

# Oxidative Stress in COPD: Molecular Background and Clinical Monitoring

Balazs Antus\* and Zsuzsanna Kardos

Department of Pathophysiology, National Koranyi Institute of TB and Pulmonology, Pihenó ut 1, H-1121 Budapest, Hungary



**Abstract:** Chronic obstructive pulmonary disease (COPD) is a major and rapidly increasing health problem associated with a chronic inflammatory response, predominantly in small airways and lung parenchyma. Oxidative stress induced by reactive oxygen and nitrogen species (ROS and RNS) plays a central role in the pathophysiology of COPD. There is evidence that several molecules formed during oxidative processes may have the potential to serve as biomarkers of oxidative stress in the airways of patients with COPD. Among these molecules carbon monoxide, ethane and pentane can be measured in the exhaled air, while 8-isoprostane, malondialdehyde, 4-hydroxyhexenal, 4-hydroxynonenal, acrolein, hydrogen peroxide, nitrogen oxides and 3-nitrotyrosine can be detected in exhaled breath condensate and/or sputum supernatant. In this review the molecular background of these processes including the formation of ROS and RNS, the biosynthesis of essential  $\omega$ -3 and  $\omega$ -6 polyunsaturated fatty acids as building blocks of lipids in the cellular membranes and their enzymatic and non-enzymatic metabolism to eicosanoids and related compounds have been summarized. Moreover, the formation of oxidative stress markers studied most commonly in the context of COPD has been briefly discussed. The associations between biomarkers and clinical variables have also been highlighted in an attempt to illustrate the potential clinical applicability of these biomarker measurements.

**Keywords:** Chronic obstructive pulmonary disease, eicosanoids, isofurans, isoprostanes, lipid peroxidation, oxidative stress, polyunsaturated fatty acids, reactive oxygen and nitrogen species.

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a complex respiratory disease characterized by progressive decline in lung function, chronic airway inflammation and impairment in quality of life [1]. The disease has relatively high prevalence rates worldwide (5-13%), and is mainly caused by the inhalation of noxious substances, predominantly cigarette smoke in the Western world. COPD is associated with high mortality and morbidity rates and a high economic and social burden, mainly due to the requirements for substantial and ongoing medical support [2].

Oxidative stress plays a pivotal role in the pathogenesis of COPD [3, 4]. Oxidative stress is not only a consequence of an irritant-induced damage of bronchial epithelial cells, but represents also an amplifying mechanism for the recruitment of inflammatory cells in the airways. Nevertheless, the molecular pathomechanism of COPD is quite different from those of asthma, as indicated by the observation that anti-inflammatory drugs such as corticosteroids have been found to be effective in patients with asthma, while they do not significantly reduce inflammatory response in patients with COPD [5].

Monitoring oxidative stress is difficult and may not be reflected by changes of lung function parameters. Theoretically, oxidative stress can be estimated by *direct* quantification of oxidation products, or *indirectly*, by quantification of by-products derived typically from lipid peroxidation in blood, urine, exhaled air, exhaled breath condensate (EBC), sputum or bronchoalveolar lavage (BAL). In recent years several biomarkers and “footprints” of oxidative/nitrosative damage have been detected in these body fluids that may assist in the clinical management of COPD [6, 7].

Several of these putative markers are present in elevated levels in blood indicating systemic oxidative stress that may or may not originate in the respiratory tract. Assessment of markers in a respiratory sample is more likely to reflect oxidative processes that occur in the lungs. However, bronchoscopy and BAL are invasive procedures. They may cause discomfort to the patients, may not be possible to apply to patients with more severe disease and repeated measurements are difficult to perform. By contrast, exhaled gases, EBC and induced sputum collection allow sampling of the airways in a non-invasive or semi-invasive fashion [6]. These techniques are safe, do not require invasive intervention and can be repeated within a short period of time. These methods offer a unique opportunity to identify pulmonary biomarkers of potential clinical utility in the management of COPD.

Among exhaled gases carbon monoxide (CO) and volatile hydrocarbons have been implicated as potentially useful

\*Address correspondence to this author at the Department of Pathophysiology, National Koranyi Institute of TB and Pulmonology, Pihenó ut 1, H-1121 Budapest, Hungary; Tel: +36 1 3913309; Fax: + 36 1 2007060; E-mail: [antusb@hotmail.com](mailto:antusb@hotmail.com)

biomarkers of oxidative/nitrosative stress in COPD. EBC and sputum supernatant may also contain several oxidants including metabolites of arachidonic acid (AA), aldehydes, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and nitrogen oxides which can provide valuable information about oxidative processes in the airways of patients with COPD. Biomarkers can be widely used as noninvasive means to make more accurate diagnoses, monitor disease progression and create personalized treatment regimes. Moreover, biomarkers have the potential to indicate an individual's disease phenotype and estimate treatment responsiveness, for example, in relation with corticosteroids.

The aim of this review is to summarize briefly the molecular background of oxidative stress associated with the pathogenesis of COPD and to give an overview on airway oxidative stress markers. Although this issue has been discussed in many reviews recently [3, 7-13], the biochemistry of free radical-initiated lipid peroxidation as a key process in the development of the disease, and the formation and the metabolism of airway oxidants have not been adequately addressed in these reports.

