



## Workshop

Protective / preventive role of bioactive food components in human health

# Measurements of oxidative stress in “in vitro” and “in vivo” systems



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IL PRESENTE MATERIALE È RISERVATO AL PERSONALE DELL'UNIVERSITÀ DI BOLOGNA E NON PUÒ ESSERE UTILIZZATO AI TERMINI DI LEGGE DA ALTRE PERSONE O PER FINI NON ISTITUZIONALI



## Biomarkers of oxidative stress

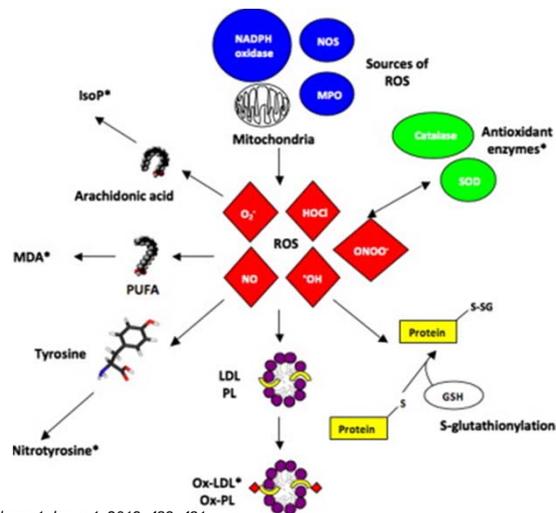
- Molecules that are modified by interactions with ROS in the microenvironment
- Molecules of the antioxidant system that change in response to increased redox stress.
- DNA, lipids (including phospholipids), proteins and carbohydrates are examples of molecules that can be modified by excessive ROS in vivo.



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## Formation pathways of selected biomarkers of oxidative stress



Ho et al. Redox Biology, Volume 1, Issue 1, 2013, 483–491

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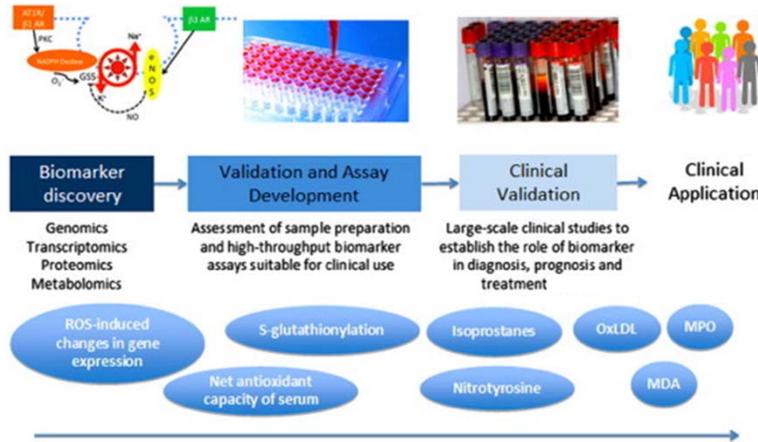
## Biomarkers of oxidative stress

- The **functional significance** or **causal role** of the oxidative modification on cell, organ and system function is recognized as a key determinant of the validity of the marker.
- Other factors influencing the clinical applicability of a ROS biomarker include:
  - ease of obtaining an appropriate biological sample
  - stability of the biomarker throughout various storage conditions and sample preparation steps
  - specificity,
  - sensitivity
  - reproducibility

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# Oxidative stress biomarker development



Schematic timeline of required steps in biomarker development, from discovery in the Laboratory to clinical application after validation in large scale clinical trials. Although many ROS biomarkers have reached clinical trials level, only few are regularly applied to patients in clinical practice.



