LOOKING FOR LIPID PEROXIDATION *IN VITRO* AND *IN VIVO*: IS SEEING BELIEVING?

Vanderbilt University School of Medicine

Jason D. Morrow MD

Question?

- Which of the following assays of lipid peroxidation may be useful and accurate for the quantification of oxidant stress *in vivo*? More than one may be correct.
  - Plasma isoprostanes
  - Urinary malonaldehyde
  - Exhaled pentane
  - Total plasma antioxidant capacity
  - Plasma glutathione levels

Question?

- In which of the following diseases has lipid peroxidation definitively been shown to be involved in disease pathogenesis?
  - Atherosclerosis
  - Scleroderma
  - Pulmonary hypertension
  - Stroke
  - All of the above
  - None of the above

Question?

- A potential disadvantage of measuring isoprostanes as an index of oxidant stress include all of the following except:
  - They can be formed ex vivo.
  - They are unstable and rapidly degrade ex vivo.
  - Accurate assay methods are complex and expensive.
  - They represent only one of a myriad of end products of lipid peroxidation.
  - Their quantification in a particular body fluid may not be index of systemic (total body) oxidant stress.
  - Immunoassay kits to measure isoprostanes are of questionable accuracy.

Goals of Lecture

- Critical appraisal of methods to quantify lipid peroxidation *in vitro* and *in vivo*.
  - Theoretical considerations regarding quantification of lipid peroxidation.
  - Assays currently available to measure lipid peroxidation.
    - » Advantages
    - » Disadvantages

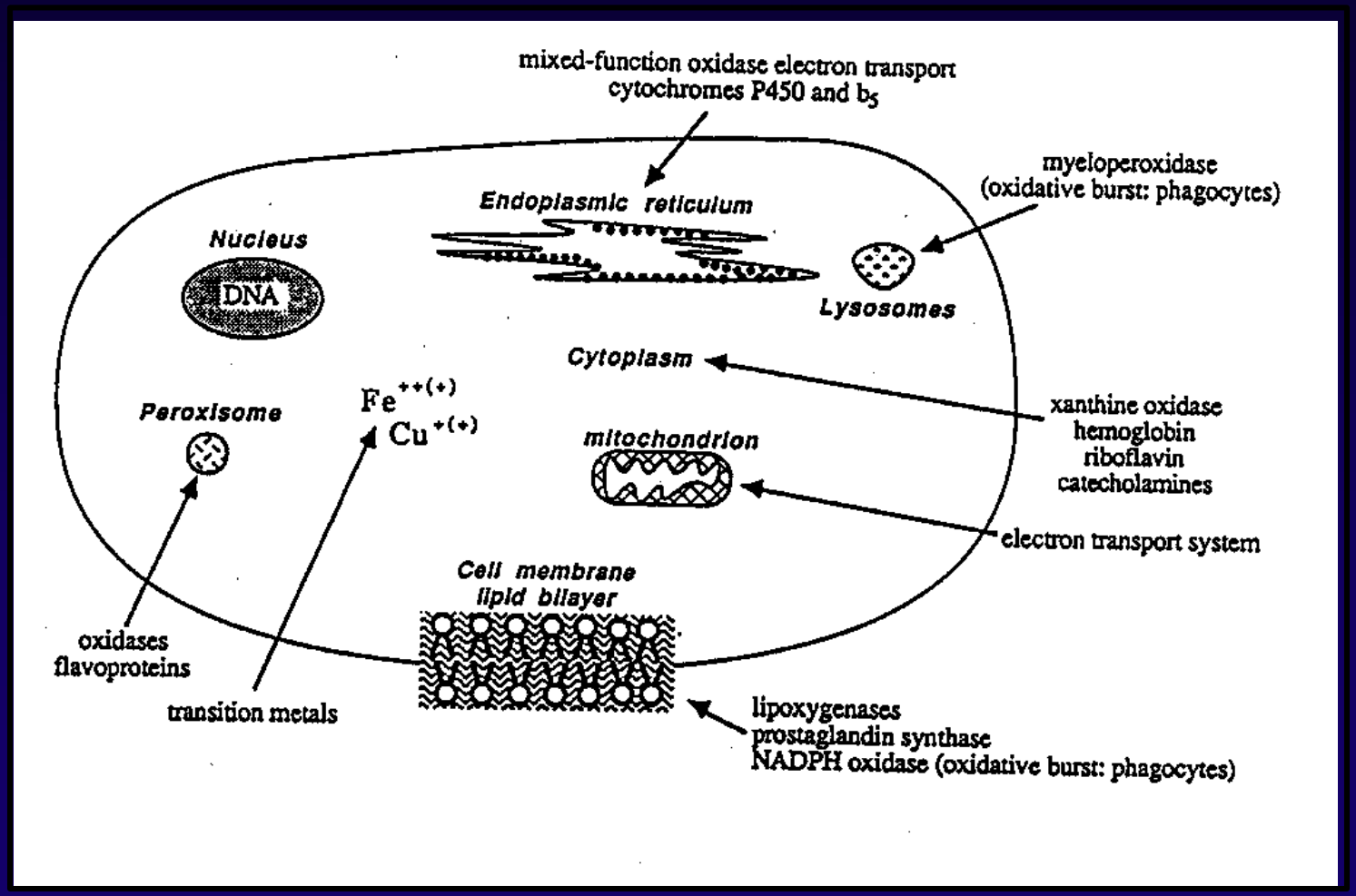
Background

- Oxygen free radicals are implicated in many diseases.
  - Ischemia-reperfusion injury
  - Cancer
  - Inflammation
  - Aging
- Underlying mechanisms responsible for sequelae of oxidant stress are poorly understood.
- Reliable methods of assessment have not been available.

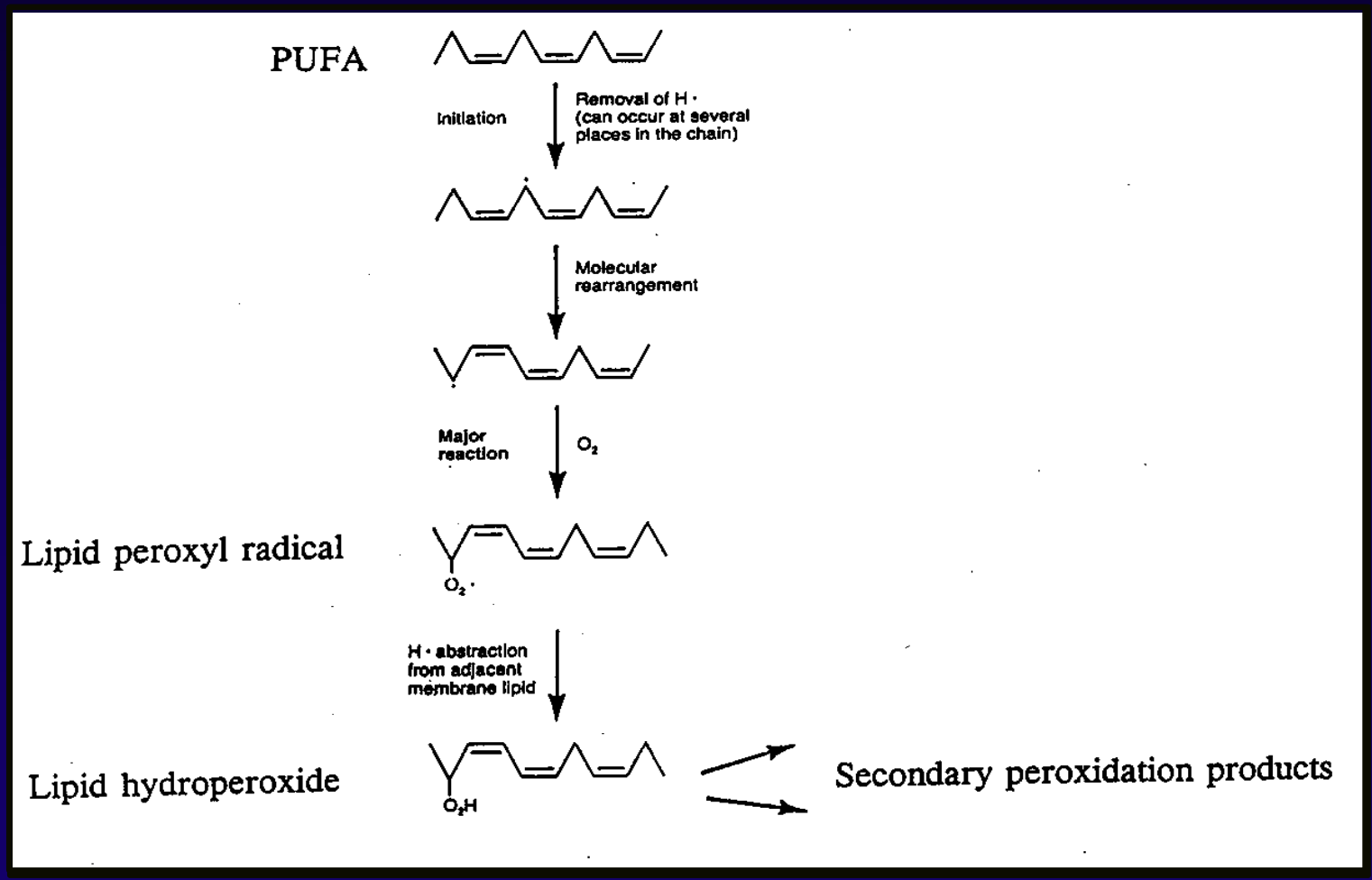
Targets of Free Radical Injury

- Lipids
- Proteins
- DNA

Cellular Sources of Free Radicals and Lipid Peroxidation Products



Lipid Peroxidation



Methods to Quantify Lipid Peroxidation

- Measurement of substrate loss.
- Quantification of lipid peroxidation products.
  - Primary end products
  - Secondary end products

The Ideal Assay of Lipid Peroxidation

- Assay is accurate, specific, and sensitive index of lipid peroxidation.
- Compounds to be quantified are stable.
- Assay applicable to *in vitro* and *in vivo* studies.
- Assay easy to perform with high throughput.
- Assay economical.

Assays of Lipid Peroxidation

- No assay is ideal.
- Most assays are more accurate when quantifying lipid peroxidation *in vitro* than *in vivo*.
- Little data exist comparing various methods in vivo.
- Few assays accurately provide an integrated assessment of lipid peroxidation in an animal or human as a whole.

Assays of Potential Use to Quantify Lipid Peroxidation *in Vitro* and *in Vivo*

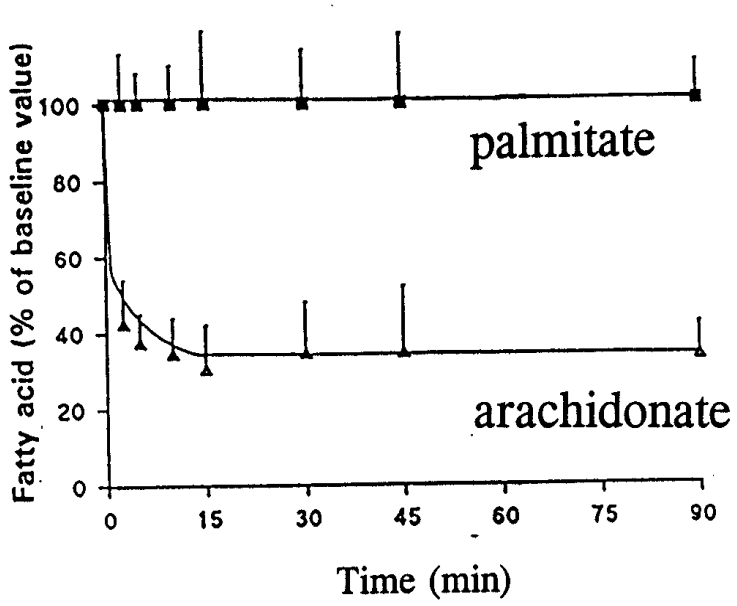
- Fatty acid analysis
- Conjugated dienes
- Lipid hydroperoxides
- Thiobarbituric acid-reactive substances (TBARS) or malondialdehyde (MDA)
- Alkanes
- F_2-Isoprostanes

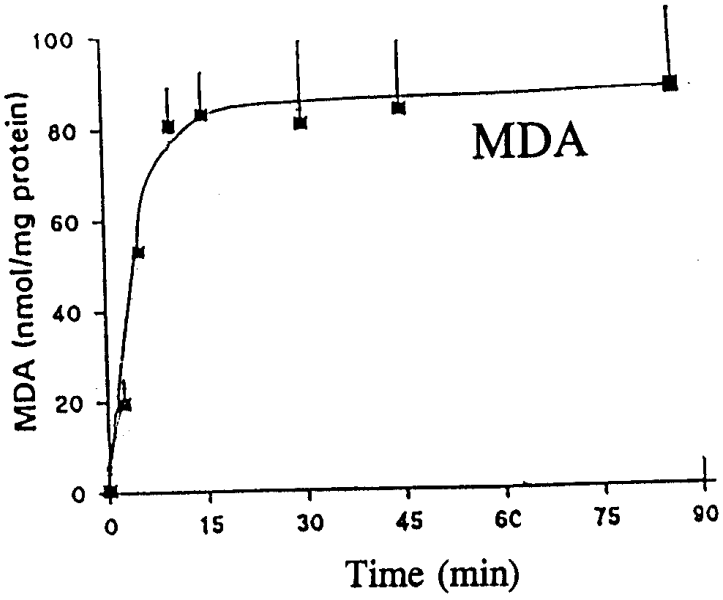
Fatty Acid Analysis

- Commonly used to assess fatty acid content in biological fluids or tissues.
- Method
  - Lipid extraction of biological fluid or tissue
  - Transmethylation of fatty acids
  - Separation by GC (or HPLC)
  - Quantification of fatty acid with flame ionization

Analysis of Fatty Acids in Peroxidizing Microsomes

- Disappearance of arachidonic acid is associated with accumulation of peroxidation end products.