### FORMATION OF REACTIVE OXYGEN AND NITROGEN SPECIES IN THE COURSE OF OXIDATIVE STRESS

Oxidative stress occurs in the tissue of mammals when the production of oxidants exceeds the antioxidant capacity of the defenses which are able to detoxify them [3, 11, 12]. It is known for a long time that free radicals of oxygen form in all organisms during physiological and pathological processes. In the course of the evolution only those organisms could survive, which developed a suitable system to defend against free radical reactions. It is also well known that aerobic life uses oxygen to oxidize carbon- and hydrogen-rich substrates (foods) to obtain energy and build up their essential biological molecules. Unfortunately, when molecules are oxidized by oxygen, oxygen is reduced resulting in water *via* reactive oxygen species (ROS), from which three are free radicals, as shown in (Fig. 1).

The molecular oxygen itself may be regarded as a "diradical" and it is only a weak oxidant due to its two unpaired electrons with parallel spins. Its true reactivity can appear when this spin restriction will be overcome by UV irradiation or additives called initiators. In the first step of this

process, one of its unpaired electrons excites to a higher energy orbital and its spin is inverted to result in singlet oxygen ( $^1\text{O}_2$ ).  $^1\text{O}_2$  can exist in two states such as the (i) delta singlet oxygen ( $\Delta^1\text{O}_2$ ) whose newly paired electrons with opposite spin occupy the same orbital and (ii) sigma singlet oxygen ( $\Sigma^1\text{O}_2$ ) in which electrons with opposite spin are on different orbitals. The later one is extremely energetic and rapidly decays to the  $\Delta^1\text{O}_2$  form in biological systems, and its surplus energy may diminish by thermal decomposition, light emission and chemical reaction. In the next step of the reduction, superoxide radical anions ( $\cdot\text{O}_2^-$ , half-life= $1 \times 10^{-6}$  sec at  $37^\circ\text{C}$ ) are formed by one electron transfer, e.g. in the course of autoxidation of various compounds in the mitochondrial respiratory pathway and the activation of membrane nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) *oxidase* as physiological electron donors (Eq. 1) [12].



The superoxide radical anion it is a weak oxidizing agent and it is able to damage several biological systems in aqueous solution at  $\text{pH}=7.4$ . Its oxidizing power can be significantly increased in the presence of iron complexes, such as cytochrome-c or copper-zinc *superoxide dismutase* (SOD) enzyme [11, 12]. In acidic media such as the vacuole of the phagocytes or the microenvironment of cell membrane, the protonation of  $\cdot\text{O}_2^-$  takes place very smoothly to give  $\text{H}_2\text{O}_2$  *via* perhydroxy radical ( $\text{HOO}\cdot$ ), which is a stronger oxidant than  $\cdot\text{O}_2^-$ , but its concentration is usually very low at  $\text{pH}=7.4$ . Since  $\text{H}_2\text{O}_2$  does not possess unpaired electron, it is also a weak oxidant and it is able to diffuse across the cell membranes. In the presence of transition metal ions such as ferrous one ( $\text{Fe}^{2+}$ ), it is transformed into hydroxyl radical ( $\text{HO}\cdot$ , half-life= $1 \times 10^{-6}$  sec at  $37^\circ\text{C}$ ), which is an extremely aggressive oxidant and can attack most biological molecules. It is also noteworthy that  $\text{HO}\cdot$  can be also produced from  $\cdot\text{O}_2^-$  in the presence of  $\text{H}_2\text{O}_2$  according to the Haber-Weiss reaction (Eq. 2) [14].



However, this reaction takes place very slowly in human cells, and thus  $\text{HO}\cdot$  is present in a very low concentration. Nevertheless, its formation can be significantly accelerated by metal catalysis as observed by Fenton in 1894 (Eq. 3-5) [15].

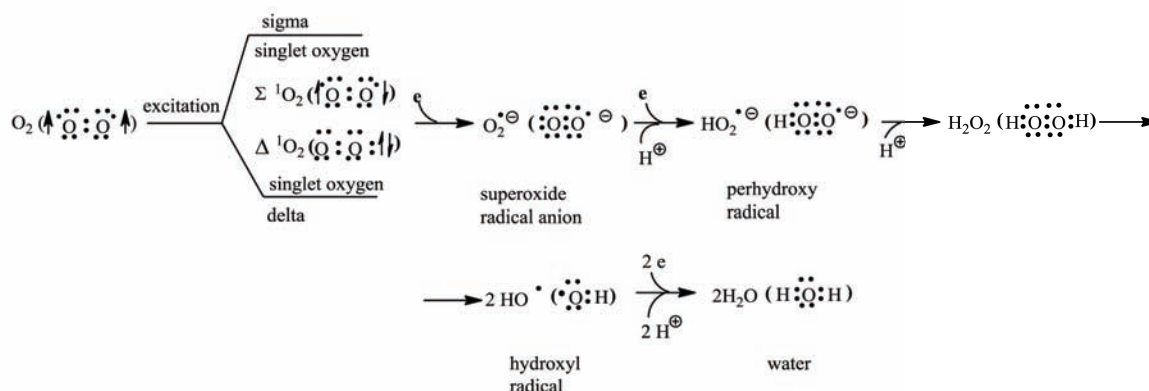
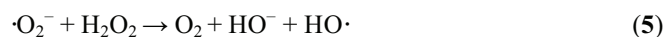


Fig. (1). Formation of ROS during reduction of molecular oxygen.