# Markers of oxidative stress

## Oxidants

**Superoxide anions**  
**Hydroxyl radical**  
**Hydrogen peroxide**  
**Peroxynitrite**  
**Other radicals**

## Antioxidants

**Glutathione**  
**Ascorbate**  
**Alpha-tocopherol**  
**Total antioxidant capacity**

## Oxidation products

**Protein carbonyls**  
**Isoprostanes**  
**Nitrotyrosine**  
**8-OH-dG**  
**4-hydroxy-nonenal**  
**Malondialdehyde**

## Antioxidant/Pro-oxidant balance

**GSH/GSSH ratio**  
**Cysteine redox state**  
**Thiol/disulfide state**  
**Other?**



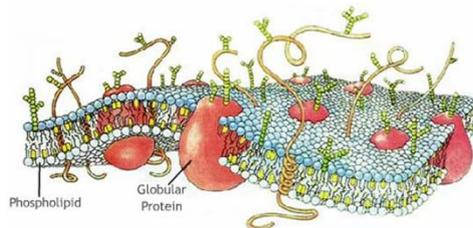
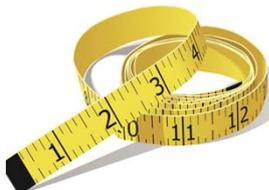
## Where to measure oxidative stress?

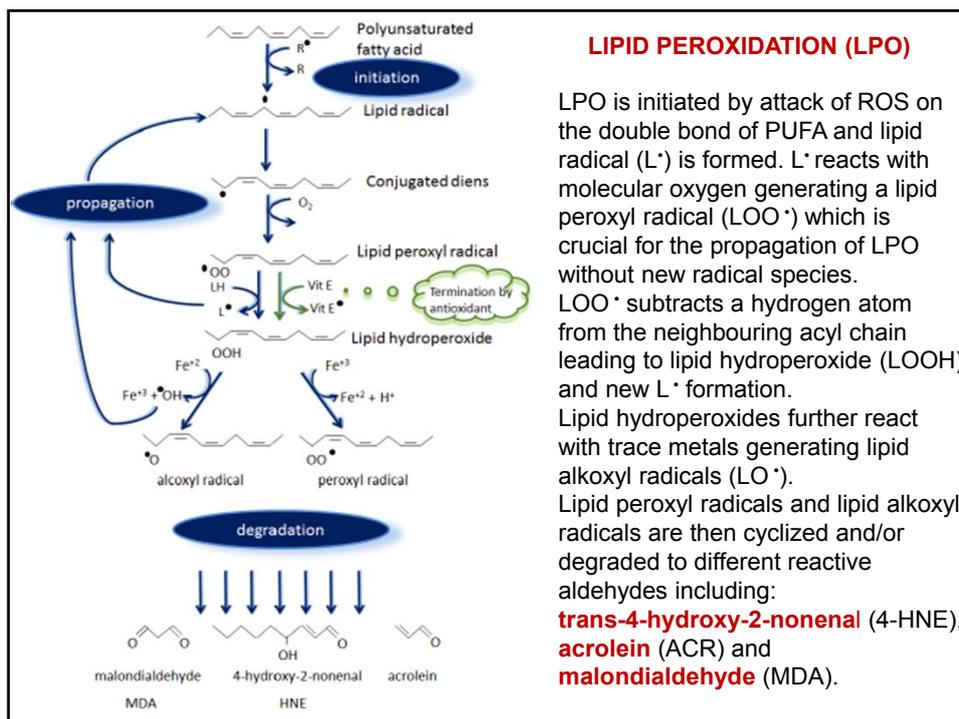
- Plasma/Serum 
- Sputum 
- Urines
- Tissue
- Cells

Sputum collections is a noninvasive procedure and, thus, it is more likely to be used in the clinical practice. These technique is safe, and can be repeated within a relatively short period of time and no specialized operators are required.



## Biomarkers of Lipid peroxidation





**Measure of MDA**

- Analytic methods for measuring MDA can be divided into:
  - **derivatization-based**
  - **label-free methodologies**
- These methods have been further coupled to separation techniques such as liquid chromatography (LC) and gas chromatography (GC).

