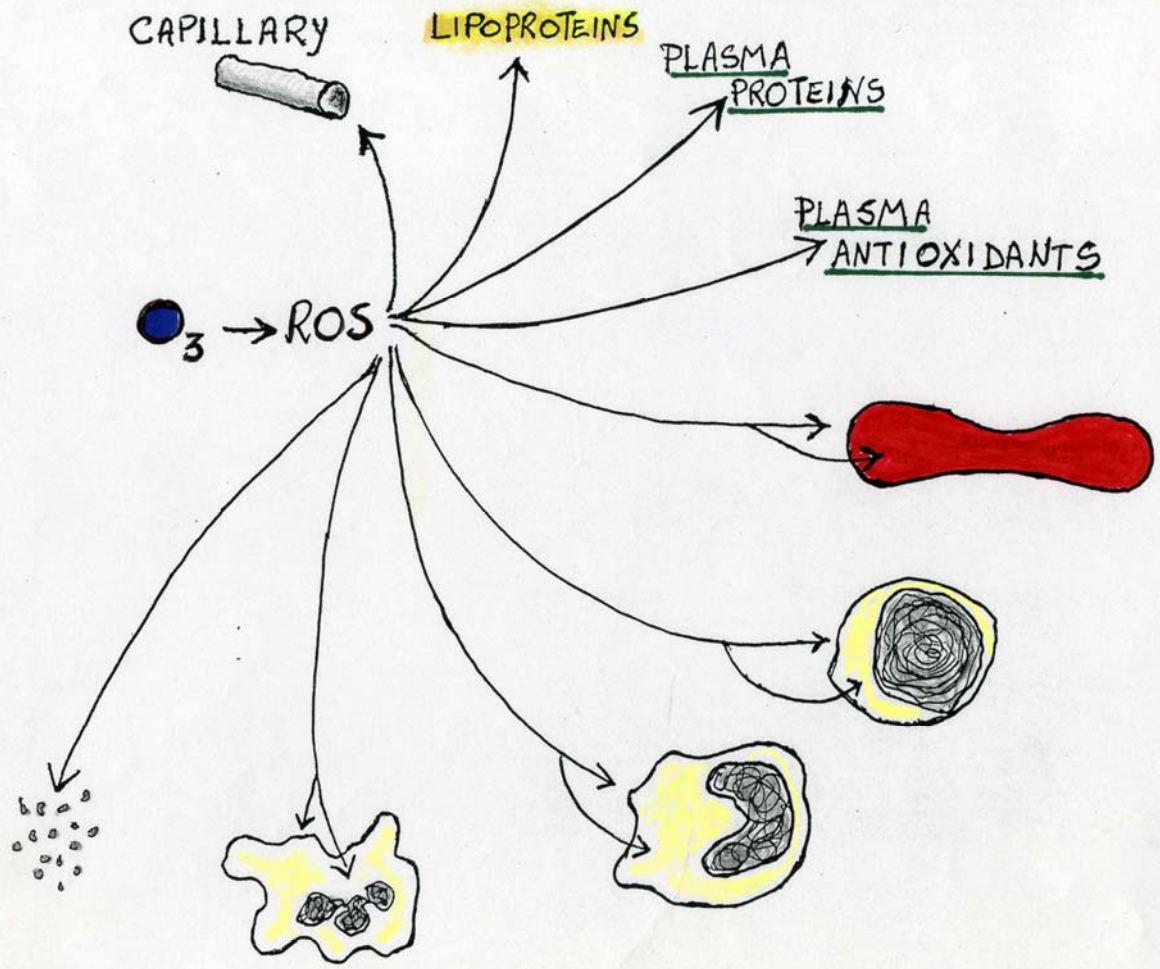
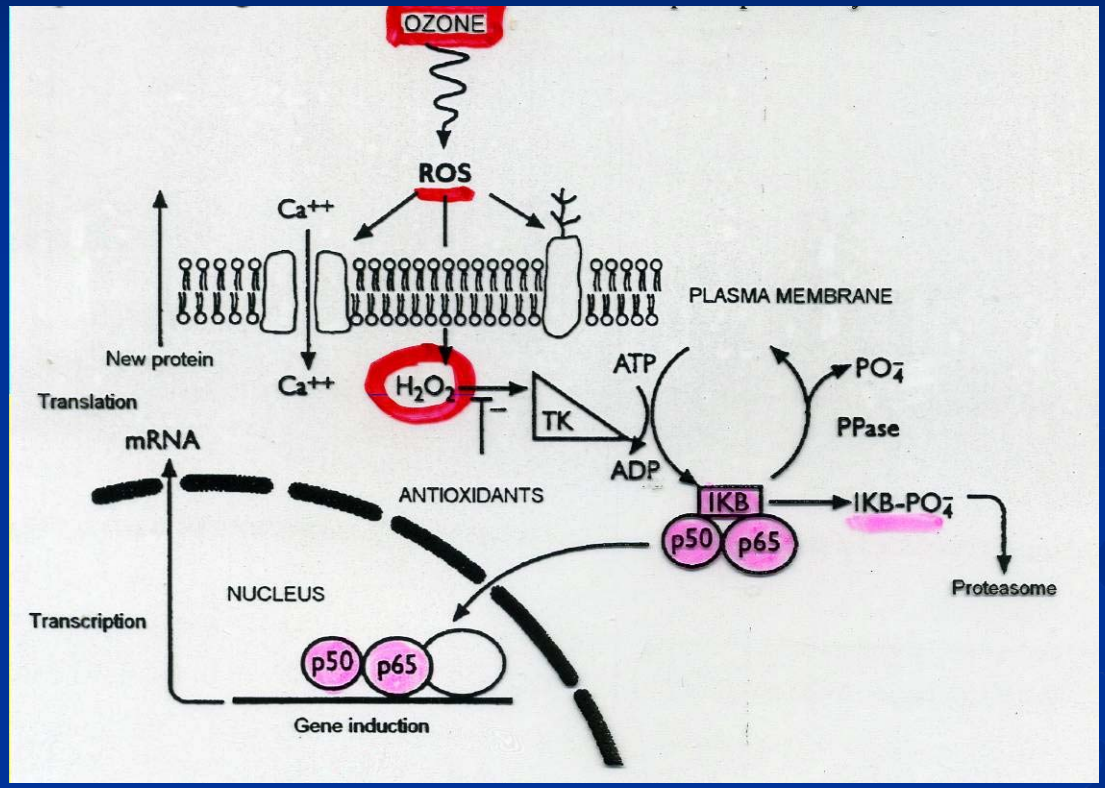