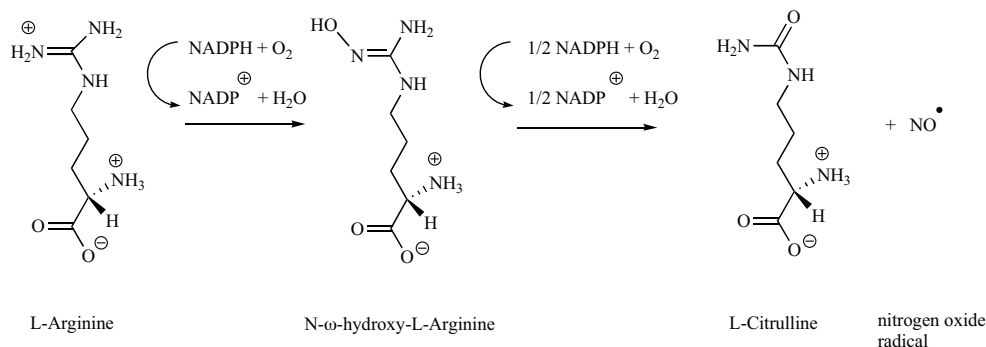
In the last step of the reduction, hydroxyl anion ( $\text{HO}^-$ ) is formed by an electron transfer and the protonation of  $\text{HO}^-$  gives water.

Oxidative stress can also be induced by nitrogen oxide radical ( $\text{NO}\cdot$ , half-time=0.09-5 sec depending on  $\text{O}_2$  concentration gradients away from blood vessel at  $37^\circ\text{C}$ ), which is produced from L-arginine by *nitric oxide synthase* (NOS) enzyme in human cells [16], as depicted in (Fig. 2). Its common source is tobacco smoke which has been estimated to contain over  $10^{15}$  free radicals per puff.  $\text{NO}\cdot$  can react with another endogenous free radical such as  $\cdot\text{O}_2^-$  to produce a reactive intermediate, the powerful oxidant peroxynitrite ( $\text{ONOO}^-$ ) [17]. In general, reactive nitrogen species (RNS) act together with ROS to damage cells causing nitrosative stress.

During the past six decades, it has been proven that free radicals play an important role in the pathogenesis of various pulmonary (bronchial asthma, COPD, etc.), hepatic (alcoholic liver damage, Dubin-Johnson syndrome, etc.) and neu-

phenolic compounds, carotenoids, vitamin A, C and E) and metal deactivators (MD, transferrin, ferritin and polyphenols) [18]. When the increased production of ROS during inflammation or the metabolism of environmental toxins including cigarette smoke cannot be detoxified by intra- and extracellular antioxidants, oxidative stress occurs which induces lipid peroxidation followed by the damage of cellular macromolecules such as deoxyribonucleic acid (DNA), lipids and peptides, particularly lung elastin [19-21].

COPD is chronic inflammatory disease characterized by airflow limitation, which is not fully reversible. The airflow limitation is usually progressive and is associated with abnormal inflammatory responses of the lungs to noxious particles and gases [1, 4]. In this process various inflammatory and structural cells (neutrophils, eosinophils, macrophages and epithelial cells) are activated in the airways by ROS-mediated lipid peroxidation. Lipids are essential components of cellular membranes to maintain the biological function of cells and control major cellular activities [19, 20]. However, lipids with PUFAs are primary targets for the attack of ROS. Moreover, some of them are also implicated as essential molecules for the biosynthesis of other inflammatory mediators in human cells.



**Fig. (2).** Formation of NO radical.

rological (Alzheimer's and Parkinson's disease, etc.) diseases. Additionally, they may have a regulatory role in different physiological (AA metabolism, synthesis of adrenocortical hormones, etc.) and pathological (enzyme, nucleic acid and cell membrane damage) processes.