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## Label-free methodologies

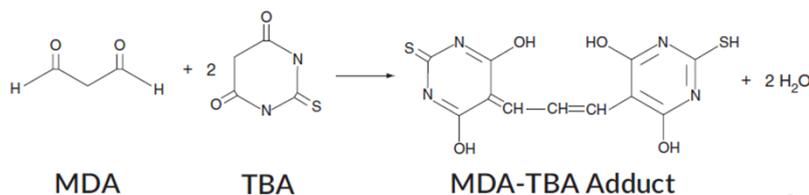
Among label-free techniques, the simple ultraviolet (UV) absorbance-based method has poor sensitivity and specificity.

Other separation techniques such as reverse-phased LC or LC-tandem mass spectrometry (MS/MS) are more advanced and accurate techniques.



## Derivatization-based method

- The **thiobarbituric acid (TBA) assay** is the most frequently applied methods.
- It consists in the condensation of two molecules of TBA with one molecule of MDA that gives a colored reaction product. This compound can then be easily measured spectrophotometrically (530-540 nm) or by fluorescence detection ( $\lambda_{\text{ex}}=532$  -  $\lambda_{\text{em}}=553$  nm).





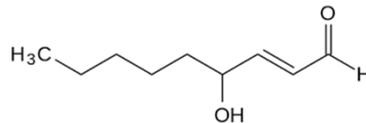
## Limitation of the method

- TBA assay is not specific for MDA, and in complex biological systems including many compounds can react with TBA to produce colored adducts.
- The assay should be combined, for example, with LC separation and fluorescence detection of the formed product.
- Other novel approaches for the specific determination of MDA include hydralazine-based derivatization methods that are again coupled with high-performance liquid chromatography (HPLC)



## Measure of 4-hydroxy-2-nonenal (4-HNE)

- **4-HNE** is a lipid peroxidation product derived from oxidized  $\omega$ -6 polyunsaturated fatty acids such as arachidonic acid.

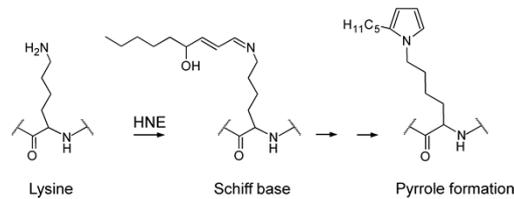


Mainly detected by ELISA assay with specific monoclonal antibody



## Measure of 4-HNE-His

- HNE has been shown to be capable of binding to proteins and forming stable adducts, also termed advanced lipid peroxidation end products.
- HNE can cause both structural and functional changes of oxidized proteins.



- Different ELISA kit measure HNE-His adduct

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## Isoprostane

- **IsoPs are a family of stable, prostaglandin-like compounds** generated from the peroxidation of arachidonic acid.
- IsoPs are subsequently released from the cell membrane into circulation by phospholipases, and can then be quantified in tissues, blood and urine.
- F<sub>2</sub>-IsoPs, so called because they contain F-type prostane rings, are the most stable of the IsoPs family and show the most potential as a biomarker.

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## Measure of isoprostane

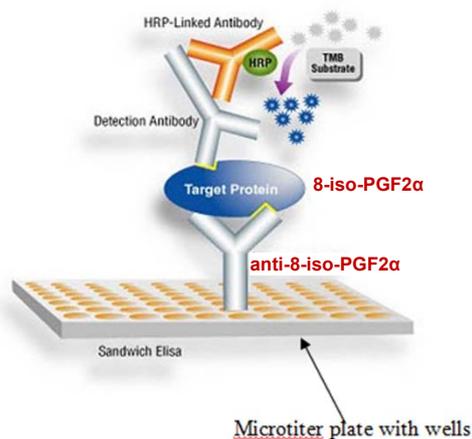
- IsoPs can be measured using gas chromatography–mass spectrometry (GC/MS), liquid chromatography–mass spectrometry (LC/MS), enzyme-linked immunosorbance assays (ELISA) and radioimmunoassay in plasma and urine samples.
- Mass spectrometric techniques are the gold standard for IsoP quantification.
- A small study revealed that the concentrations of F2-IsoPs in human plasma measured by GC/MS at 0 and 24 h ex vivo were similar, but significant ex vivo artefactual generation of F2-IsoPs occurred in plasma stored on ice for 36 h.