Figura 39: Variazione dei livelli di 2,3 DPG in 4 pazienti, nel corso di terapia con EBOO. Sono riportati i livelli basali (BASALE), dopo circa 6-7 trattamenti (INTERMEDIO), e alla fine dei trattamenti (FINALE).

SITES OF ACTION OF OZONE IN BLOOD



IN THE CELL, THE EFFECTIVE STIMULUS MATERIALIZES WHEN THE CONCENTRATION OF ROS *transiently* SURPASSES THE CAPACITY OF THE CELLULAR ANTIOXIDANT DEFENCE SYSTEM


OZONE
ROS
Ca++
PLASMA MEMBRANE
New protein
Translation
Ca++
H_2O_2
ATP
PO_4^-
TK
PPase
mRNA
ADP
ANTIOXIDANTS
IKB
IKB-PO_4^-
p50
p65
Proteasome
NUCLEUS
Transcription
p50
p65
Gene induction

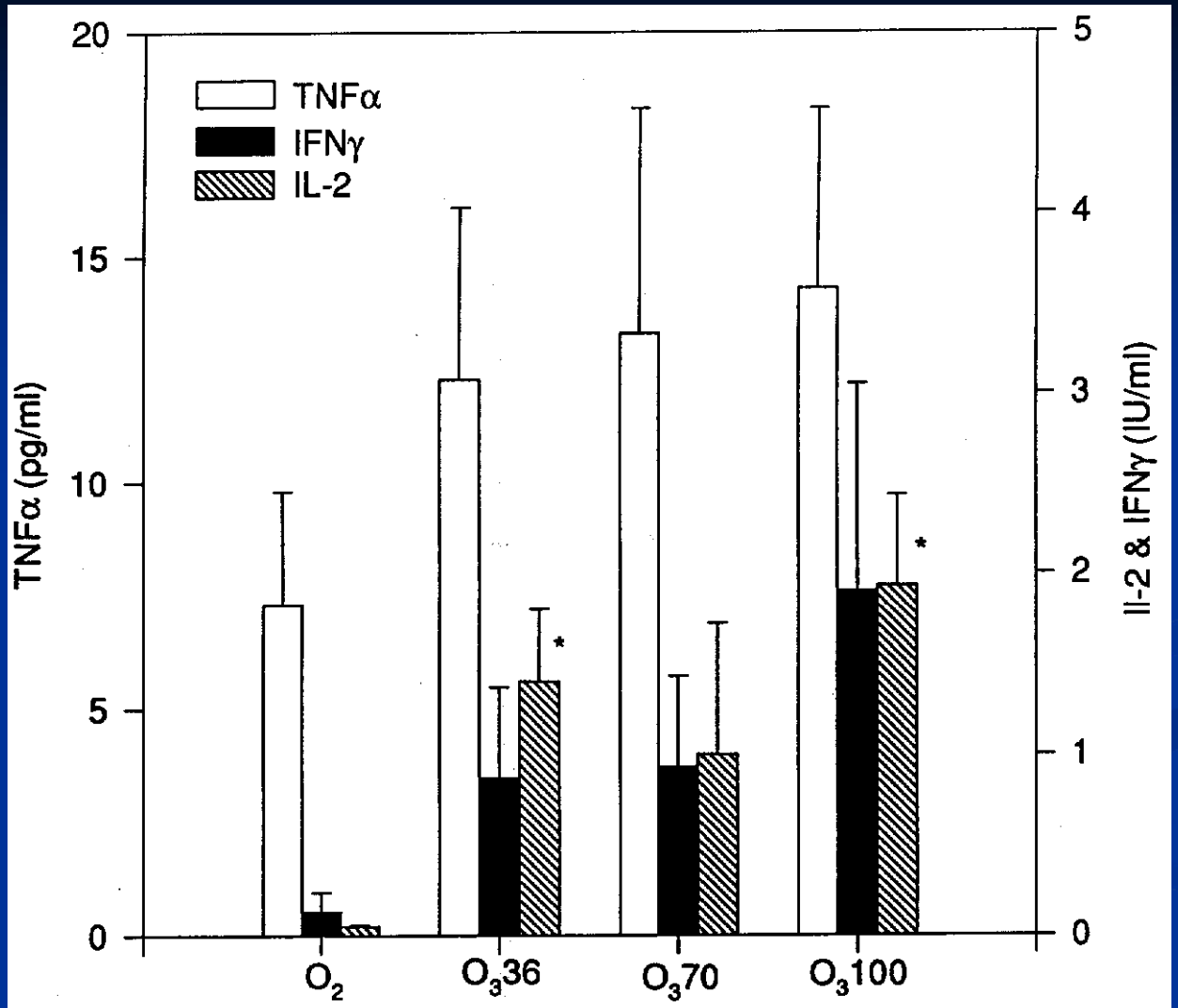


Fig. 5 Effect of different doses of O_3 (36, 70 and 100 μg/ml per g blood) on the production of TNFα, IFNγ and IL-2 by human blood samples (n=3) after incubation for 9 hours.