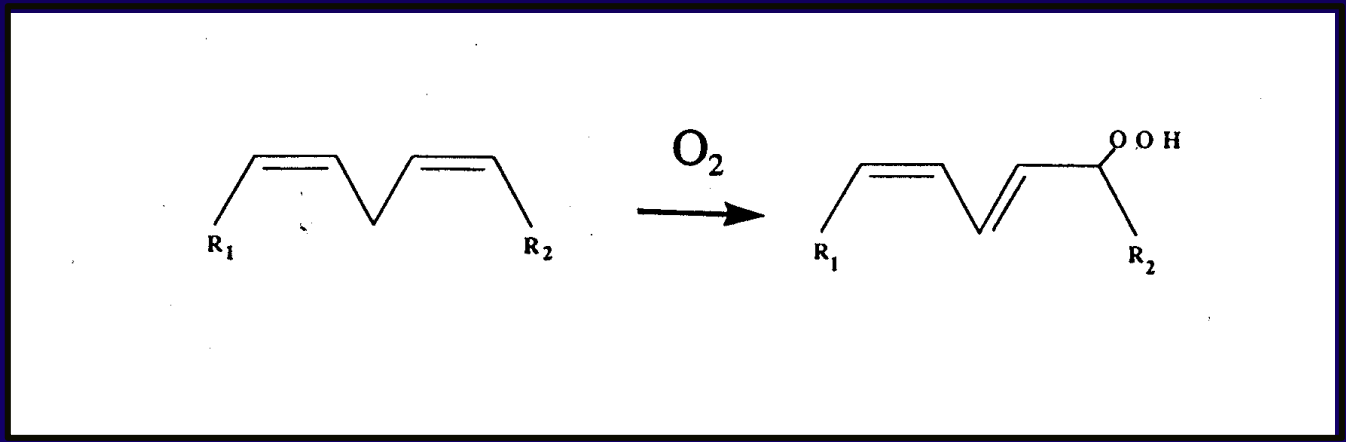


Fatty Acid Analysis

- Advantages
  - Easy to perform.
  - Equipment readily available.
  - Data complement generation of peroxidation products *in vitro* and *in vivo*.
- Disadvantages
  - Impractical for a number of *in vivo* situations.
  - Provides information on disappearance of substrate only.
  - May see very little change associated with oxidation *in vivo*.

Conjugated Dienes

- Peroxidation of unsaturated fatty acids results in the formation of conjugated diene structures that absorb light in the wavelength of 230-235 nm which can be quantified spectrometrically.



Conjugated Dienes

- Useful for studying oxidation of lipids in vitro.
  - Enhanced assay sensitivity using second derivative spectroscopy, HPLC, or GC/MS.
- Studies have shown increases in animals and humans associated with oxidant stress.
- Inaccurate index of lipid peroxidation when applied to complex biological fluids.
  - A primary diene conjugate from human body fluids is an non-oxygen containing isomer of linoleic acid, octadeca-9-cis-11-trans-dienoic acid.
  - Other compounds-purines, pyrimidines, heme proteins absorb at 234 nm.

Conjugated Dienes

- Advantages
  - Relatively easy to perform
  - Provides useful information primarily regarding oxidation of pure lipids *in vitro*.
- Disadvantages
  - Virtually useless for analysis of lipid peroxidation products in complex biological fluids.

Lipid Hydroperoxides

- Primary products of lipid peroxidation.
- Several quantitative methods exist (iodometric, electrochemical, mass spectrometric)
  - Most accurate-chemiluminescence-based HPLC detection.

$$\mathrm{LOOH} \xrightarrow{\text{microperoxidase}} \mathrm{LO}\cdot \quad (1)$$

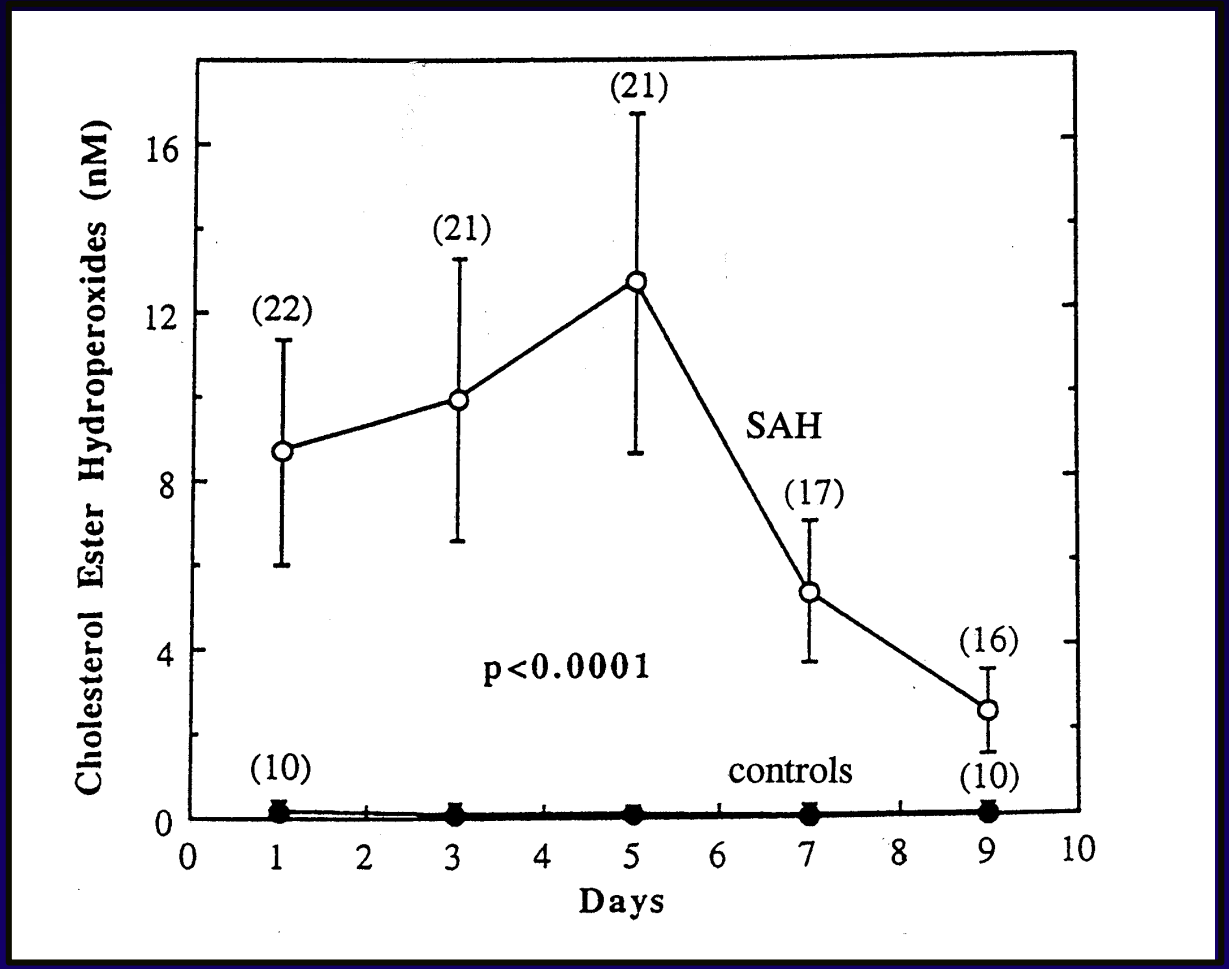
$$\mathrm{LO}\cdot + \text{isoluminol } (\mathrm{QH}^-) \rightarrow \mathrm{LOH} + \text{semiquinone radical } (\mathrm{Q}^{\cdot-}) \quad (2)$$

$$\mathrm{Q}^{\cdot-} + \mathrm{O_2} \rightarrow \text{quinone (Q)} + \mathrm{O_2}^{\cdot-} \quad (3)$$

$$\mathrm{Q}^{\cdot-} + \mathrm{O_2}^{\cdot-} \rightarrow \text{isoluminol endoperoxide} \rightarrow \text{light} \quad (\lambda_{max}\ 430\mathrm{nm}) \quad (4)$$

  - » Sensitive (pmol or less) and specific
  - » Information regarding which lipid class is oxidized can be obtained.

Plasma Lipid Hydroperoxides in Patients with Subarachnoid Hemorrhage



Polidori *et al.* Free Rad. Biol. Med. 23: 762-7, 1997

Lipid Hydroperoxides

- Advantages
  - Assays more specific and sensitive than for other peroxidation products.
- Disadvantages
  - Products are unstable.
  - *Ex vivo* oxidation a major concern.
  - Lack of detectable levels in some fluids and tissues.
  - Equipment expensive.

Thiobarbituric Acid-Reactive Substances (TBARS)/MDA

- Most commonly used method to assess lipid peroxidation.
  - Measures malondialdehyde (MDA) which is a breakdown product of lipid peroxidation.
- Method:
  - Sample to be tested is heated with thiobarbituric acid at low pH and a pink chromogen (believed to be a TBA-MDA adduct) is formed.
  - Quantification-absorbance at 532 nm or fluorescence at 553 nm.

TBARS/MDA

- Quantification of TBARS is an accurate measure of peroxidation in oxidizing systems *in vitro*.
- TBARS quantification in body fluids is inaccurate.
  - Substances other than MDA form chromogens at 532 nm.
  - MDA is formed during the assay procedure.
  - Antioxidants can interfere with the assay.
  - MDA can be derived from the diet.

TBARS/MDA

- Assays exist to measure TBARs by HPLC.
- MDA, HNE, and other aldehydes can be quantified by HPLC or GC/MS.
- These assays are generally more specific than TBARs although not necessarily more accurate as an index of lipid peroxidation.

TBARS/MDA

- Levels of TBARS vary widely.
  - Plasma levels
    - » Regular assay 4-35 uM.
    - » HPLC-coupled 0-0.18 uM.
- TBARS increased in various disorders.
  - Hypercholesterolemia (Chirico *et al.,* Free Rad. Res. Comm. **19:**51, 1993).
    - » Controls 0.10 ± 0.08 uM
    - » Hypercholesterolemics 0.61 ± 0.25 uM

TBARS/MDA

- Summary
  - The TBARS assays are important because they are easy to perform and widely available.
  - They are a reasonably accurate index of lipid peroxidation in a number of in vitro oxidizing systems.
  - They are less reliable as an index of lipid peroxidation in complex biological fluids or in vivo.

Alkanes

- Volatile hydrocarbons generated from scission of oxidized lipids.
  - Pentane (n-6 fatty acids)
  - Ethane (n-3 fatty acids)
- Method of quantification
  - Collection of gas from an *in vitro* incubation or exhaled air from an animal or human.
  - Concentrating and filtering of samples to remove water and carbon dioxide.
  - Analysis of product by GC (capillary).

Alkanes

- Advantages
  - Is an integrated assessment of peroxidation in vivo.
- Disadvantages
  - Collection of exhaled air for in vivo studies cumbersome and can take several hours to obtain an adequate sample for analysis.
  - Oxygen tension alters alkane formation.
  - Atmospheric contamination of collected gases.
  - Different researchers report a 1000-fold difference in normal levels of pentane generated in humans (4.1-4900 pmol/L).