### ROLE OF FREE RADICAL REACTIONS IN COPD

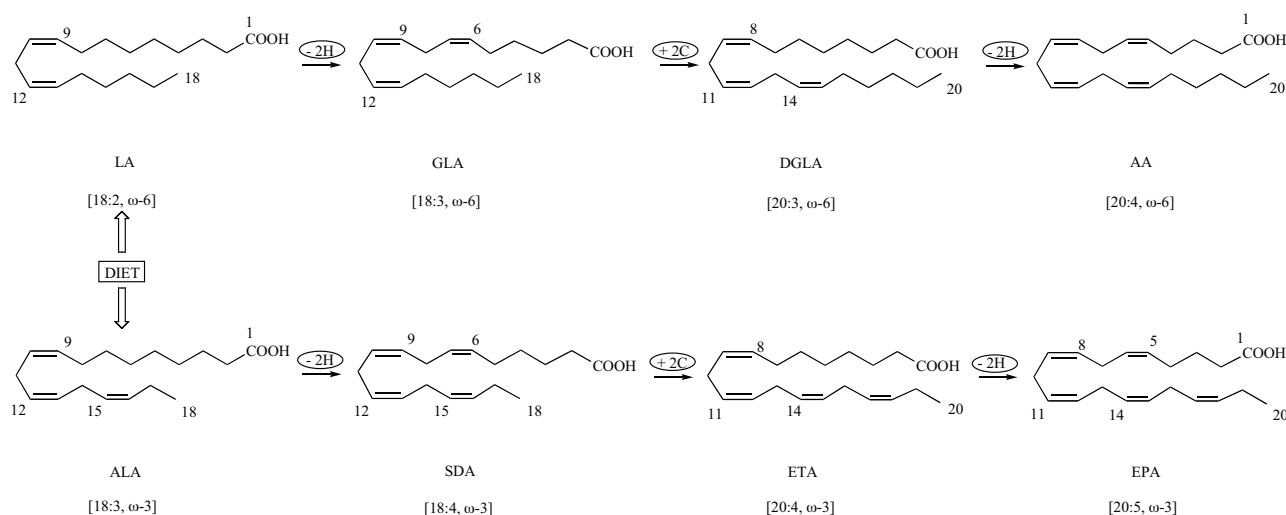
COPD is generally diagnosed when lung function parameters have become significantly reduced and a major part of the lung has been damaged resulting in serious pathological changes, especially within the small airways [4]. Its pathogenesis is strongly associated with ROS which are generated constantly *in vivo* from the molecular oxygen but also during a variety of endogenous processes including normal mitochondrial aerobic respiration, phagocytosis of bacteria or virus-containing cells and peroxisomal-mediated degradation of polyunsaturated fatty acids (PUFAs) [3, 4].

The overproduction of ROS is normally detoxified by antioxidant defense systems such as antioxidant enzymes (*superoxide dismutase* [SOD], *catalase* [CAT] and *glutathione peroxidase* [ $\text{GP}_x$ ]), low molecular weight antioxidants and vitamins (uric acid, glutathione, flavonoids and related poly-

### BIOSYNTHESIS OF ARACHIDONIC AND EICOSAPENTAENOIC ACID

PUFAs are the fundamental building blocks of lipids stored as glycerophospholipid and cholesteryl esters in the endothelial cell membrane of humans. Since mammalian cells are not able to introduce a double bond in fatty acids beyond C-9, all fatty acids containing a double bond beyond C-9 have to be supplied in the diet. These molecules include linoleic (LA)<sup>1</sup> and  $\alpha$ -linolenic (ALA) acids, which are called as essential fatty acids (EFA) for mammals. Other PUFAs such as AA and eicosapentaenoic acid (EPA) are synthesized from LA and ALA in the liver, respectively, as outlined in (Fig. 3) [22].

<sup>1</sup>According to the nomenclature of PUFAs linoleic acid is designated as LA:18:2,  $\omega$ -6. The first number specifies the number of carbon atoms of PUFAs, while the second the number of double bonds. The position of the terminal double bond is denoted in the form of  $\omega$ -x where x indicates the number carbon atoms from the terminal double bond assuming that all the other double bonds are methylene-interrupted.



**Fig. (3).** Biosynthesis of AA and EPA.

In the first step of the biosynthesis of AA and EPA, a *cis*-double bond at C-6 is introduced in LA and ALA by the dehydrogenation with  $\Delta^6$ -desaturase enzyme to afford  $\gamma$ -linolenic (GLA) and stearidonic (SDA) acids whose chains are subsequently elongated with two carbon units by malonyl-CoA as a carbon atom donor to result in dihomogamma-linolenic (DGLA) and eicosatetraenoic (ETA) acids, respectively. In the last step, the repeated dehydrogenation at C-18 gives the target molecules such as AA and EPA. AA and EPA are stored in the endothelial cell membrane of mammals as glycerophospholipid and cholesterol esters or docosahexaenoic acid (DHA), respectively. Moreover, it is also remarkable that the activity of the  $\Delta^6$ - and  $\Delta^5$ -desaturase enzymes are rather low and can be influenced by ingested GLA from dietary sources. Due to the limited activity of the  $\Delta^5$ -desaturase, most of the DGLA formed from GLA is inserted into membrane phospholipids at the same C-2 position as AA.

### BIOSYNTHESIS OF EICOSANOIDS

Eicosanoids ( $C_{20}$ -compounds, *eicosa*=20) consist in prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs) and lipoxins (LXs) [12, 23, 24]. The PGs and TXs are collectively called as prostanoids<sup>2</sup>. Prostaglandins were originally shown to be synthesized in the prostate gland, thromboxanes from platelets (thrombocytes) and leukotrienes from leukocytes. The lipoxins are anti-inflammatory eicosanoids synthesized through *lipoxygenase* interactions. All mammalian cells except erythrocytes synthesize eicosanoids. These molecules are extremely potent and able to cause profound physiological effects at very low concentrations. All eicosanoids function locally at the site of synthesis, through receptor-mediated G-protein linked signaling pathways.