*Significant difference ($P < 0.05$) compared with samples treated with O_2.

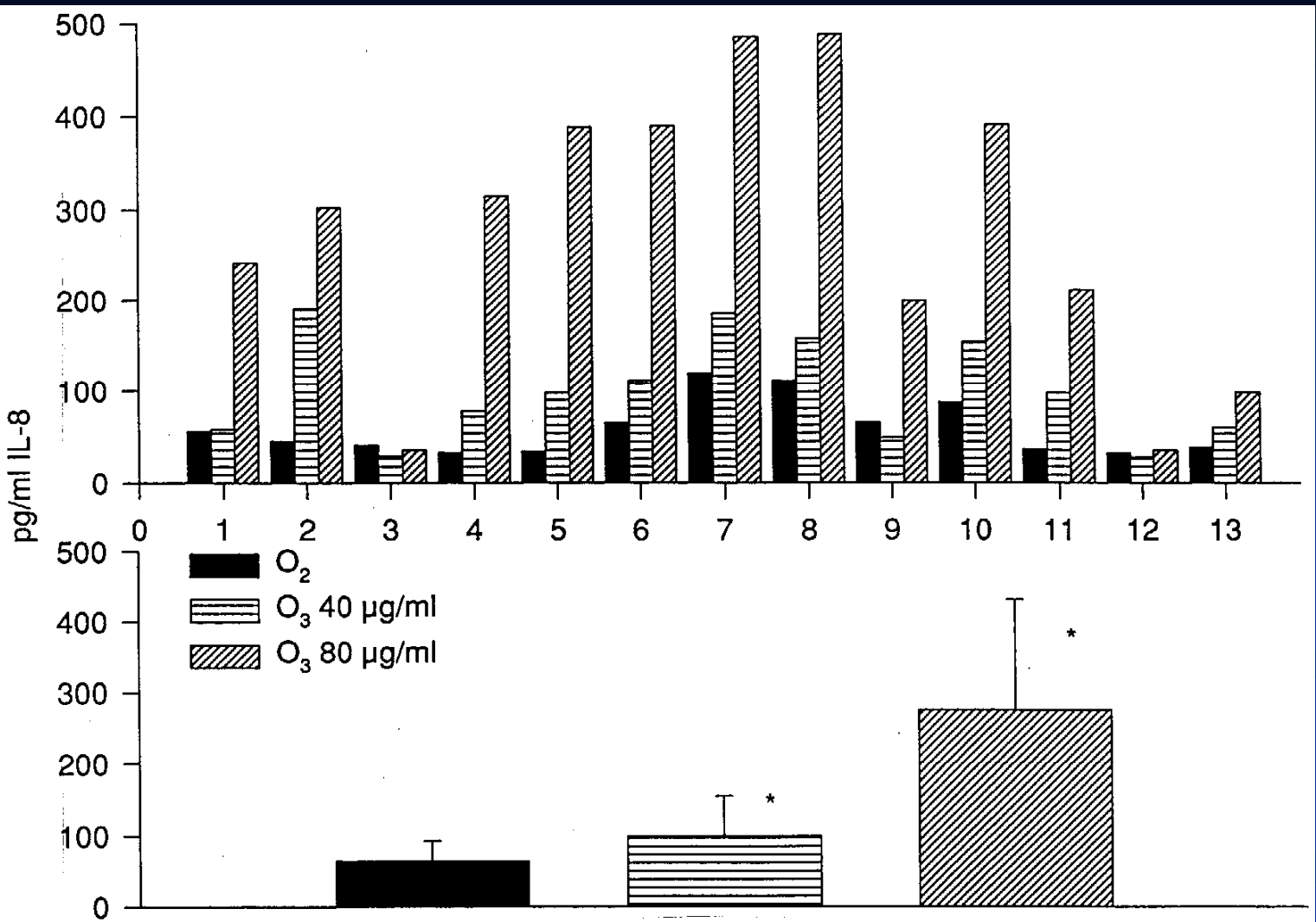
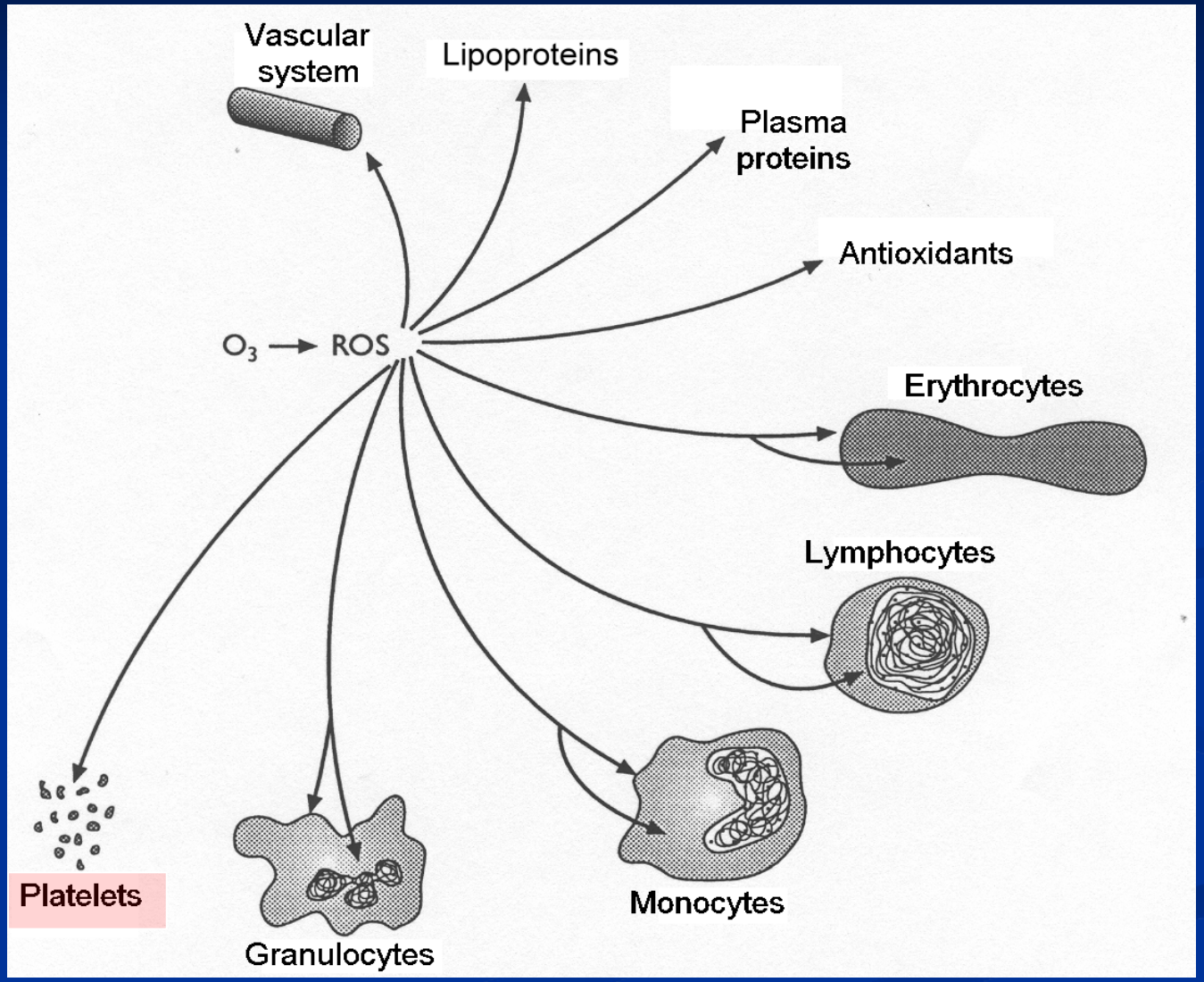