Formation of Alkanes in Peroxidizing Microsomes

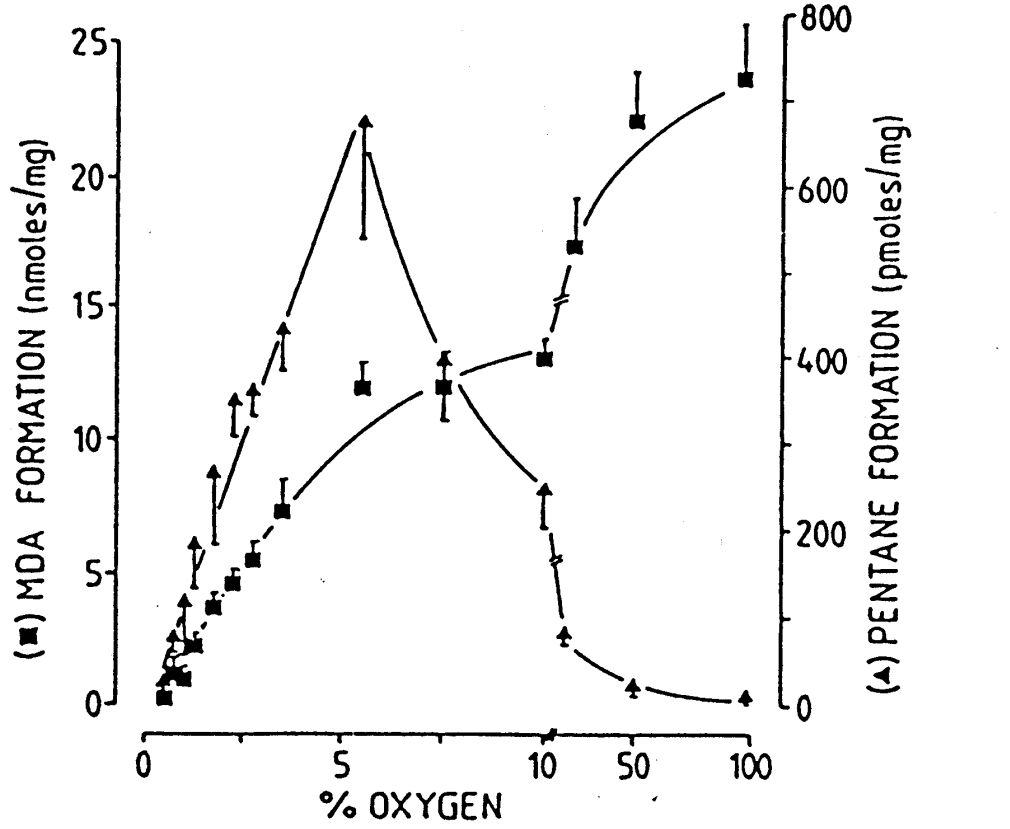


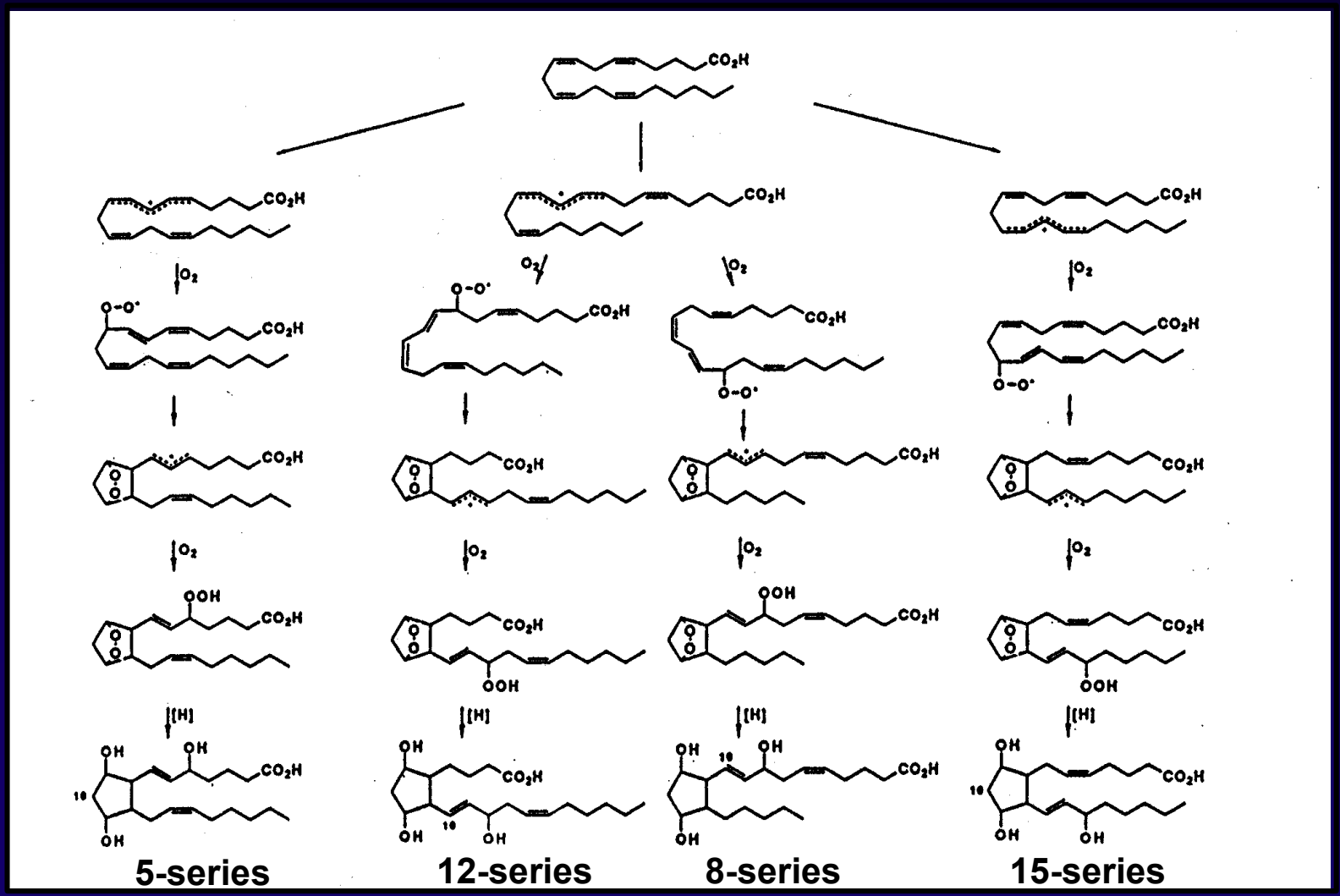
Fig. 2. Oxygen dependence of malondialdehyde (MDA) (■) and pentane (▲) formation in the incubations described in Fig. 1.

Reiter R *et al.* Biochem. Pharm. **36**: 925-9, 1987.

F_2-Isoprostanes

- Arachidonyl-containing lipids are peroxidized to PGF_2-like compounds, termed F_2-isoprostanes.
- Formed independent of the cyclooxygenase by peroxidation of arachidonate.
- Generated in large amounts *in vivo*.
- Exert potent biological activity.

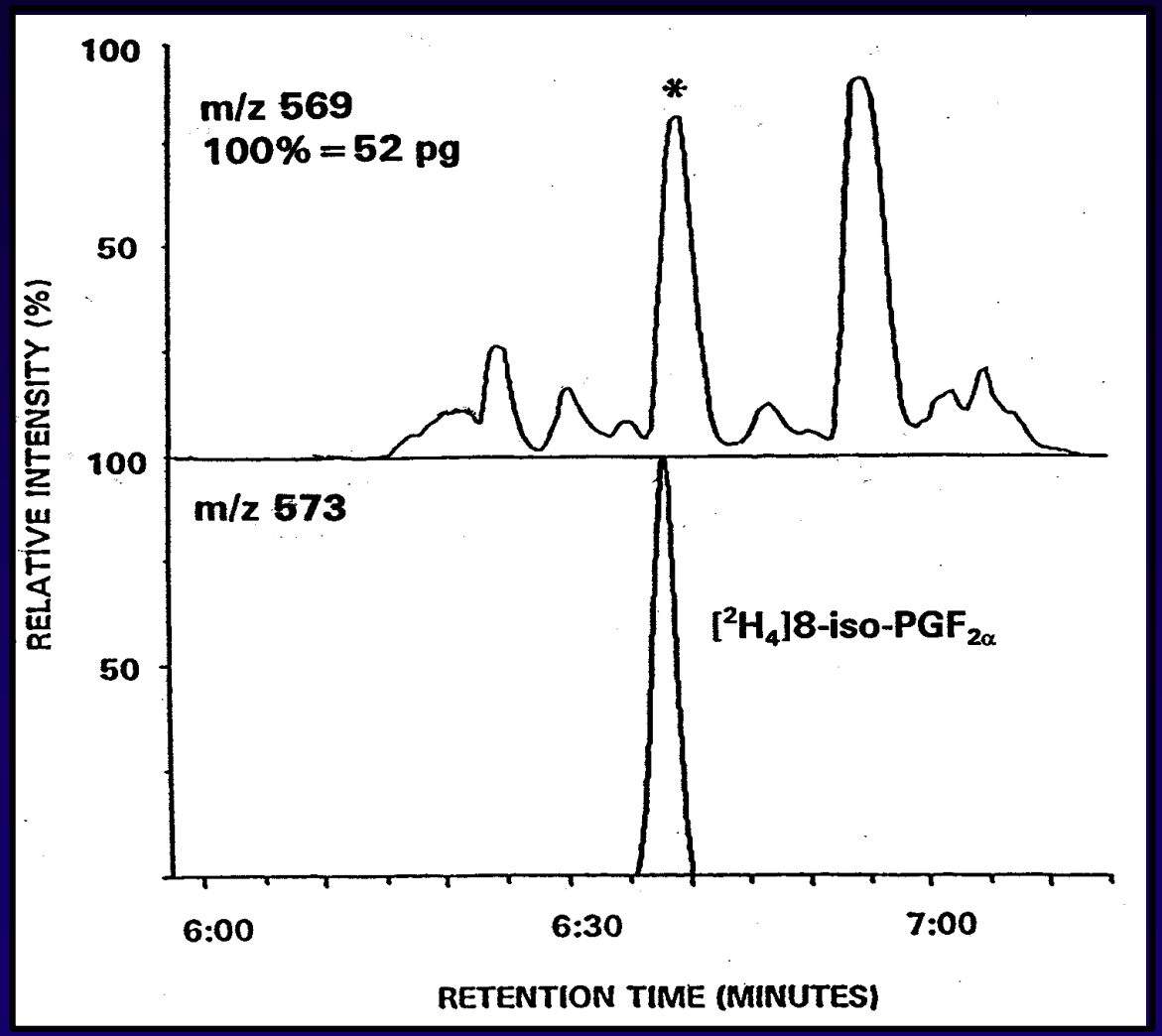
Pathway of Isoprostane Formation



Analysis of F_2-Isoprostanes

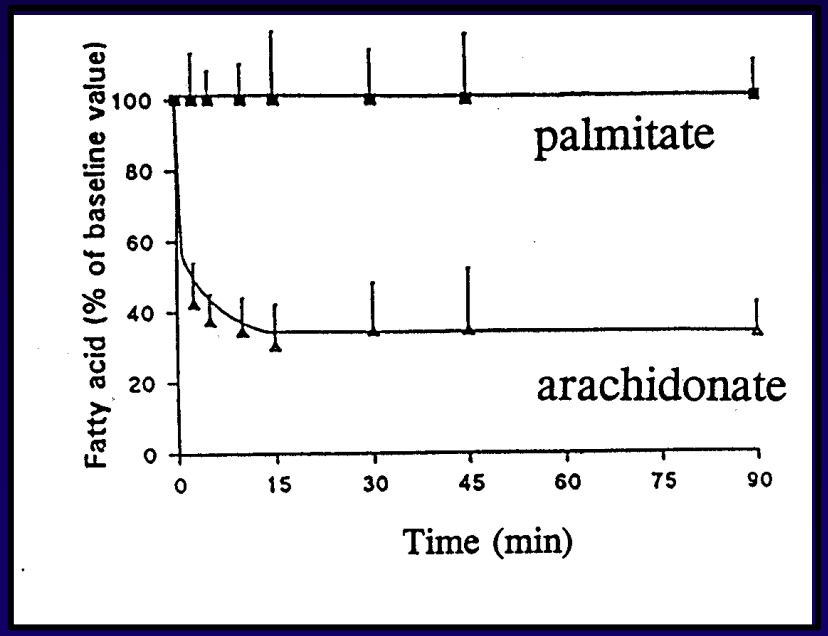
- Measured either free or after liberation from tissue lipids.
- Purified by Sep-Pak extraction and TLC and derivatized to PFB ester, TMS ethers.
- Analyzed using stable isotope dilution techniques employing a deuteriated standard by gas chromatography/mass spectrometry.