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## Measurment of isoprostanes

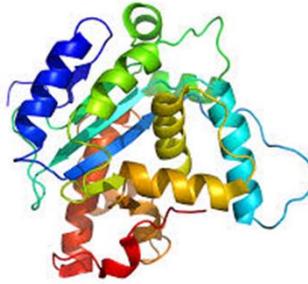
8-iso-PGF<sub>2</sub>α standards or treated samples are added to the well. Then a secondary antibody-HRP conjugate is added. After a substrate to the HRP is added. The HRP activity results in color development that is directly proportional to the amount of 8-iso-PGF<sub>2</sub>α bound to the plate



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## Biomarkers of oxidative protein modification

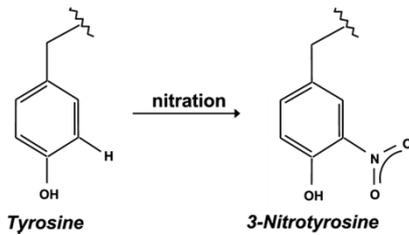
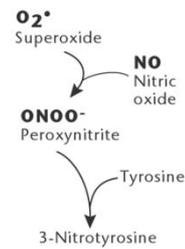


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## Oxidative protein modifications

Protein tyrosine nitration is mediated by reactive nitrogen species such as peroxynitrite ( $\text{ONOO}^-$ ) and nitrogen dioxide ( $\text{NO}_2$ ), and results in a nitro group adduct on susceptible tyrosine residues.



Nitration of tyrosine residues appears to inactivate numerous enzymes and prevent kinase substrate phosphorylation

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## How to measure 3-NT

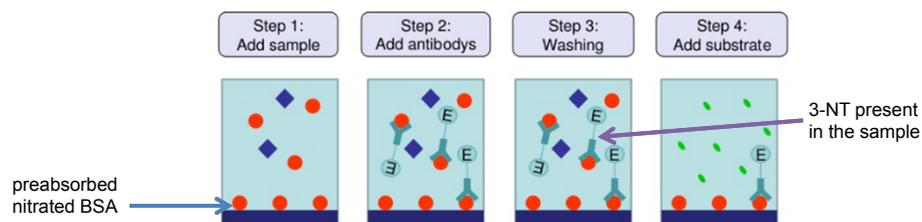
- ELISA kit
- GC-MS/MS
- LC-MS/MS
- HPLC



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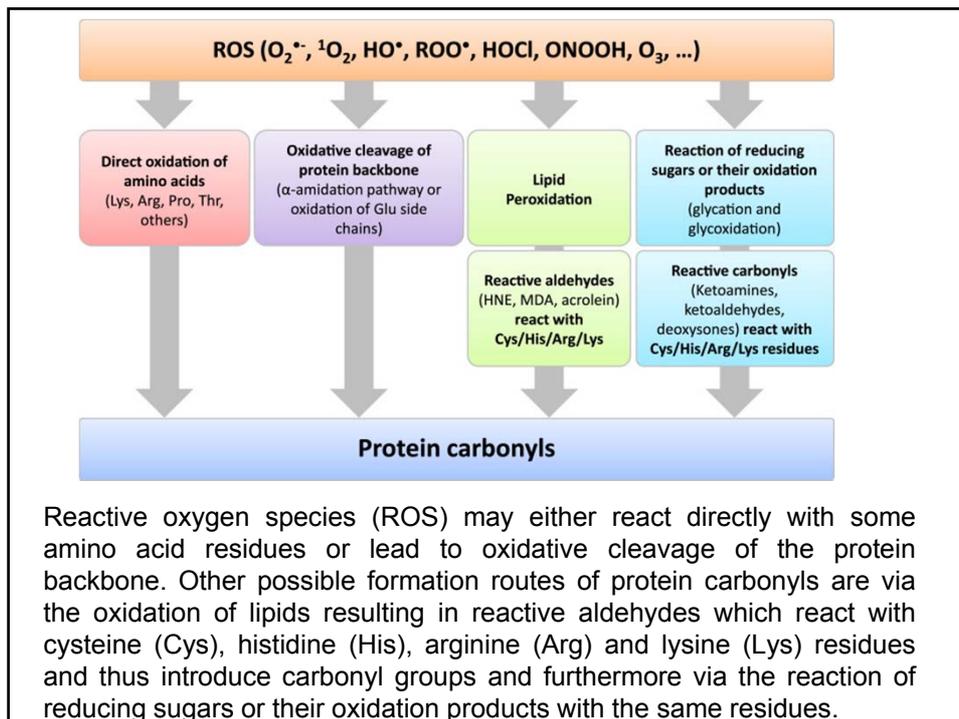