### ENZYME CATALYZED AND FREE RADICAL MEDIATED TRANSFORMATIONS OF ARACHIDONIC ACID AND RELATED COMPOUNDS

PGx, TXs and LTs play an important role in the mediation and the resolution of inflammation. Numerous stimuli (e.g. epinephrine, thrombin, bradykinin) may activate *phospholipase* enzyme ( $PLA_2$ ), which hydrolyzes AA in membrane phospholipids. Although AA can also be re-esterified by phospholipides and cholesterol or converted reversibly to arachidonyl ethanolamide (anandamide) [25], as shown in (Fig. 4), it is more likely that it undergoes fast metabolism to result in oxygenated metabolites. The main pathways mediated by enzymes such as *cyclooxygenases* (COXs), *5-lipoxygenase* (5-LOX) and *cytochrom P450* (CYP) include the formation of (a) prostacyclins ( $PGI_2$ ), PGs and TXs; (b) LTs and (c) hydroxyeicosatetraenoic acids (HETEs), dihydroxy (DHETs)- and epoxy-(EETs) eicosatrienoic acids in optically pure form [23].

However, starting from AA, the oxidation process initiated by ROS can also take place in a completely different manner resulting in isoprostanes (IsoPs) and isofurans (IsoFs) and a number of other degradation products such as alkanes, alkenes, aldehydes, ketones and NO-containing substances with diverse pathophysiological actions [24, 26]. Furthermore, in contrast to the biosynthesis of PGs, IsoPs and IsoFs are formed in racemic form without regio- and diastereoselectivity due to the radical mechanism of their synthesis. IsoPs, IsoFs and related compounds are formed primarily from esterified phospholipids and are subsequently hydrolyzed by  $PLA_2$  enzyme, while prostanoids are enzymatically synthesized from free AA released from phospholipids upon stimulations [27].

Ingold's and Barclay's research groups have established that the free radical oxidation of phospholipids in the lipid membrane (lipid bilayer or liposome) follows the same kinetic law as in homogenous system [28-30]. Considering this, and for the sake of simplicity, enzyme catalyzed and free radical mediated routes of the oxidation of PUFAs will be discussed parallel above.

<sup>2</sup>The nomenclature of prostanoids includes a subscript number which refers to the number of carbon-carbon double bonds that exist in the molecule. The majority of the biologically active PGs and TXs referred to as series 2 molecules due to the presence of two double bonds. The predominant LTs are series 4 molecules due to the presence of four double bonds in the molecule.

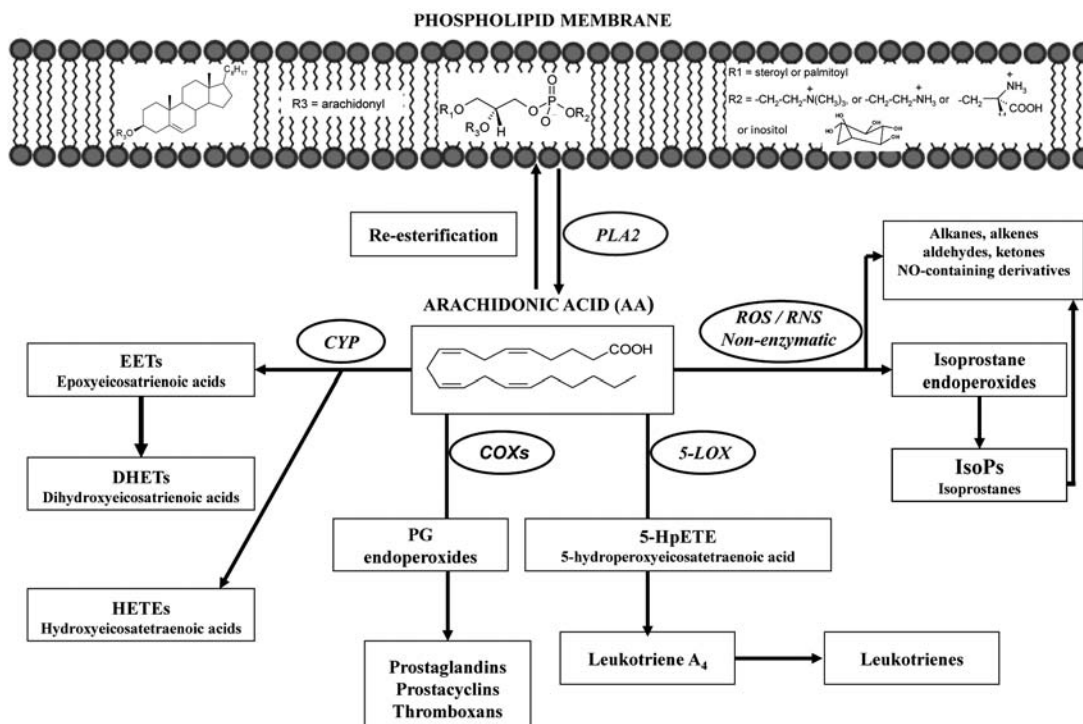


Fig. (4). Major metabolic pathways of AA.