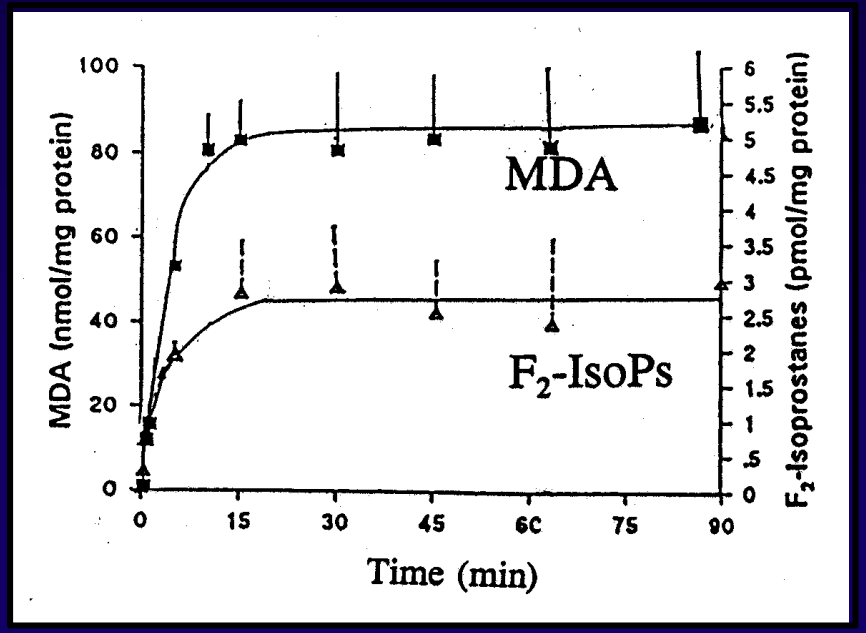
Analysis of F_2-Isoprostanes in Human Plasma



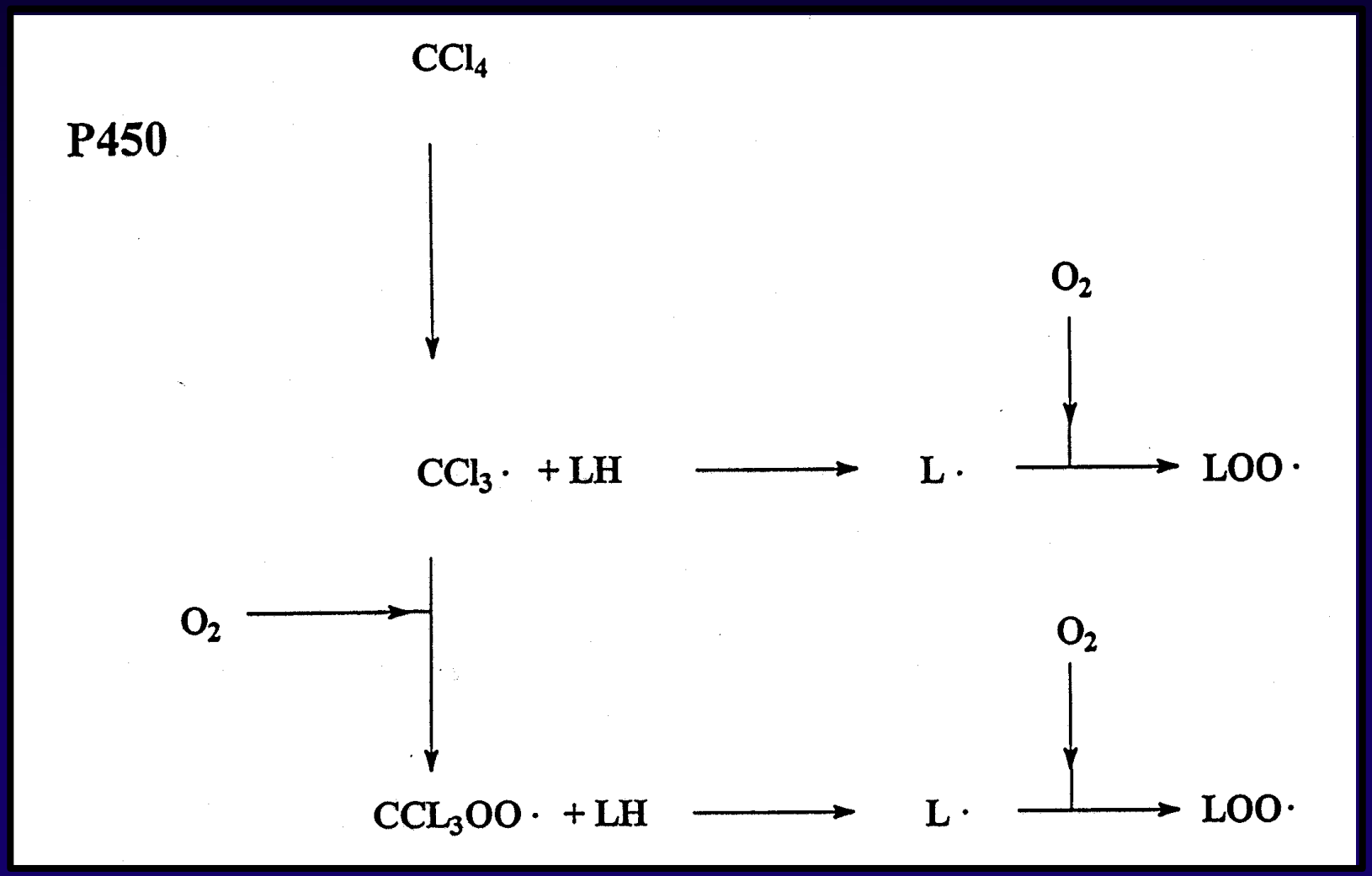
Formation of F_2-Isoprostanes in Peroxidizing Microsomes

- F_2-IsoP formation correlates with disappearance of arachidonate and generation of MDA.



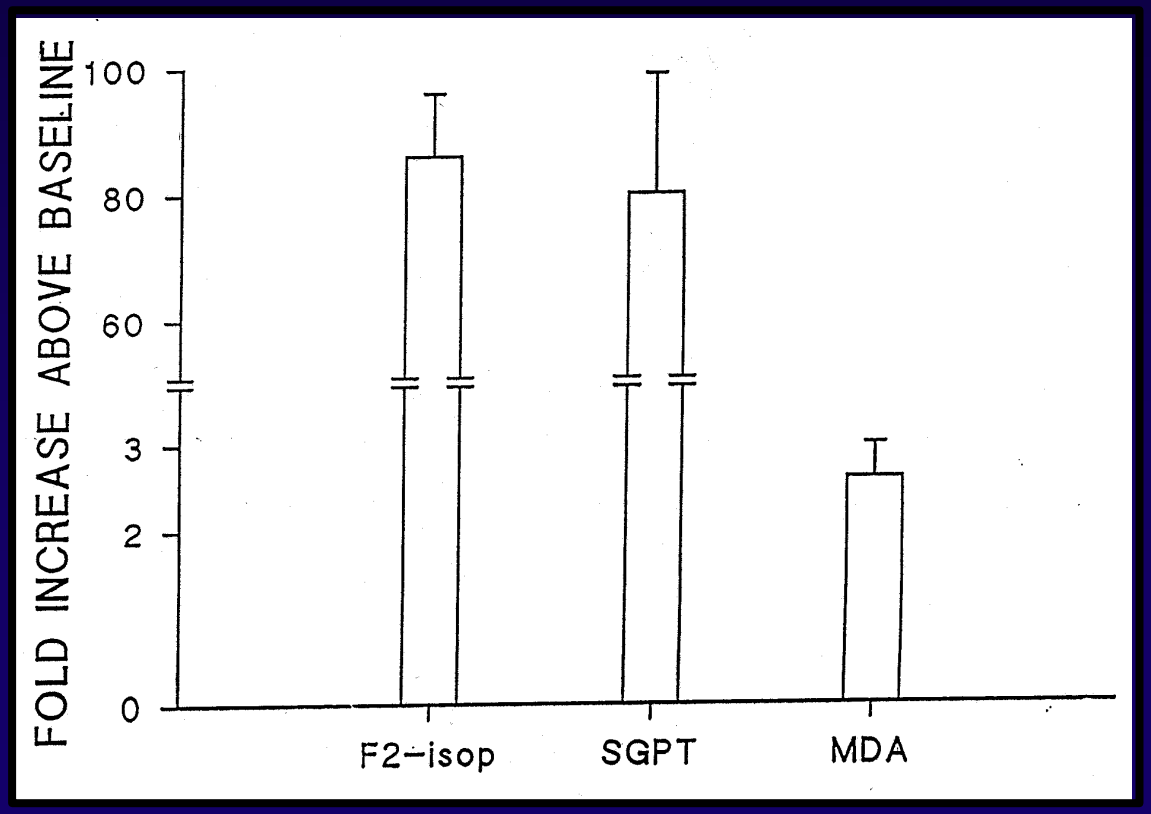


CCl_4-Mediated Oxidant Injury



F_2-Isoprostanes Are a Reliable Marker of Lipid Peroxidation in Vivo

- Comparison of formation of MDA and F_2-IsoPs with hepatic injury in CCl_4-treated rats.



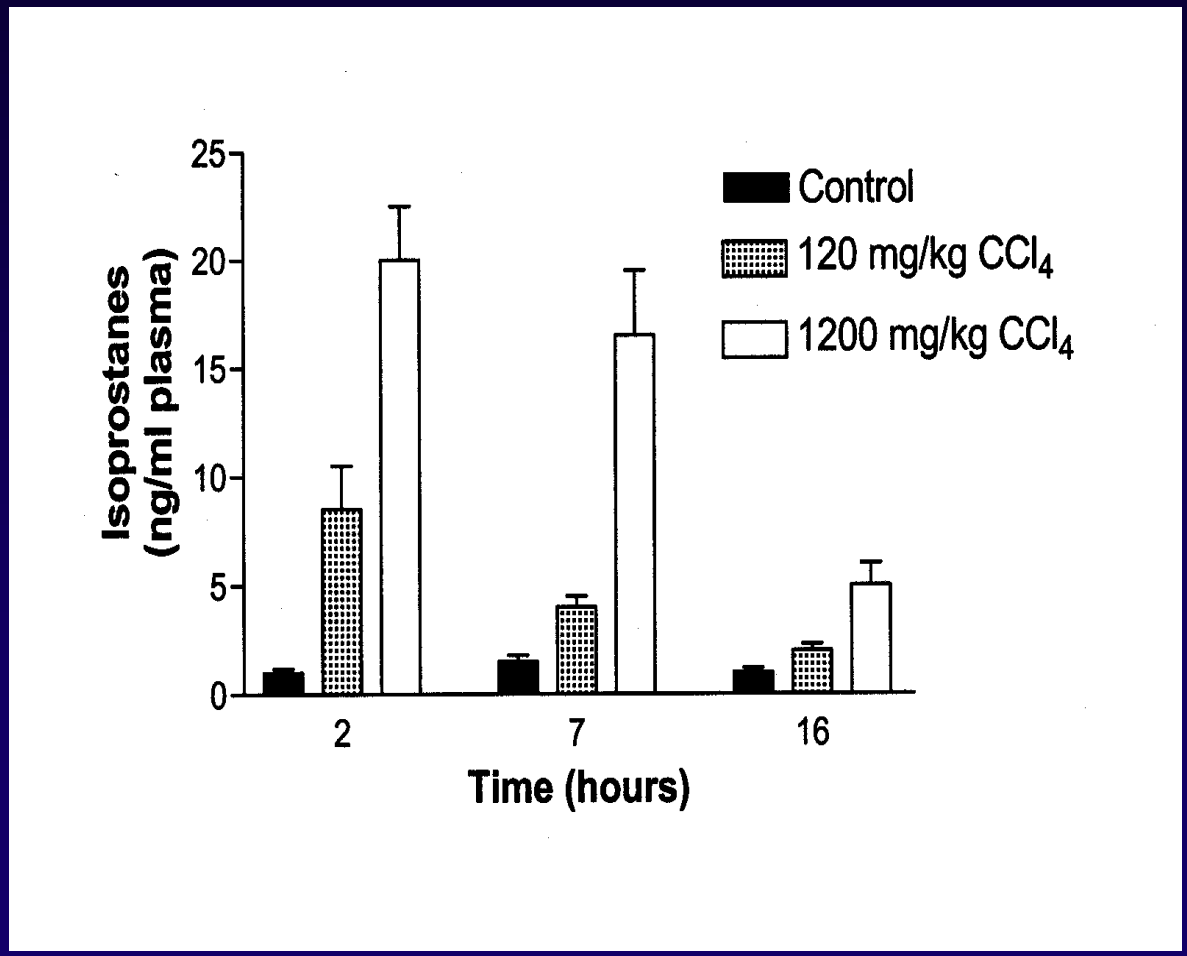
F_2-IsoPs as a Measure of Oxidant Stress

- BOSS study (2000)-IsoPs most accurate measure of oxidant stress in CCl_4-treated rats.
- Deficiencies in antioxidants *in vivo* are associated with increased IsoP formation.
- Antioxidants decrease IsoP levels in animals and humans.
- IsoP levels are increased in animal models of human diseases and human disorders associated with oxidant stress.