Fig 4 Effect of 1 min exposure of either O_2 or O_3 (40 µg/ml) or O_3 (80 µg/ml) on the production of IL-8 after 8-hours incubation of the same thirteen blood samples of Fig. 2 and 3. Average values are reported in the lower panel after subtracting control values.

*Significant difference ($P < 0.01$) compared with samples treated with O_2.


Vascular system
Lipoproteins
Plasma proteins
Antioxidants
O_3 → ROS
Erythrocytes
Lymphocytes
Platelets
Granulocytes
Monocytes

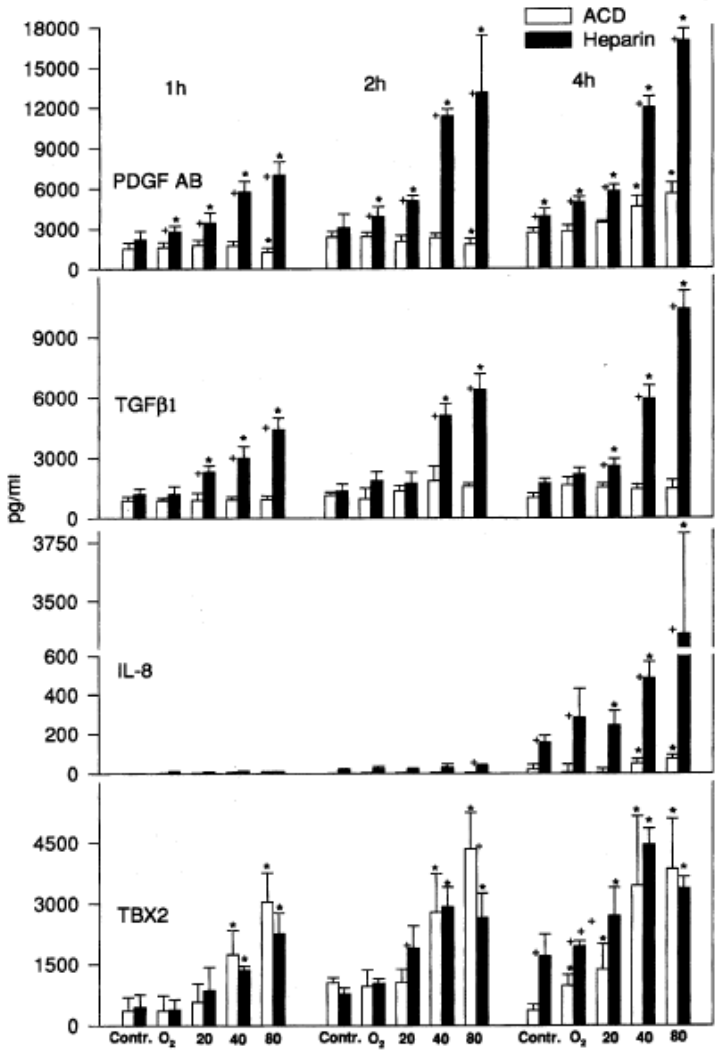
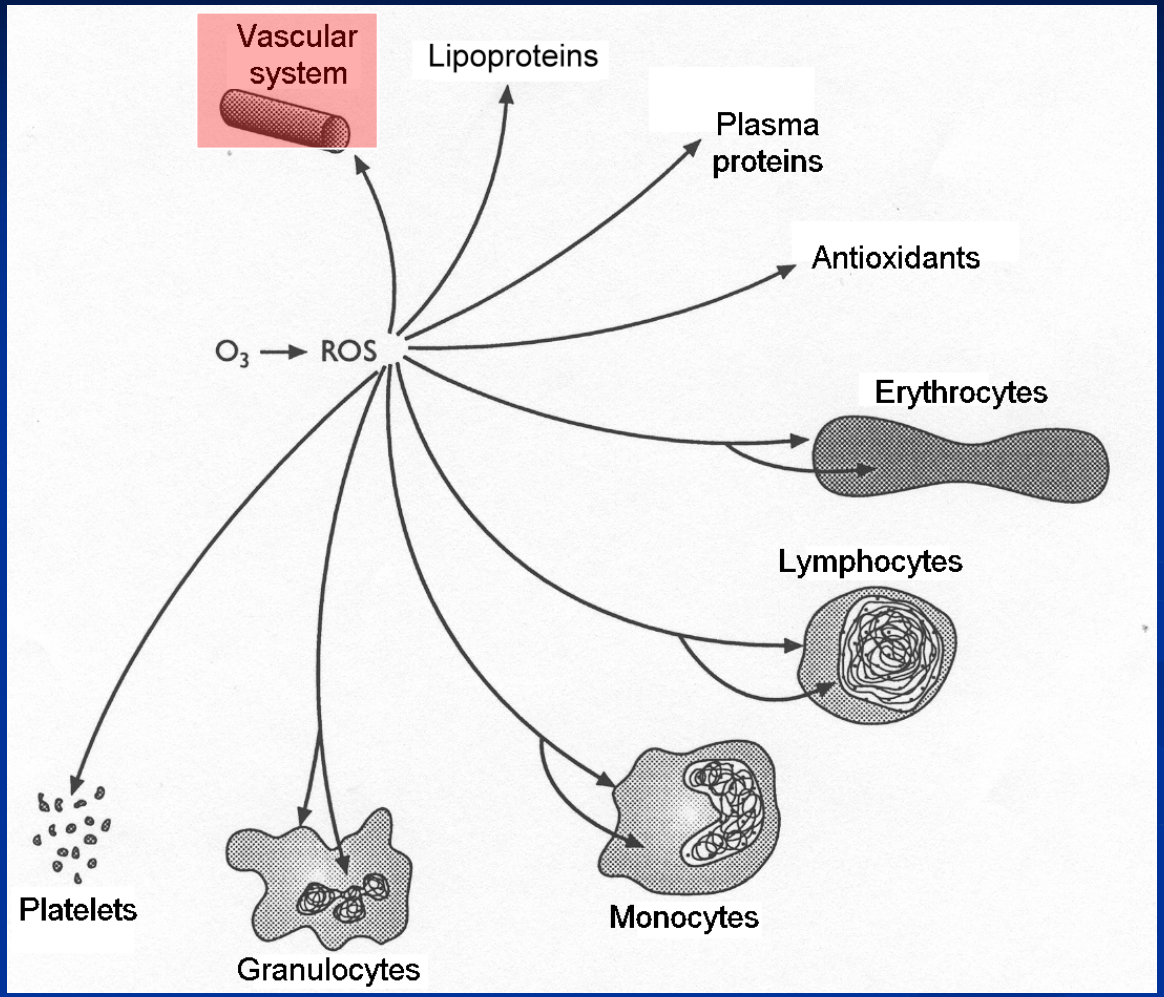
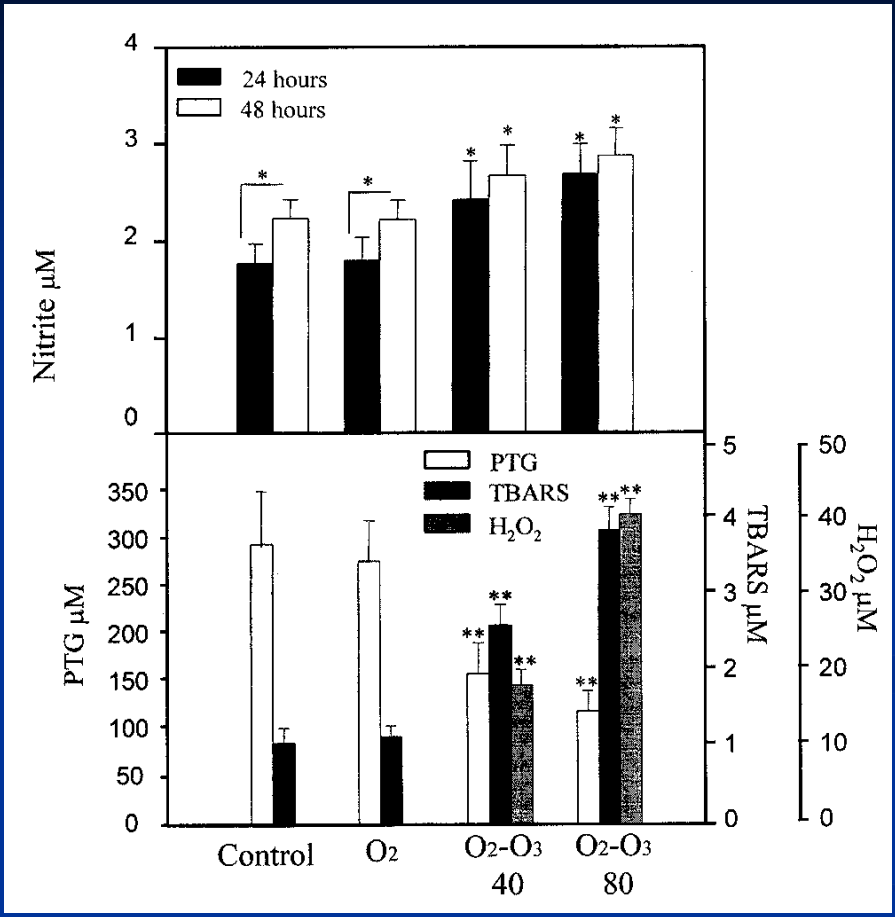