## Competitive ELISA kit for 3-NT



This is an example of competitive ELISA. The unknown protein nitrotyrosine sample or nitrated BSA standards are first added to a nitrated BSA preabsorbed plate. After a brief incubation, an anti-nitrotyrosine antibody is added, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in unknown sample is determined by comparing with a standard curve that is prepared from predetermined nitrated BSA standards.

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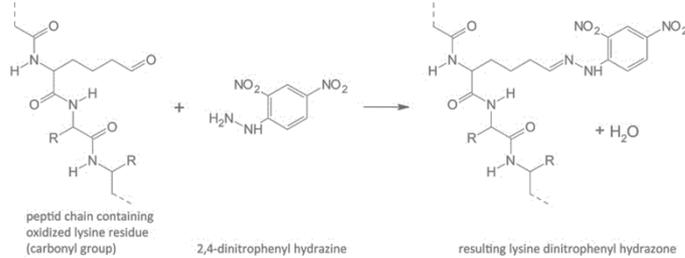
## Measure of protein carbonyls

- Protein carbonyls can be detected by various methods, all relying on the derivatization of the carbonyl group.
- The reduction with radiolabeled borohydride introduces a measurable radiolabel into the protein, whereas several hydrazine derivatives, most commonly 2,4-dinitrophenylhydrazine (DNPH) or biotin hydrazine, introduce detectable functional groups into the oxidized protein.



## Measure of protein carbonyl

- The most often used procedure to detect protein carbonyls is after their derivatization with DNPH.



The nucleophilic addition, also called condensation reaction, resulting in a 2,4-dinitrophenyl hydrazone is shown for an oxidized lysine residue (aminoaldehyde).



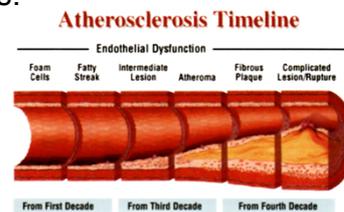
## Measure of protein carbonyl

- The amount of protein-hydrazone produced could be quantified:
- spectrophotometrically (360-585 nm)
- HPLC
- Immunoblotting
- ELISA
- The general drawbacks of the spectrophotometric assay are that the method is rather work-intensive, time-consuming and high throughput measurement is not possible.