Biomarkers of Oxidative Stress Study

- CCl_4-induced oxidant stress in rats.
- Markers quantified and compared to hepatic histology/enzyme leak:
  - Plasma and urine IsoPs
  - Plasma antioxidants
  - Plasma GSH and GSSG
  - Protein carbonyls and specific amino acid oxidation products
  - 8-hydroxy-deoxyguanosine

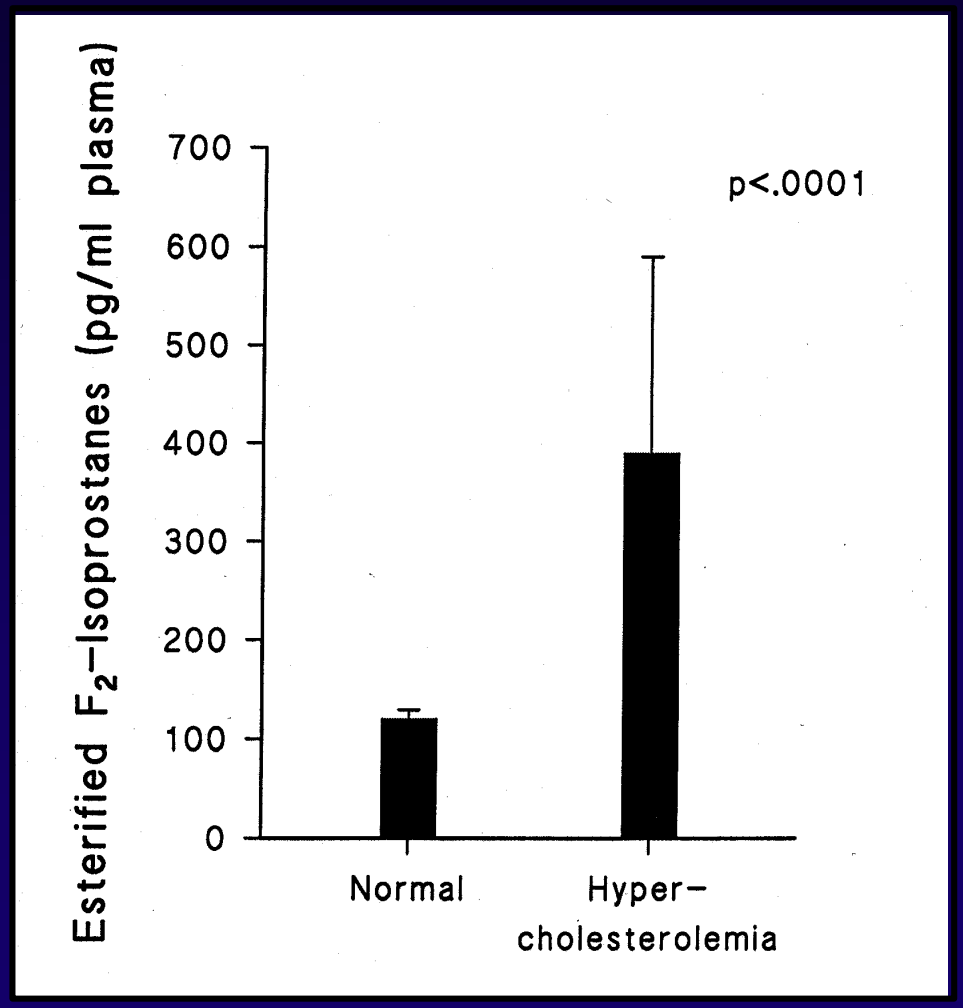
Biomarkers of Oxidative Stress Study



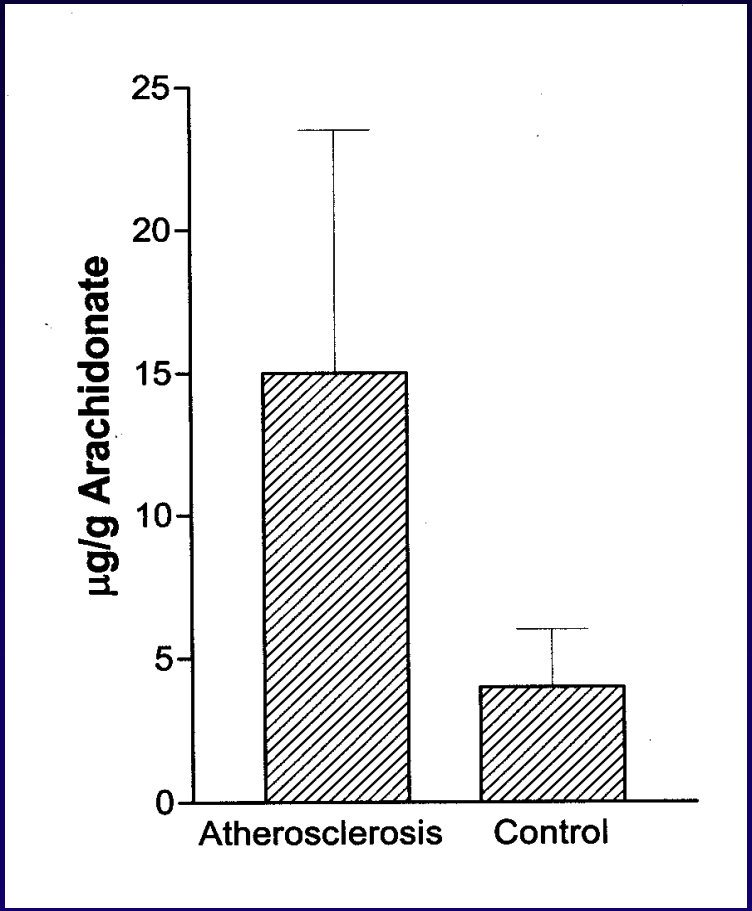
Isoprostanes as an Index of Oxidant Stress in Humans-Atherosclerosis

- Oxidation of lipoproteins plays a pivotal role in the development of atherosclerotic lesions.
- Levels of IsoPs in oxidized LDL correlate with other markers of lipid peroxidation.
- Risk factors for atherosclerosis are associated with increased levels of IsoPs *in vivo* in humans.
  - Chronic cigarette smoking
  - Hyperhomocysteinemia
  - Diabetes mellitus

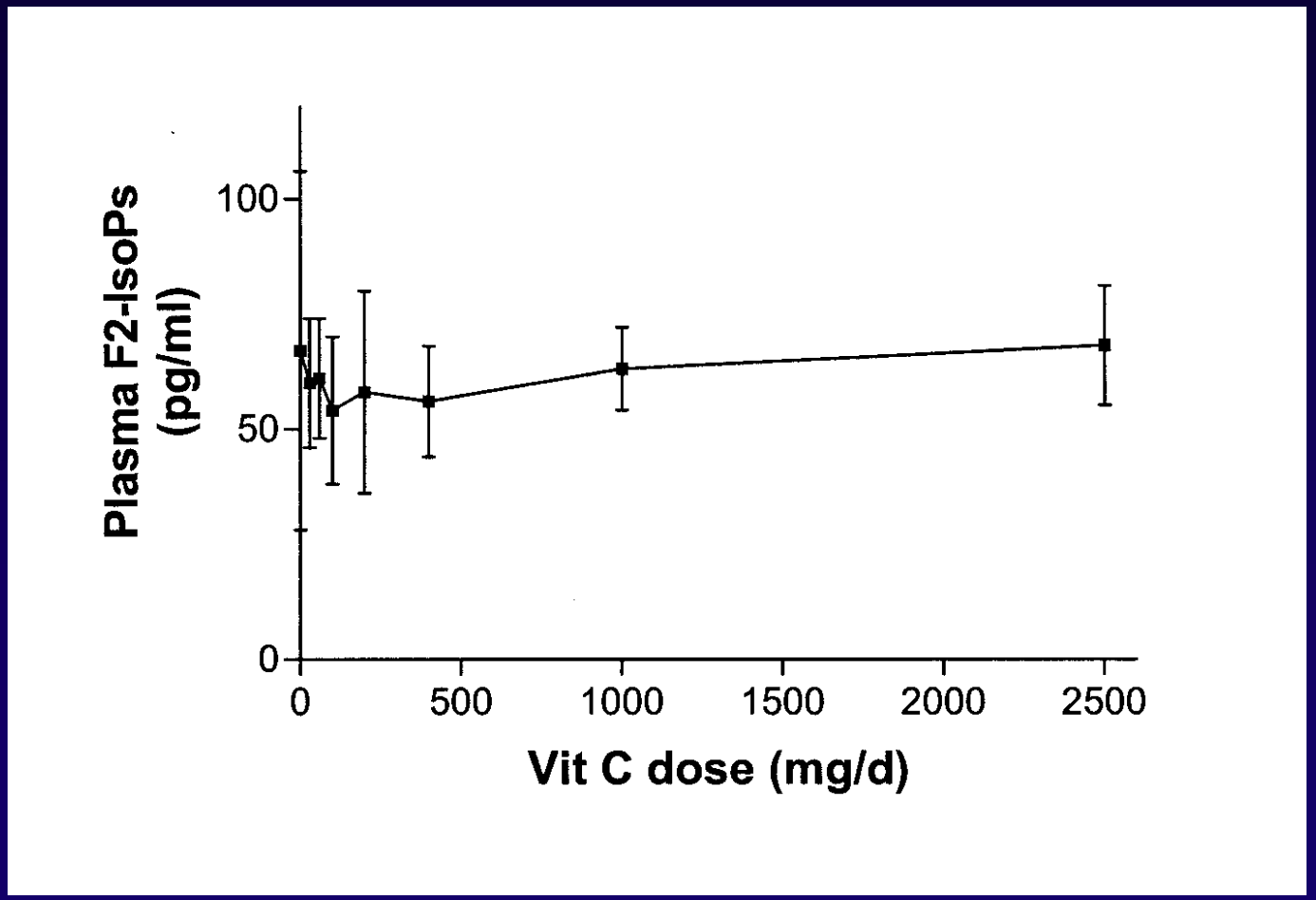
F_2-IsoPs Are Increased in Humans With Hypercholesterolemia



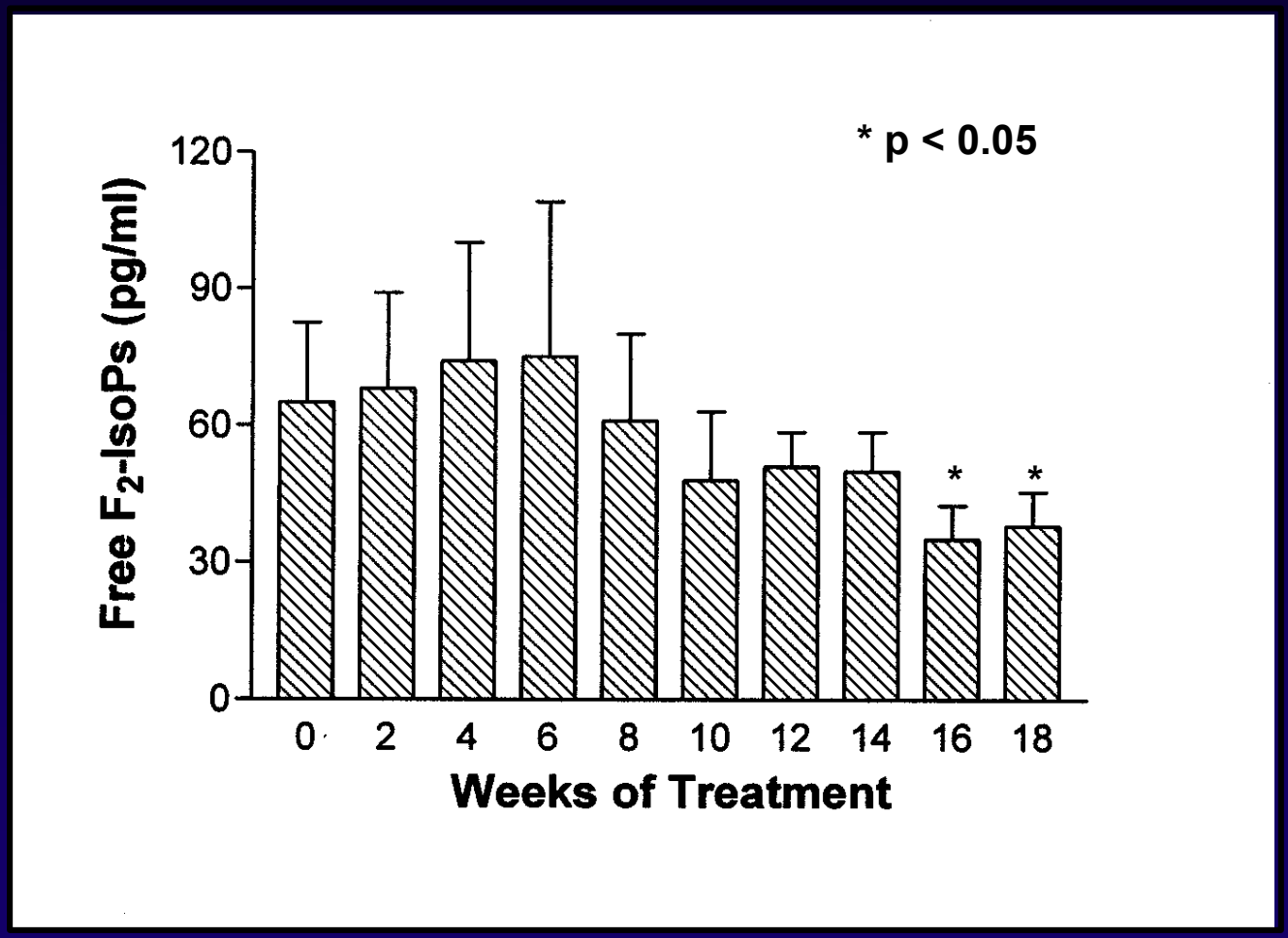
Levels of IsoPs in Human Atherosclerotic Arteries