Figure 65. Release of factors from human platelets during 1, 2 and 4 hr incubation. The same PRP samples collected either in heparin or ACD were not exposed (control), or exposed to O_2 alone or to O_2-O_3 at concentrations of 20, 40 and 80 μg/ml for 30 sec before incubation. Statistical significance is indicated by (*) for intergroup analysis and (+) for intragroup analysis

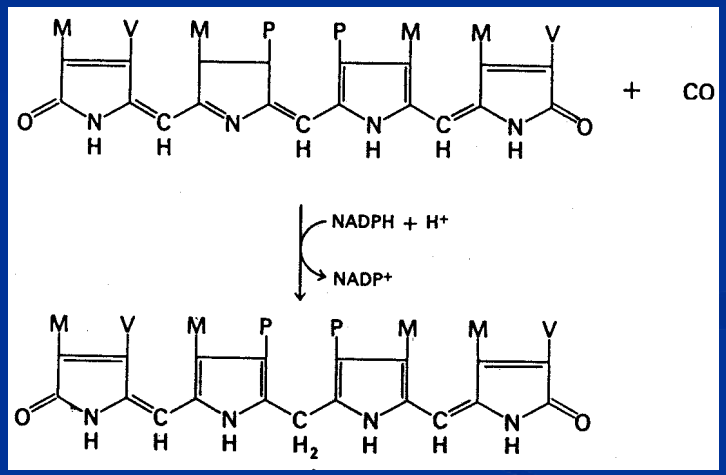

Vascular system
Lipoproteins
Plasma proteins
Antioxidants
O_3 → ROS
Erythrocytes
Lymphocytes
Platelets
Granulocytes
Monocytes



Effect of different concentrations of ozone on the production of nitrite by HUVECs, 24 and 48 hr after addition of ozonized human serum (top panel). Effect of either oxygen or ozone on PTG, TBARS and H_2O_2 levels in the serum before addition to HUVECs. The data are presented as the M ± SD of 6 different experiments

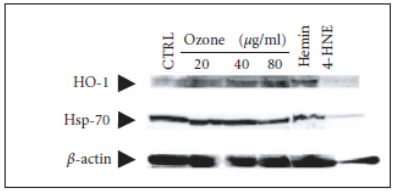
Haeme oxygenase I
HO-I
(HSP-32) → HAEME group from haemoglobin → Biliverdin +CO +Fe^{++}

Biliverdin reductase →

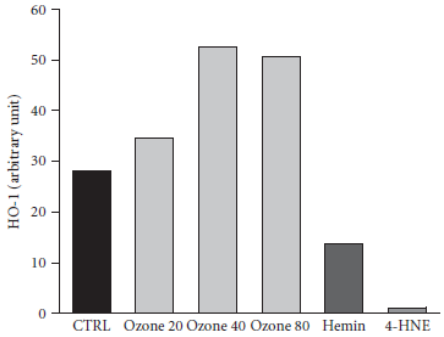


2 $ROO^{\bullet}$ + bilirubin → 2 ROOH + biliverdin

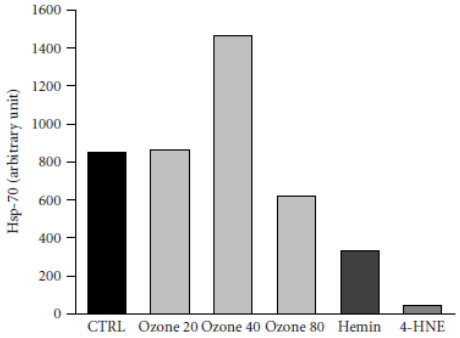
+ H^{+}



(a)



(b)



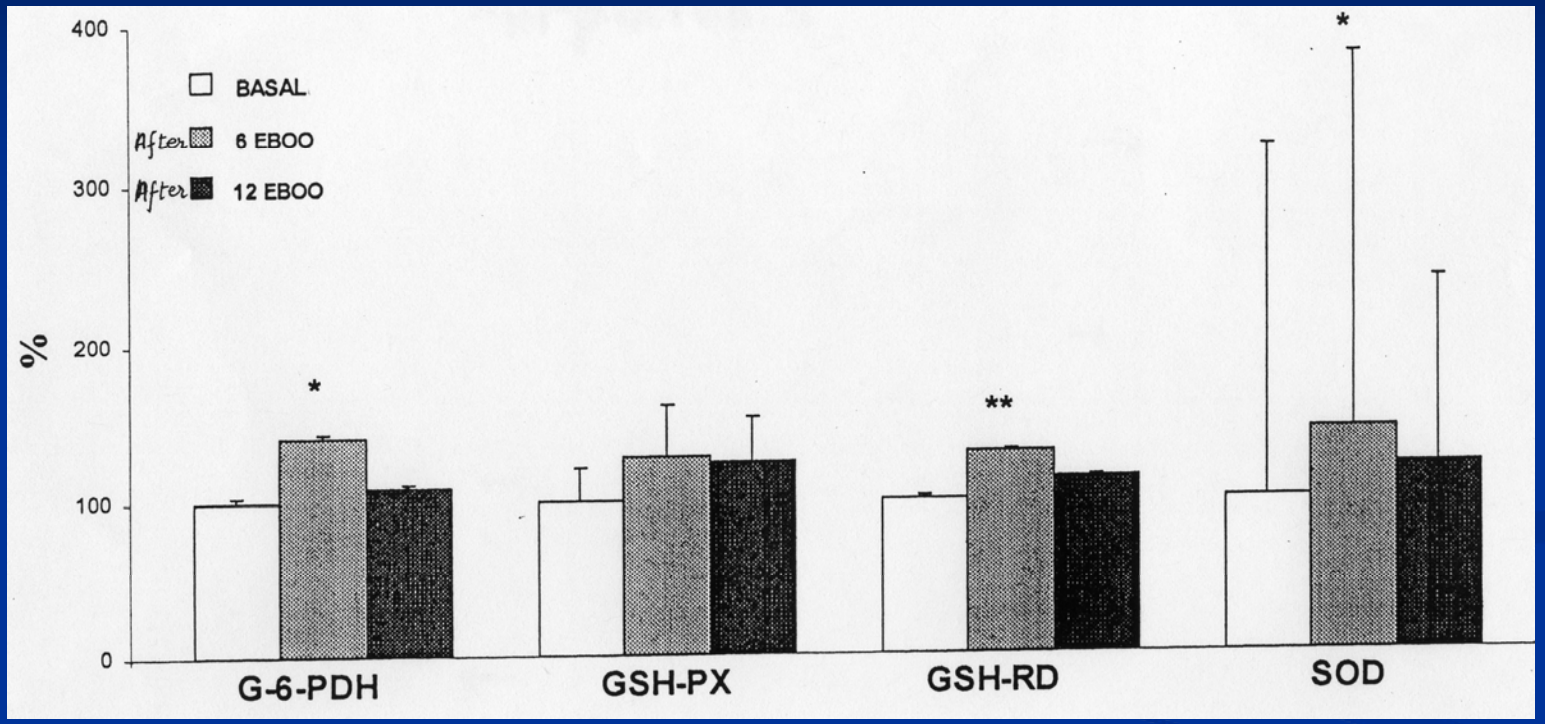
(c)