## Oxidized low-density lipoprotein

- The oxidation and glycation of LDL plays a central role in the pathogenesis of atherosclerosis, with the adducts being both proatherogenic and proinflammatory.
- The oxidation of LDL can occur non-enzymatically or can be catalyzed by enzymes such as 12/15-lipoxygenase
- OxLDL formation occurs primarily within vascular walls where it is taken up by macrophages via scavenger receptor pathways to form foam cells.
- Accumulation of OxLDL within the vascular walls also stimulates the overlying endothelial cells to produce proinflammatory cytokines

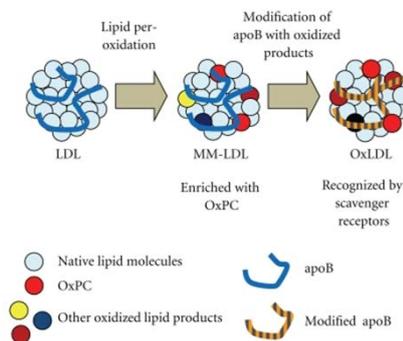


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## Measure of OxLDL

- OxLDL is frequently detected using specific monoclonal antibodies that directly recognize unique oxidation specific epitopes.
- ELISAs for OxLDL are available



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## Biomarkers of oxidation to DNA/RNA



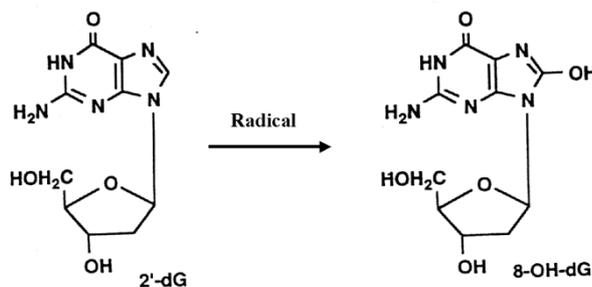
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## Marker of DNA/RNA damage 8-OHdG

Guanine is the base most prone to oxidation that leads to the formation of

- 8-hydroxy-2'-deoxyguanosine (8-OHdG) from DNA,
- 8-hydroxyguanosine from RNA,
- 8-hydroxyguanine from either DNA or RNA.



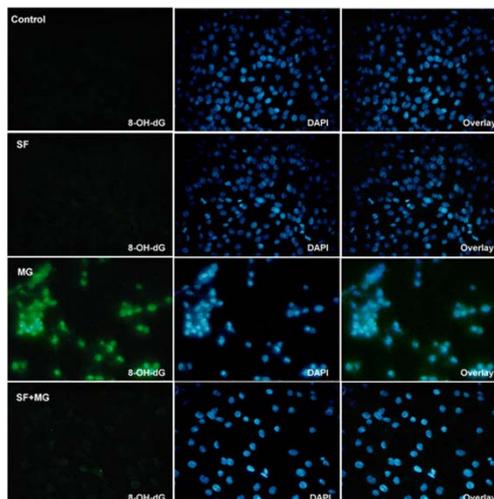
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## How to measure 8-OHdG

- ELISA kit
- Immunoblotting

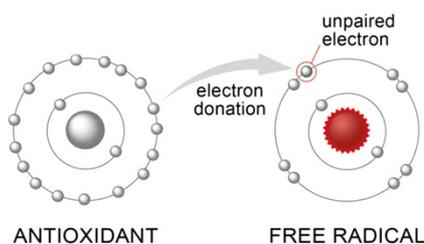
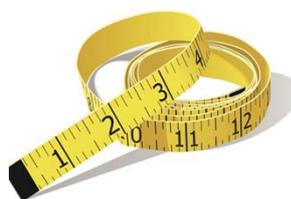
Green: anti-8OHdG  
Blue: DAPI



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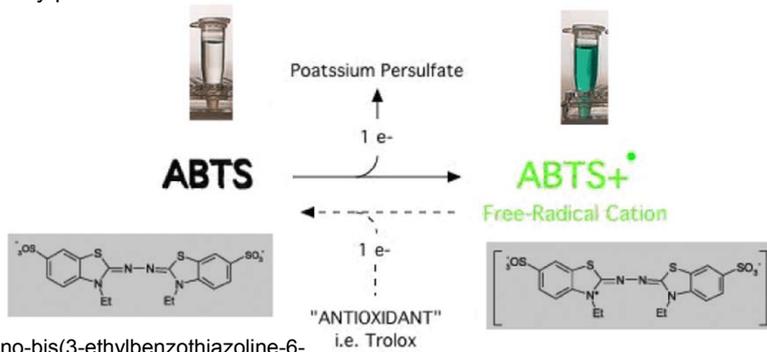
## Biomarkers of the impairment of the antioxidant system