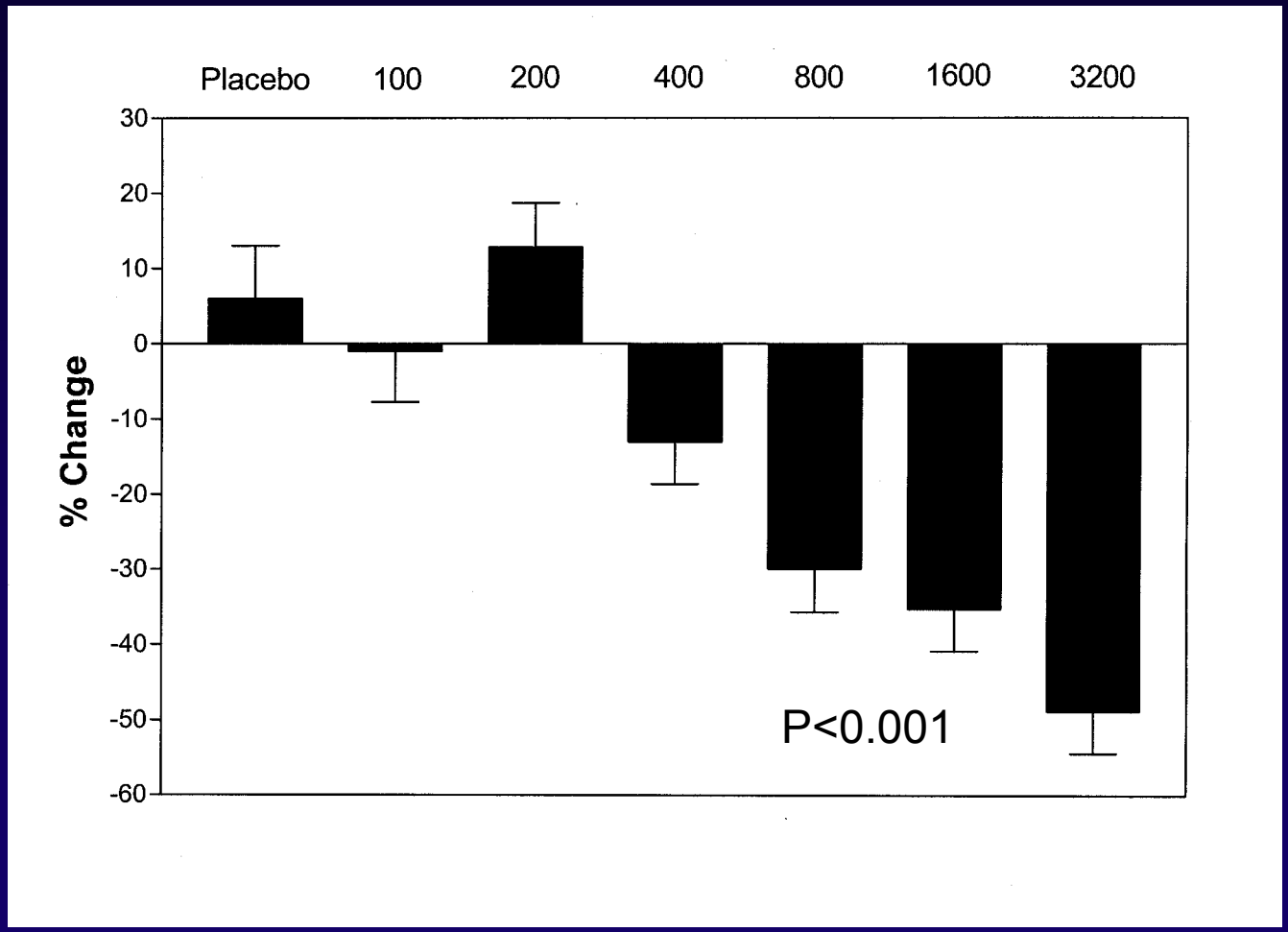
Vitamin C Does Not Alter Isoprostane Levels in Healthy Humans



Vitamin E Decreases IsoP Levels in Hypercholesterolemic Humans



Vitamin E Reduces Isoprostane Formation in Humans– Effect of Dose



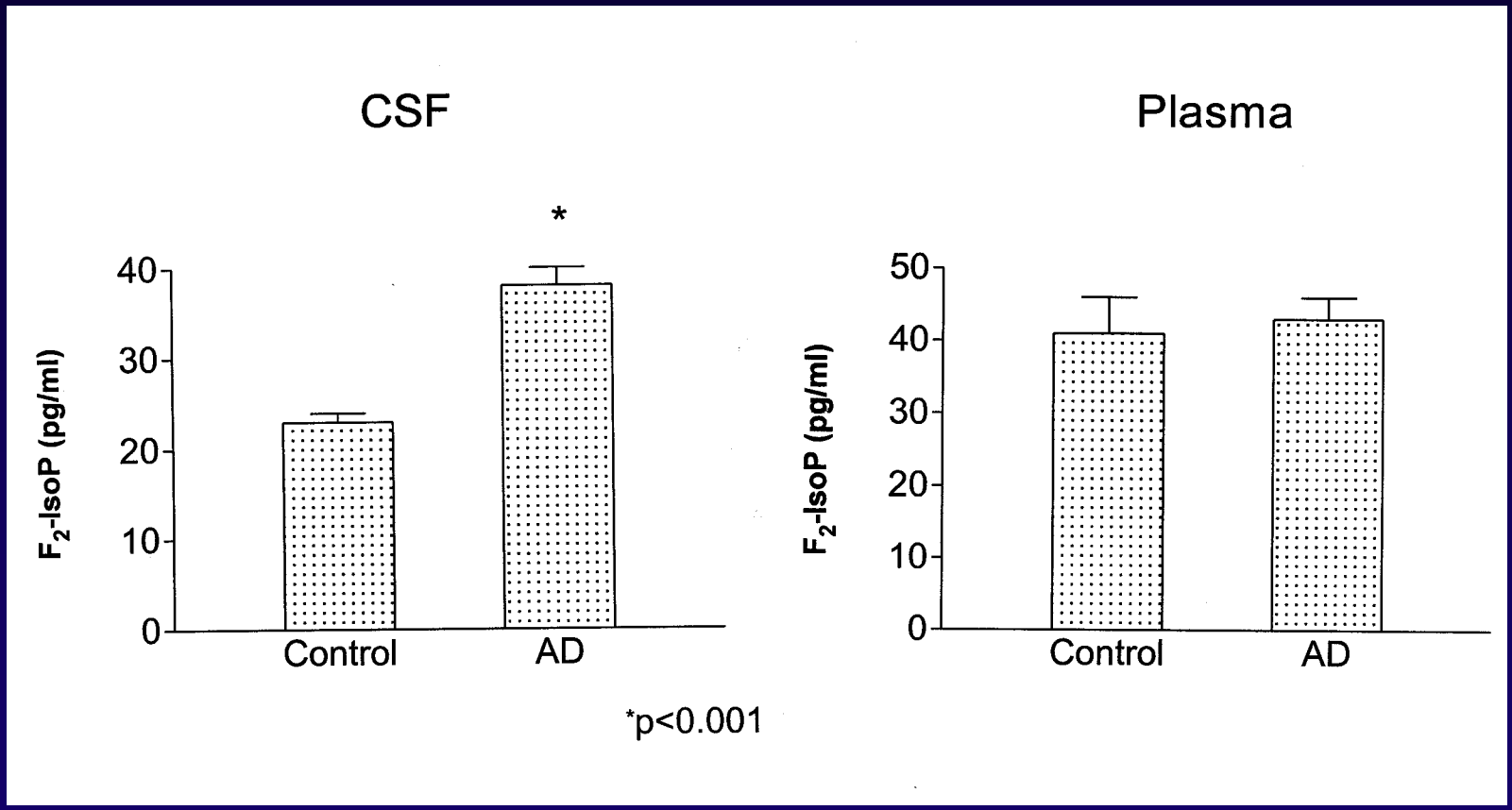
Advantages of Isoprostane Quantification to Assess Oxidant Stress

- Isoprostanes are stable molecules.
- The assay is highly precise and accurate.
- IsoPs can be detected in all fluids and tissues.
  - Normal ranges can be defined.
  - Allows for studies to evaluate the effects of interventions on endogenous lipid peroxidation.

Disadvantages of Isoprostane Quantification to Assess Oxidant Stress

- Samples must either be analyzed immediately or stored at –70^{o} C.
- Increases in IsoPs locally in tissues or fluids aren't detected by measuring systemic oxidant stress.

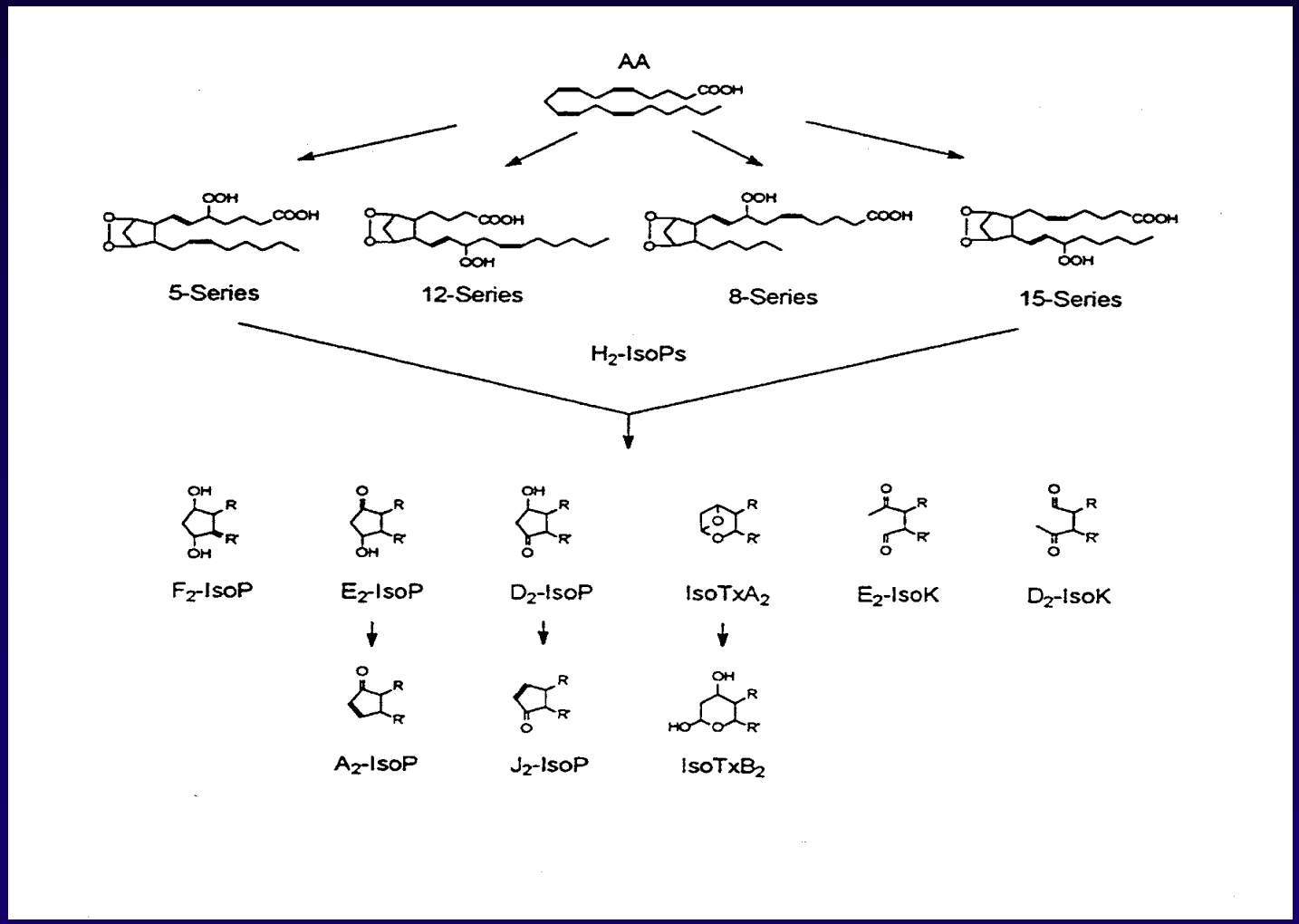
Isoprostanes Are Increased Selectively in the Central Nervous System of Humans with AD