Most of the ROS outlined in (Fig. 1) participate in the metabolism of AA *in vivo*. AA contains a *cis* double bond at C-5, -8, -11 and -14 separated by methylene groups, as shown in (Fig. 5), and thus, three equivalent bisallylic units are present in this molecule. Its bisallylic hydrogens are very sensitive to ROS due to their low dissociation enthalpy (approximately  $78\text{-}80\text{ kcal}\cdot\text{mol}^{-1}$ ) [31]. According to this characteristic feature of AA, its main chemical transformations are bisallylic peroxidation, epoxygenation of the double bonds and hydroxylation at  $\omega$ - or  $\omega$ -1-4 carbon atoms [32].

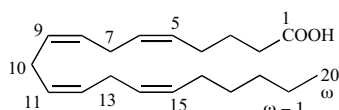


Fig. (5). Structure of AA.

## BISALLYLIC PEROXIDATION OF ARACHIDONIC ACID

This transformation is a so-called chain reaction consisting of three steps: initiation, propagation and termination [28-30,32].

### Initiation

Abstraction of a hydrogen radical from the bisallylic system of AA and its esters such as cholesteryl arachidonate takes place by exogenous chemical agents such as air pollution, UV-light, singlet oxygen and smoke or endogenous enzymatic systems such as NADPH oxidase, xanthine oxidase and CYP enzymes as well as by an electron transport chain (ETC) in mitochondria whose reaction with oxygen produce  $\cdot\text{O}_2^-$  and other ROS, as shown in (Fig. 1). AA and its esters possess

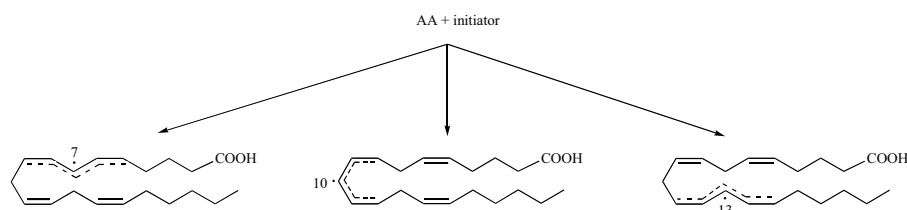
three bisallylic positions (C-7,-10,-13) as possible sites for initial abstraction of a hydrogen radical<sup>3</sup> (Fig. 6).

### Propagation

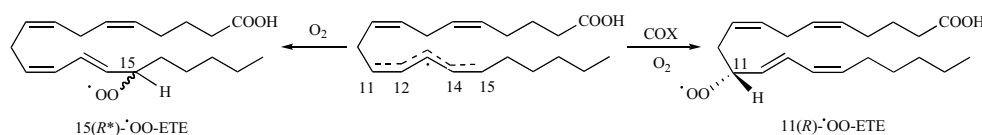
- Oxygen addition*: Reaction of bisallylic radicals formed from AA or its esters with molecular oxygen results in a mixture of four racemic peroxy-eicosatetraenoate radical isomers possessing a conjugated (*E,Z*)-dien unit (rac-5-, 9-, 11-,15-  $\cdot\text{OO-ETE}$ ), two racemic isomers possessing a conjugated (*Z,Z*)-dien units (8-,12-  $\cdot\text{OO-ETE}$ ) and three racemic isomers possessing isolated (*Z*)-double bonds (rac-7-,10,-13- $\cdot\text{OO-ETE}$ ) [28,32]. For simplicity they are illustrated only with rac-15-  $\cdot\text{OO-ETE}$ , as shown in (Fig. 7). If the reaction is catalyzed by COX enzyme only 11-  $\cdot\text{OO-ETE}$  is formed region- and enantioselectively whose absolute configuration is (*R*)<sup>4</sup>.

<sup>3</sup> Hydrogen abstraction at C-7, C-10 or C-13 of AA leads to the corresponding bisallylic radicals whose unpaired electron is not localized at these carbon atoms due to the presence of the neighboring two double bonds. Thus, these  $\pi$ -electron systems are delocalized at the corresponding five membered carbon chain possessing the highest electron density at the central and the outside carbon atoms.

<sup>4</sup> The term chirality is derived from the Greek word for hand (*cheir*) which is a very common object and not identical to its mirror image. Thus, a chiral molecule possessing one stereogenic center has two stereoisomers which are called enantiomers. All physical data of enantiomers are identical, except of the sign of their optical rotation which can be used to differentiate them. The absolute configuration (arrangement of the ligands at the stereogenic center) of a stereogenic center may be (*R*) or (*S*) according to the Cahn, Ingold, Prelog (CIP) rule [33]. The 1:1 mixture of enantiomers is the racemic form of a chiral molecule which is signed by ( $\pm$ ), or rac-, or (*R/S*), or (*R\**) as well as (*S\**).