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## Total antioxidant capacity

The ABTS Antioxidant Assay can be used to determine the total antioxidant capacity of biological fluids, cells, and tissue. The assay measures ABTS<sup>•+</sup> radical cation formation induced by potassium persulfate. Trolox, a water soluble vitamin E analog, serves as a positive control inhibiting the formation of the radical cation in a dose dependent manner. The antioxidant activity can be normalized to equivalent Trolox units to quantify the composite antioxidant activity present.



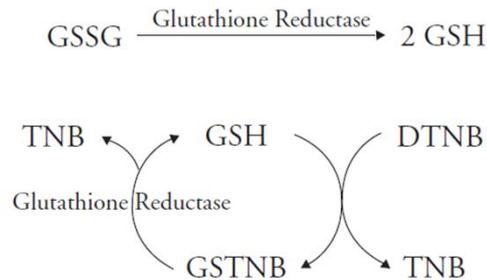
## Glutathione (GSH)

- Glutathione (GSH) is the most abundant antioxidant in aerobic cells
- GSH is critical for protecting cells from oxidative stress, acting as a free radical scavenger and inhibitor of lipid peroxidation.
- GSH also participates in the detoxification of hydrogen peroxide by various glutathione peroxidases.
- The ratio of reduced GSH to oxidized GSH (GSSG) is an indicator of cellular health, with reduced GSH constituting up to 98% of cellular GSH under normal conditions.
- It can be measured by HPLC or spectrophotometrically



## Total GSH measurement

The sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB.



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## Reduced GSH measurement

The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of GSH in the sample.

Quantification of GSSG, exclusive of GSH, is accomplished by first derivatizing GSH with 2-vinylpyridine

$$\text{Reduced GSH} = \text{Total GSH} - \text{GSSG}$$

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## Antioxidant enzymes

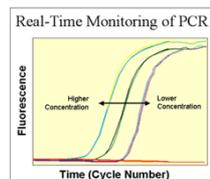
The main antioxidant enzymes studied are:  
superoxide dismutase (SOD),  
catalase (CAT),  
GSH related enzymes like  
GSH reductase,  
GSH-S transferase  
GSH peroxidase,  
NAD(P)H quinone dehydrogenase (NQO1),  
thioredoxin reductase (TR)

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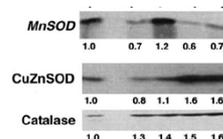


## How to measure antioxidant enzymes

- Expression by RT-PCR



- Protein level by western blot analysis



- Activity by spectrophotometric or spectrofluorimetric methods



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## Glutathione S-Transferase (GST) activity

- The method to evaluate GST activity utilizes 1-Chloro-2,4-dinitrobenzene (CDNB) which is suitable for the broadest range of GST isozymes.
- GST catalyzes the conjugation of L-glutathione to CDNB through the thiol group of the glutathione.

### **GST**



- Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample.

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## Glutathione peroxidase activity

This method is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH

### **GPx**



### **GR**



The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP<sup>+</sup> is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions

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## Summarizing

- Oxidative stress likely plays a role in several diseases, yet very few oxidative stress markers have made it into routine clinical use
- The properties of the oxidative modifications, such as the labile nature of modifications or their low abundance poses significant challenges to translate them into a high-throughput, cost-effective clinical diagnostic.



## Summarizing

- Stable oxidative modifications, such as protein carbonyls, certain lipid oxidation products, DNA/RNA oxidation, and 3-nitrotyrosine, certainly circumvent the first issue, which likely contributes to some of their positive clinical findings.
- Another limitation is methodology. While MS provides sensitivity and specificity and has become more accessible, antibody-based methods remain, for now, the clinical standard. However some of these methods fall short on specificity.