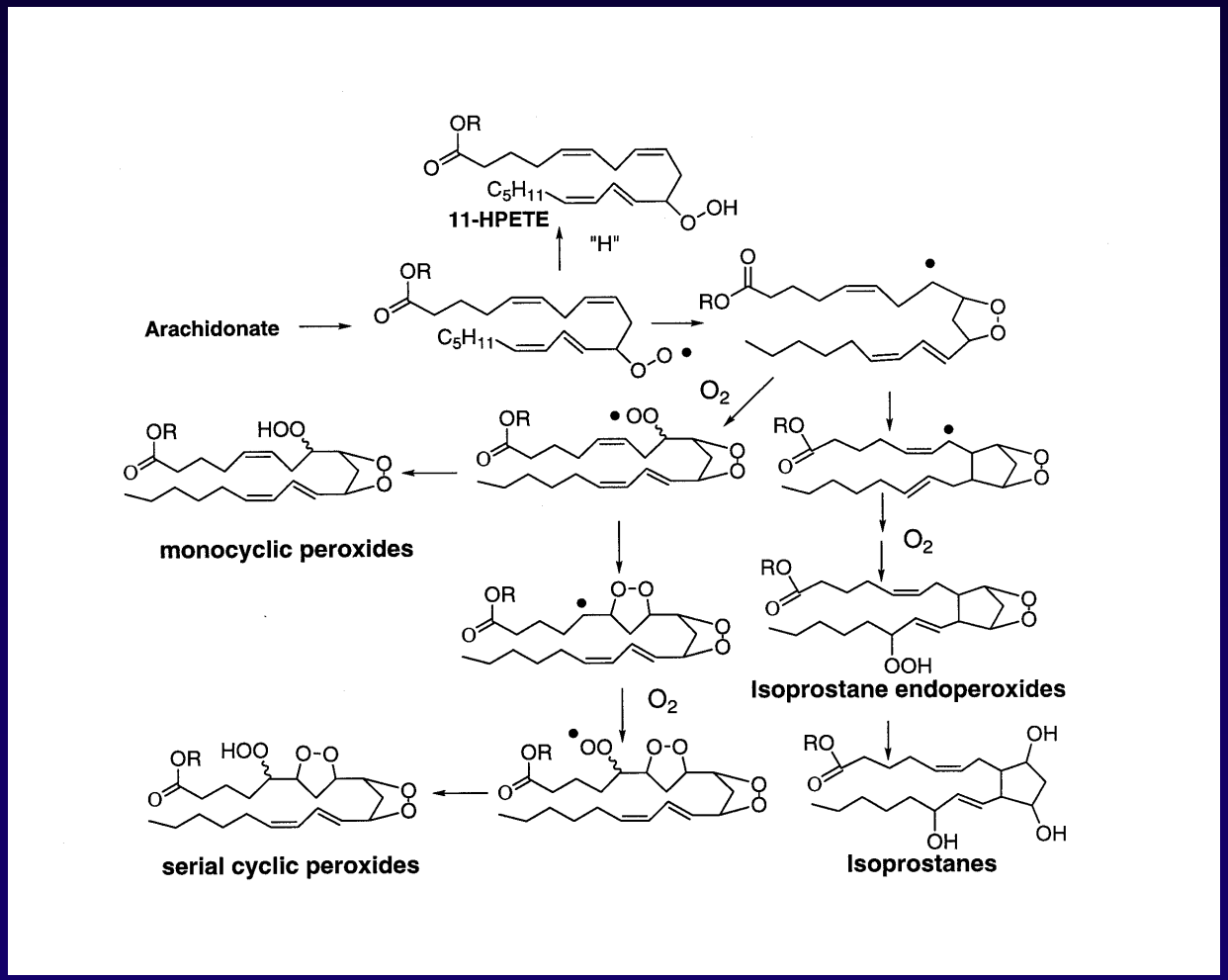
Disadvantages of Isoprostane Quantification to Assess Oxidant Stress

- Samples must either be analyzed immediately or stored at –70^{o} C.
- Increases in IsoPs locally in tissues or fluids aren't detected by measuring systemic oxidant stress.
- F_2-IsoPs represents only one of a myriad of arachidonate oxygenation products.

Classes of IsoPs



Unified Pathway of Arachidonate Oxidation



Disadvantages of Isoprostane Quantification to Assess Oxidant Stress

- Samples must either be analyzed immediately or stored at –70^{o} C.
- Increases in IsoPs locally in tissues or fluids aren't detected by measuring systemic oxidant stress.
- F_2-IsoPs represents only one of a myriad of arachidonate oxygenation products.
- Analysis is labor intensive and requires expensive equipment.

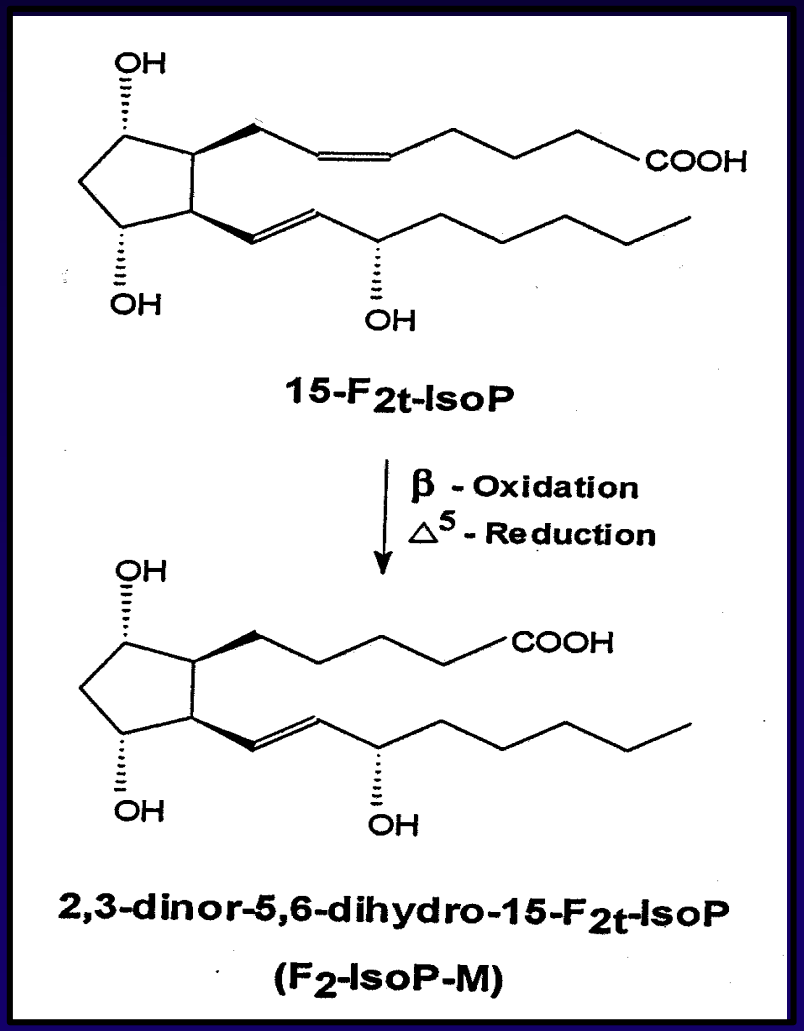
Immunoassay Methods to Quantify IsoPs

- Immunoassays advantageous because they are more economical and less labor intensive.
- Polyclonal antibodies have been made by several investigators and are commercially available.
- Accurate quantification using immunoassays requires initial compound purification.
- Amounts measured by immunoassays often differ from those obtained by mass spectrometry.
- We are currently collaborating with Unilever Ltd. in the generation of highly specific monoclonal abs.

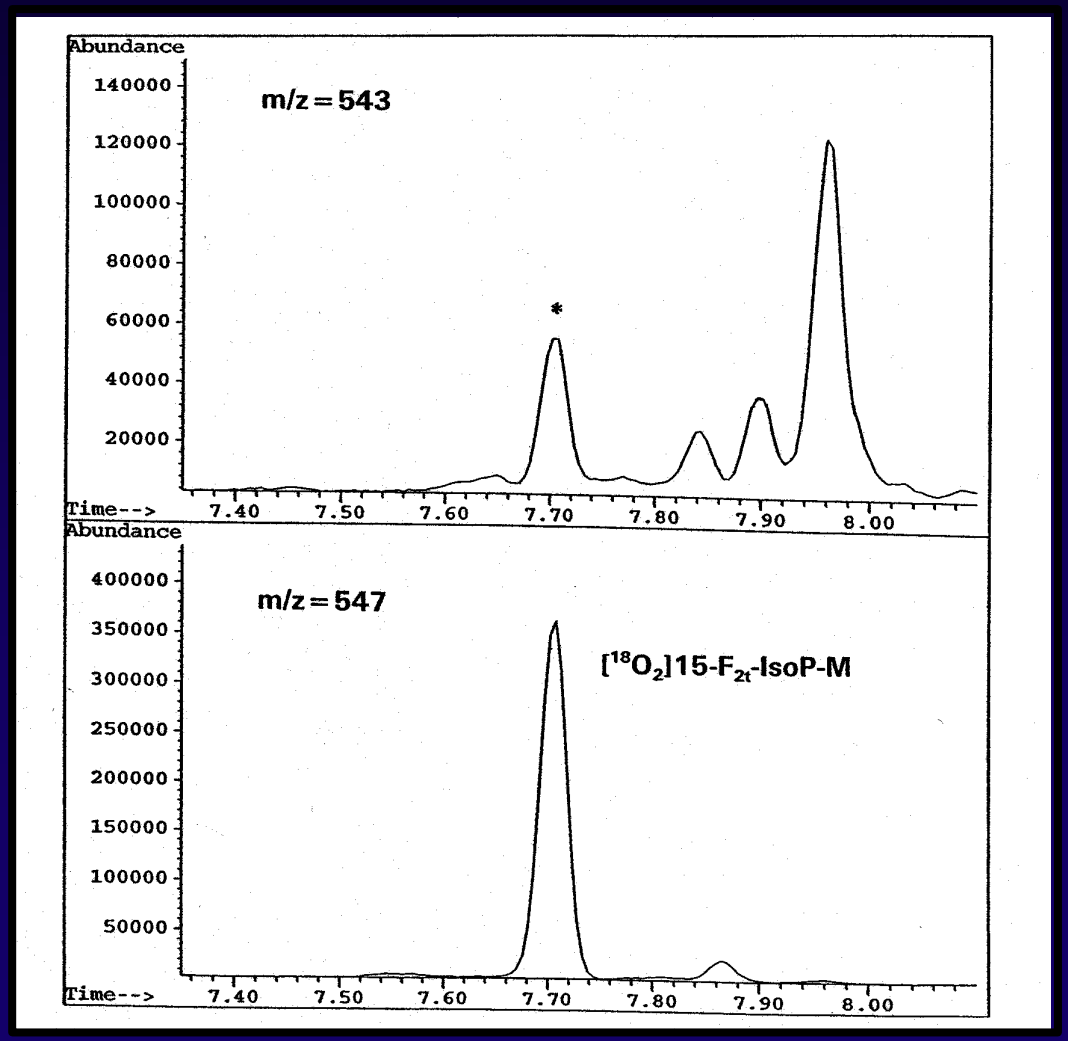
Disadvantages of Isoprostane Quantification to Assess Oxidant Stress

- Samples must either be analyzed immediately or stored at –70^{o} C.
- Increases in IsoPs locally in tissues or fluids aren't detected by measuring systemic oxidant stress.
- F_2-IsoPs represents only one of a myriad of arachidonate oxygenation products.
- Analysis is labor intensive and requires expensive equipment.
- Urinary IsoPs are not a valid measure of systemic oxidant stress since a major source of urinary IsoPs is likely the kidney.