FIGURE 2: Ozonated human plasma, particularly at the medium ozone concentration of 40 μg/mL per mL of blood activates both HO-1 and Hsp-70 in human endothelial cells. The signals of both protein levels were determined by densitometric analysis of the scanned images. Data are expressed as arbitrary units. One representative Western blot of a typical experiment is shown in the top panel.

Evaluation of G6PDH activity in total, "young" and "old" red blood cells in ARMD patients after an ozonetherapy cycle (mean ± standard deviation) n=4.

	G6PDH activity +			% "Young"	% "Old"
	Total RBCs	**"Young" RBCs**	**"Old" RBCs**	**RBCs***	**RBCs***
Before treatment	356.8 ±90.7	550.3 ±157.5	310.7 ±127.3	3.1 ±2.8	96.9 ±2.8
After treatment	406.2 ±40.4	748.2 ±181.9	434.8 ± 86.7	3.4 ±3.0	96.6 ±3.8

+G6PDH activity expressed as nmoles/h/mg Hb hi whole erythrocyte population and in young and old fractions before and after 13 O_2/O_3 treatments;
*percentage of young and old erythrocytes obtained from whole blood by isopycnic


400
300
200
100
0
%
BASAL
After 6 EBOO
After 12 EBOO
*
**
*
G-6-PDH
GSH-PX
GSH-RD
SOD

2ND PART
NEW METHODOLOGIES AND PROBLEMS

FOLLOWING THE OPEN LETTER OF PROF. E. NAZAROV AND MY ANSWER AND SOME WORRYING INFORMATION FROM Dr . A. SCHWARZ

It is useful to continue the discussion on the issues of clarifying and using the best technological advances

1) HAS THE POSTULATION THAT A MINOR AHT PERFORMED WITH BLOOD SUBJECTED TO A SUPER-OZONE OXIDATION, PLUS UVI AND PLUS HEAT STRESS USED IN CHD COMPROMISED THE FUTURE OF A CORRECT OZONETHERAPY

The procedure used an expensive device (VC7000A system, Celacade™, Vasogen Inc, Mississauga, ON, Canada) where it was able to deliver an enormously toxic dose of ozone (107.5 mg per mL of blood) plus a further blood oxidation due to UV irradiation at 42.5 °C. The final ozone dose is about 15.000-fold higher than the average ozone dose used during the classical ozonated autohemotherapy [69] and the extremely high oxidation of blood causes a complete denaturation of blood components [4]. Two IM treatments were given on consecutive days, followed by a third on day 14. Subsequent treatments were given at 4 week (28 days) intervals for at least 22 weeks, for a total of 8 injections.

2) Plastic bags regularly used for blood storage are unsuitable as, in the presence of ozone, they release phthalates and plastic microparticles into blood. Neutral glass bottles are idoneous.

3) A German ozone generator producer publicizes the use of a direct IV infusion of oxygen-ozone. This procedure is probably used by naturopathics or charlatans . It has not a rationale and is potentially dangerous. It will further discredit ozonetherapy.

4) The same producer publicizes the use of a direct intraperitoneal insufflation of oxygen-ozone in human cancer before a definite pre-clinical data. In no-expert hands, it may be dangerous and useless.