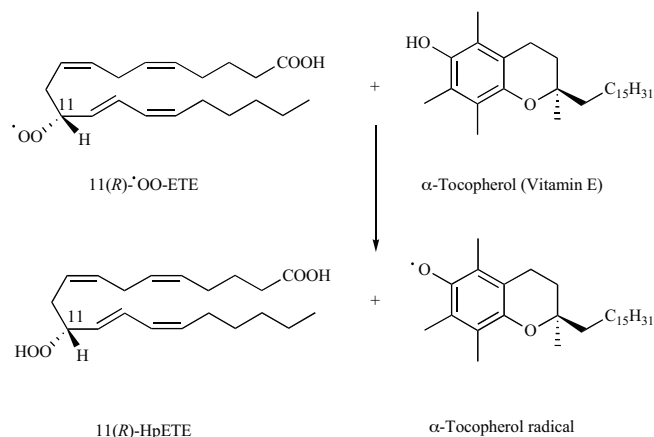


**Fig. (6).** Formation of bisallylic radicals from AA.



**Fig. (7).** Formation of 15(*R*\*)- and 11(*R*)-peroxyl-EETE radical.

- b) *Hydrogen atom transfer*: In the presence of a good hydrogen atom donor such as  $\alpha$ -tocopherol (vitamin E), peroxyl-EETE radicals can easily withdraw a hydrogen to form the corresponding hydroperoxyeicosatetraenoates (HpETEs) and  $\alpha$ -tocopherol radical, as depicted in (Fig. 8). It should be noted that (*R*) absolute configuration of 11(*R*)- $\cdot$ OO-EETE is preserved during this transformation, because the hydrogen transfer does not affect the chirality center of the molecule [32].



**Fig. (8).** Formation of 11(*R*)-hydroperoxy-EETE in the presence of vitamin E.

- c) *Peroxy radical cyclization*: When the peroxy radical is not trapped by an abstraction of a hydrogen, it can also undergo intramolecular cyclization and the oxygen tension may become the dominant factor that determines the subsequent product distribution [28,32]. It is well known that atmosphere contains about 21% molecular oxygen. In humans, the gradient of its tension (partial pressure) decreases from 150 mmHg in the environment to approximately 110 mmHg in the lung, to 95 mmHg in the arterial system and down to only 30 mmHg in most tissue [34]. In the course of the formation of 15-bicyclic endoperoxides (Fig. 9) the 5-*exo* cyclization gives ( $\pm$ )-8-carbon radical whose repeated cyclization followed by addition of a molecular oxygen results in ( $\pm$ )-15-bicyclic endoperoxide (type IV). Finally, its stabilization by reduction affords rac-PGF<sub>2 $\alpha$</sub> , as is shown in (Fig. 9). The

formation of bicyclic endoperoxide type-I, -II and -III takes place in a similar manner, as depicted in (Fig. 10).

Thus, PGF<sub>2 $\alpha$</sub>  possesses five stereogenic centers (C-8, C-9, C-11, C-12 and C-15) and therefore,  $2^5 = 64$  stereoisomers would theoretically be possible. Due to the 5-*exo* connection of the bicyclic ring-system of ( $\pm$ )-8-carbon radical, its absolute configuration at C-9 and -11 must be (*R,R*) or (*S,S*) [(*R,S*) or (*S,R*) is not possible], thus, the number of possible stereoisomers is reduced to 32.<sup>5</sup> Since the ring closure of AA catalyzed by COX enzyme takes place enantioselectively resulting *R,R* absolute configuration at C-9 and -10, the number of possible stereoisomers is 8, as shown in (Fig. 11). This has been experimentally observed by Morrow and co-workers [35, 36].

The stereoisomers whose cyclopentane ring is substituted by side chains in *cis* position are preferentially formed (e.g. 8-*epi*-PGF<sub>2 $\alpha$</sub> ) compared to those of the *trans* substituted ones such as natural prostaglandins PGF<sub>2 $\alpha$</sub>  whose side chains have *trans* geometry. The product's selectivity can be understood on the basis of the cyclization of 9(*R*),11(*R*)-peroxyl-8-carbon radicals to 9(*R*),11(*R*)-peroxyl-13-carbon radicals *via* a Beckwith-Houck's transition state [37, 38]. Thus, the transition state in which both side chains of the cyclopentane moiety of bicyclic endoperoxides are *equatorially* oriented is energetically more favored than those possessing one of the side chains in *axial* orientation. This result is also consistent with the observation of O'Connor's and Corey's groups [39, 40]. Furthermore, it is also noteworthy to mention that in the course of the metabolism of AA catalyzed by COX in human cells (+)-8(*R*),9(*R*),11(*R*),12(*R*),15(*S*)-PGF<sub>2 $\alpha$</sub>  is formed as a sole product in optically pure form. It should be noted

<sup>5</sup> If two stereogenic centers are present in the molecule, than  $2^2 = 4$  stereoisomers are possible (A:1*R*, 2*R*; B:1*S*, 2*S*; C:1*S*, 2*R*; D: 1*R*, 2*S*). Among them A and B as well as C and D are enantiomers each other. A and C or D as well as B and C or D are diastereomers each other. In case of molecules possessing more than two stereogenic centers the corresponding diastereomers used to be called as epimer as well. (e.g. A:1*R*, 2*R*, 3*R* and its diastereomers are B:1*R*,2*R*,3*S* and C:1*R*,2*S*,3*S* etc., B may called as 3-*epi*-A and C may called as 2,3-*bi-epi*-A.