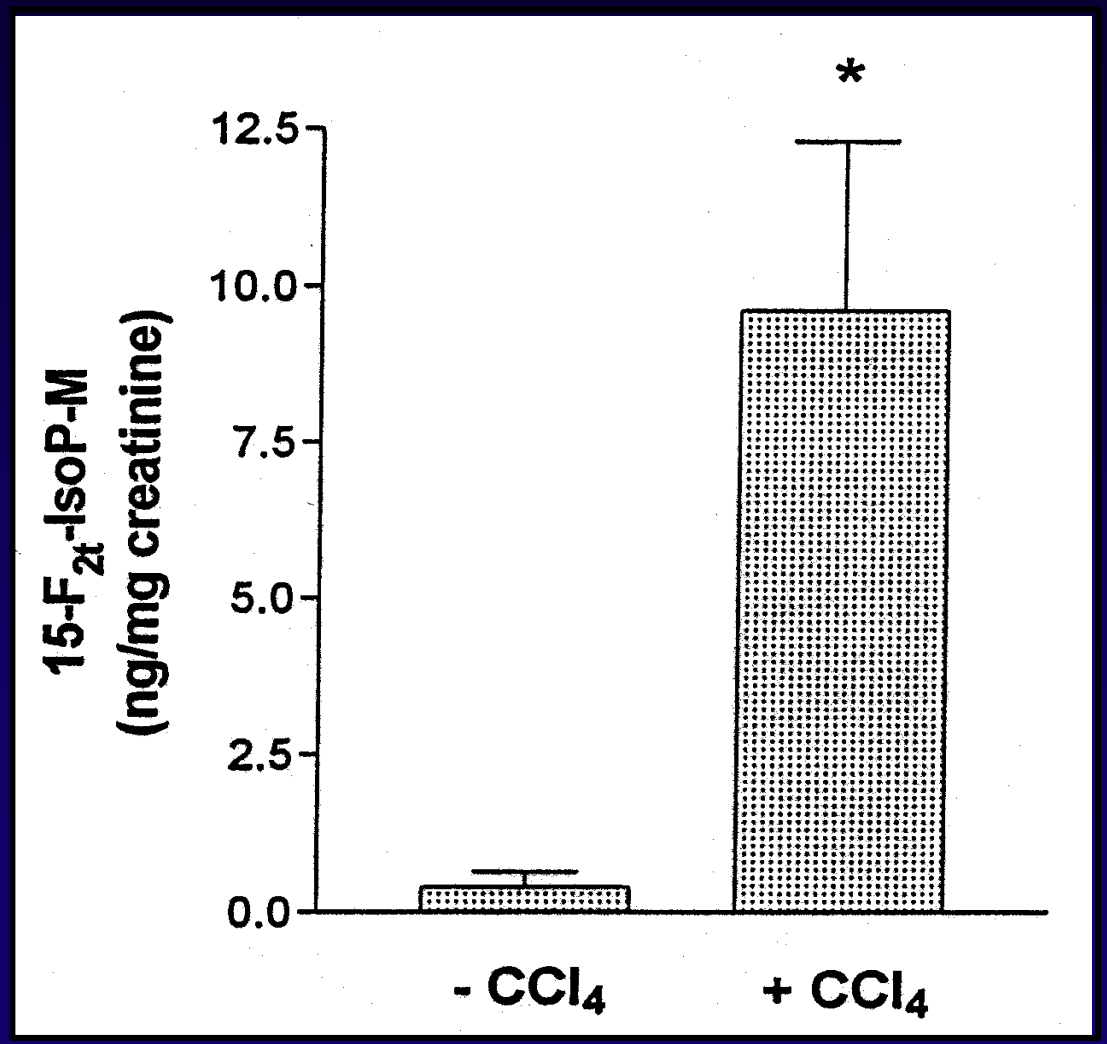
Metabolic Fate of 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) in Humans



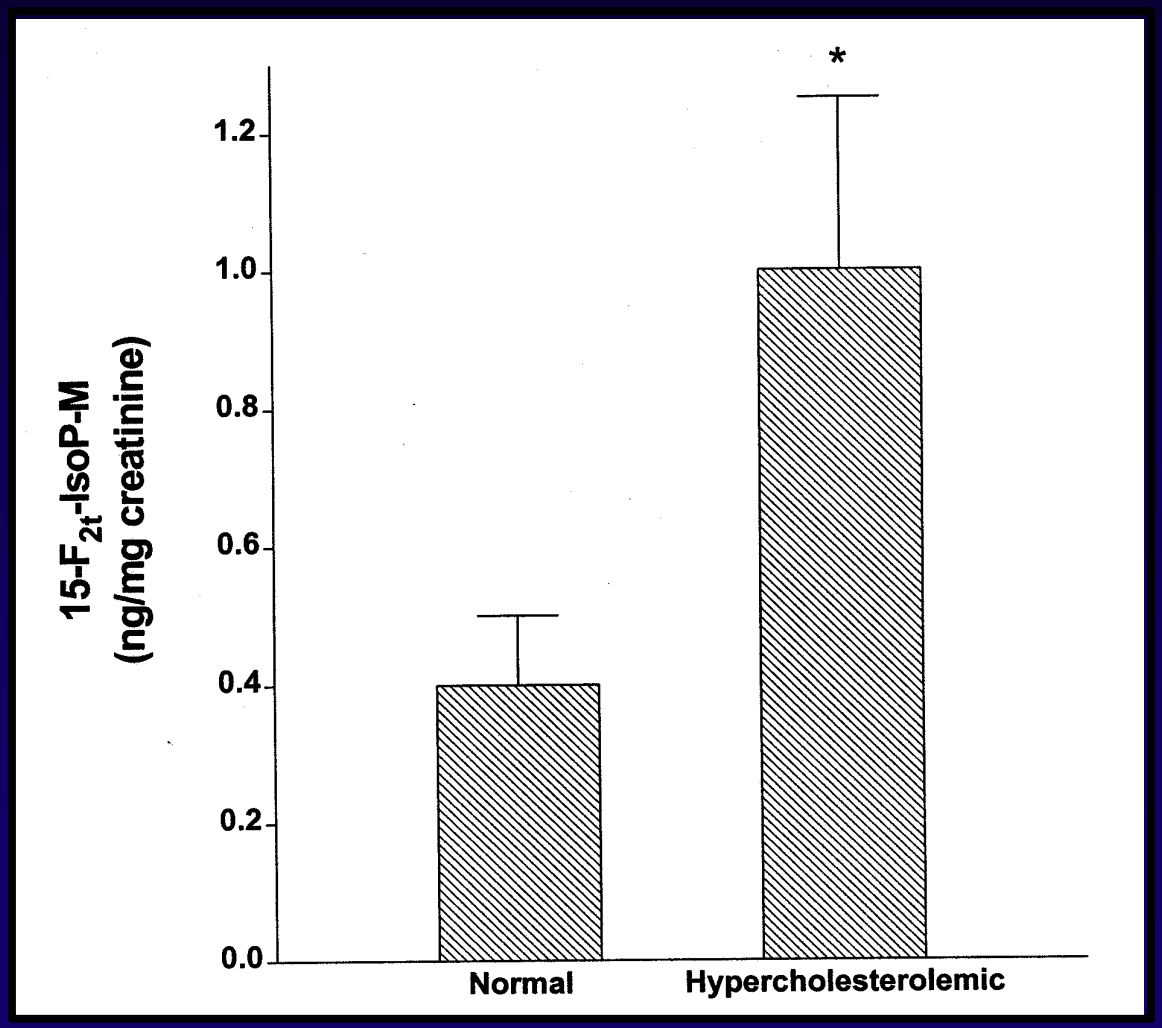
Analysis of Human Urine for F_2-IsoP-M



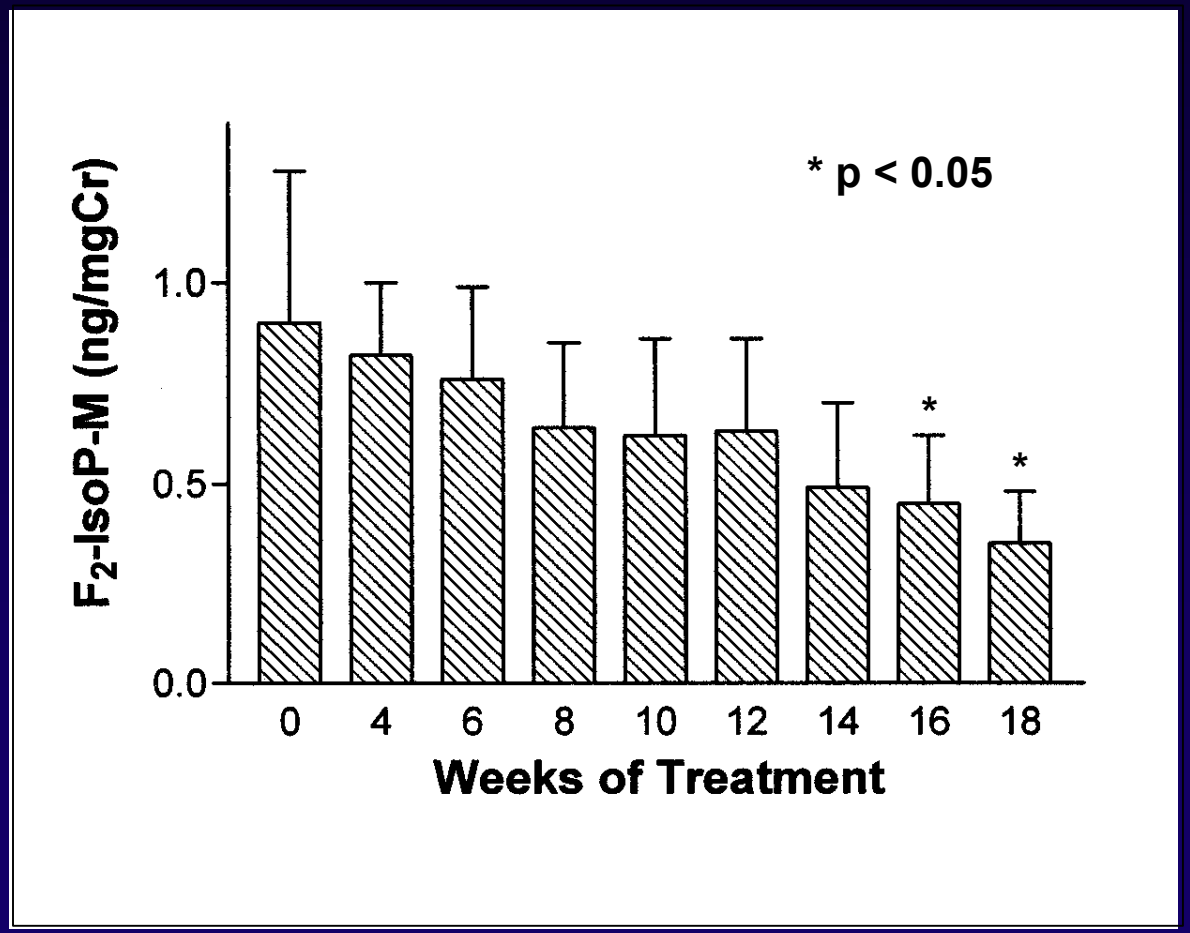
Levels of F_2-IsoP-M Are Increased in Rats Administered CCl_4



Levels of F_2-IsoP-M in Normal and Hypercholesterolemic Humans



Vitamin E Lowers F_2-IsoP-M Excretion in Hypercholesterolemic Humans



Summary

- Quantification of F_2-isoprostanes is an accurate measure of oxidant injury and lipid peroxidation *in vitro* and *in vivo*.
- Current methods employ mass spectrometry and immunoassays.
- The development of an assay to measure F_2-IsoP-M may offer certain advantages over the quantification of parent F_2-IsoPs.

Conclusions

- Numerous methods exist to quantify lipid peroxidation.
- Most assays currently available are accurate measures of lipid peroxidation *in vitro*.
- Studies suggest that these assays are either less accurate measures of lipid peroxidation *in vivo* or too little information exists to accurately assess them.
- Quantification of isoprostanes represents a significant advance in our ability to quantify lipid peroxidation *in vivo*.