5) It has now become fashionable to use the IV infusion of ozonated saline

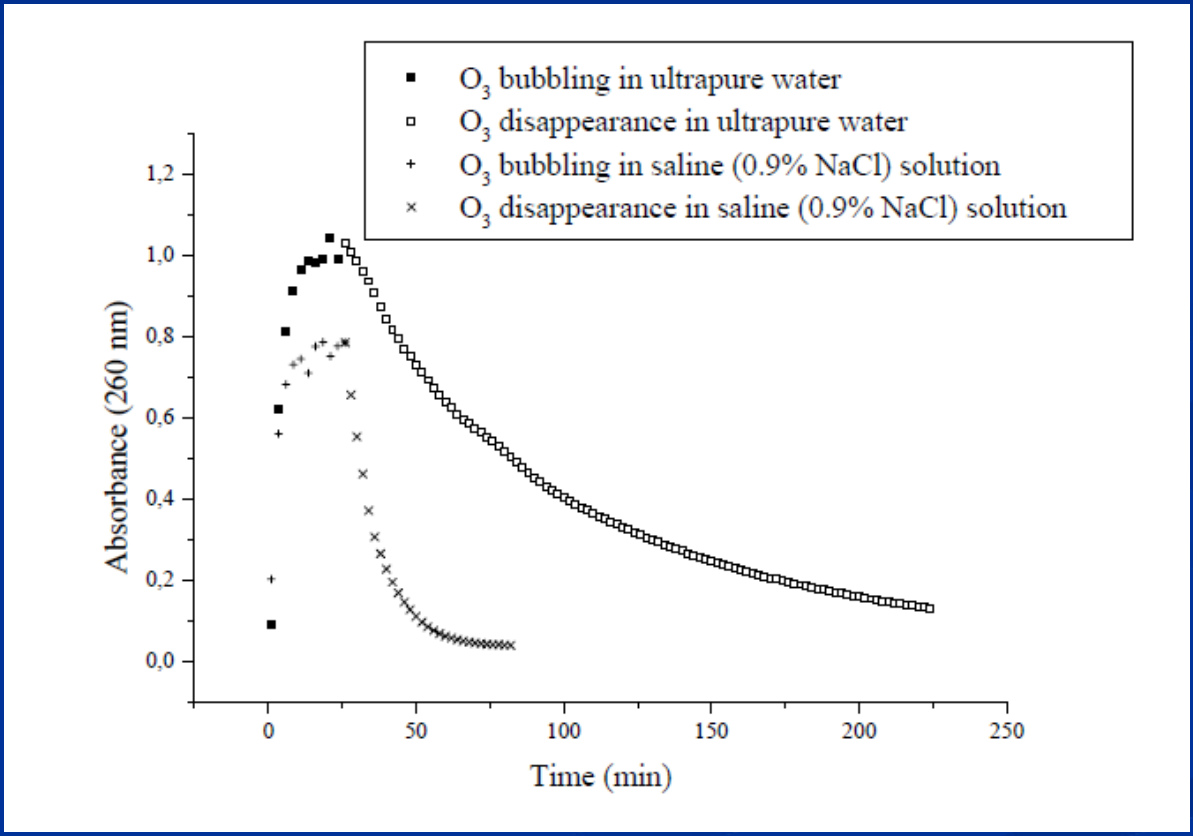
As a physician, having practised for years in a charity clinic, I vividly remember how busy an expert dialysis technician and myself were in performing no more than a dozen O_3-AHTs in a afternoon.

In the case of Cubans, to apply the rectal O_3 insufflation to all patients, which, for several reasons, is another unreliable approach.

It is unfortunate that the practice of using ozonated saline has become common in Russia and is widely used because it is inexpensive and less time-consuming than major AHT and simultaneously applicable to many patients. As it could be foreseen, physicians have started to use it also in Italy, Spain, Greece and Turkey. Ikonomidis et al. (2005) in Greece, have reported that they maintain the saline solution under a constant flow of O_3 during transfusion but they warned that the maximum amount of O_3 daily administered is usually 4-5 mg and should never exceed 8-10 mg. In their publication they also stated "if we exceed these rates, the over coagulation syndrome starts" and they strongly recommended to perform coagulation tests before starting therapy. These precautions reinforce our preliminary objection to this approach.Moreover, Foksinski et al. (1999) have measured 8-oxodeoxyguanosine, a typical oxidative DNAdamage in lymphocytes of atherosclerotic patients after the IV infusion of ozonated saline, that is aresult never detected after O_3-AHT.

Fortunately to the best of our knowledge, Russian physicians ozonize the saline with very low O3 concentrations (2-3 μg/mL) and this precaution certainly reduces toxicity but it leaves open the aspect of therapeutic efficacy.

However if the water contains NaCl, the extremely high reactivity of O_3 induces a complex series of reactions with the possible progressive formation of H_2O_2, unstable OCl- , $NaClO_4$, ·OH, $^1O_2$ and some unstable O_3. Razumovski, Ershov et al (2008); Bocci et al, (2009) have evaluated the complexity of O_3 reactions and rapidity of its decomposition. Here we enclose our diagram.



1) For human use it would be unwise to use O_3 concentration over 3 µg/mL (3 mg/L). Moreover it is essential **to establish the volume per minute of the gas mixture O_2-O_3.** The problem is that different ozone generators have variable gas output: if it is **1 L per minute**, the O_3 delivered to 200 mL of saline would be **3 mg/L** but, if the output per minute is equivalent to **3 litres of gas**, then the actual dose of O_3 delivered will be **9 mg/L**! As a consequence one must properly instruct the ozonetherapist in relation to the owned ozone generator as otherwise one risk to poison the patient.

2) **The period of ozonation time** also ought to be well defined in relation to the volume of saline because in the case of saline solution an ozonation time of **20 min** appears enough to reach a plateau.

3) Another aspect to be clearly defined **if gas bubbling will continue or not during the IV** infusion period. This is because, as soon as the gas bubbling is stopped, the concentration of **H_2O_2** remains fairly stable but the O_3 concentration will halve during the next 30 min and this affects the therapeutic result.

4) **Moreover the blood flow in the cubital vein varies considerably in different patients and in women and this implies that a fairly constant infusion of ozonated saline versus a variable blood flow and content of antioxidants implies an uncertain blood/H_2O_2**

5) Owing to the fact **that H_2O_2 is one of the most important ROS generated by O_3,** since 2005

CONCLUSIONS

This is a good opportunity to ponder on the future of ozonetherapy.
The FDA, although well and continuously informed, will not accept ozonetherapy unless randomized and controlled clinical studies performed with ozonated autohemotherapy and published in reliable peer-reviewed journals show the validity and atoxicity of this approach.

In Germany, ozonetherapy is accepted as a *NON CONVENTIONAL TREATMENT.*
Thus, in spite of good basic studies; we are still *in limbo.*
You may be satisfied with the results in your own practice but you must realize that official medicine disregards and objects our work.

THANKS FOR YOUR ATTENTION