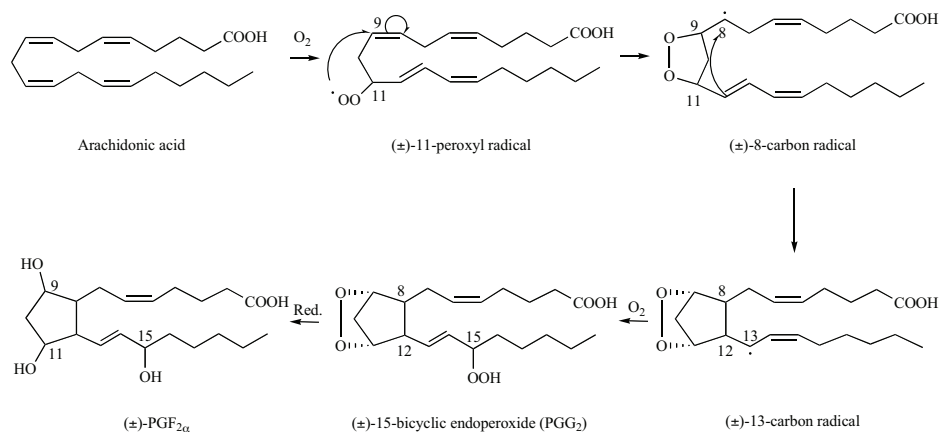


Fig. (9). Formation of racemic bicyclic endoperoxides type IV by non-enzymatic oxidation of AA and cleavage its peroxide bound.

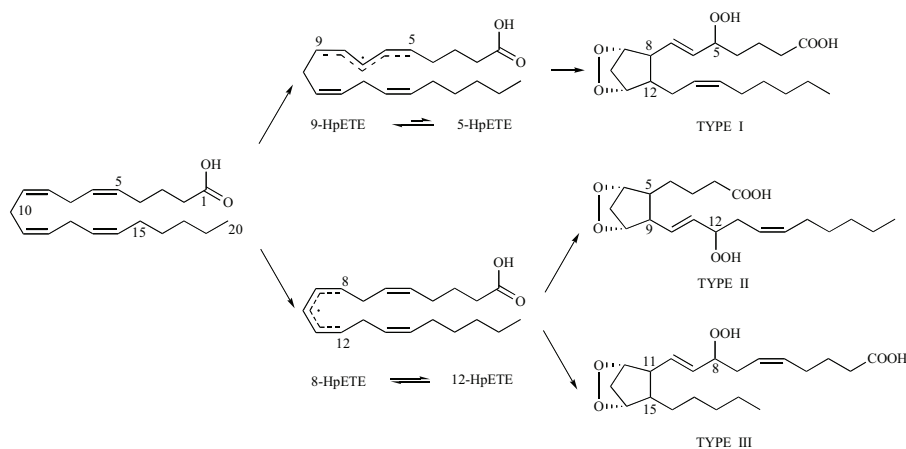


Fig. (10). Formation of G<sub>2</sub>-Isoprostans of type I-III from AA by free radical oxidation.

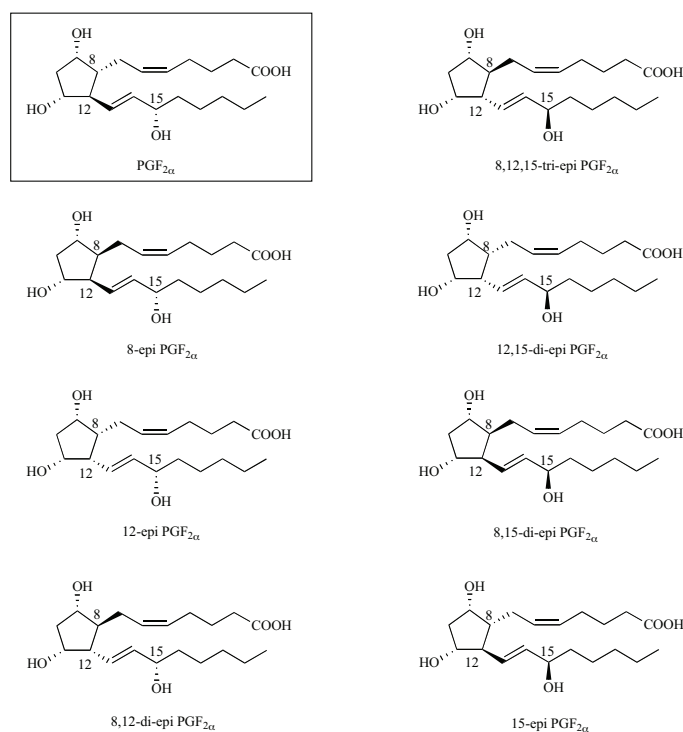


Fig. (11). Structure of (+)-8(R),9(R),11(R),12(R),15(S)-PGF<sub>2α</sub> and its diastereomers.

that beside rac-PGF<sub>2α</sub>, synthesized by uncontrolled free radical peroxidation of AA, its stereoisomers are also formed in different amounts and have various pathophysiological effects in a wide range of diseases including COPD. The observation that F<sub>2</sub>-IsoPs, a group of signaling molecules which cytotoxic effects [41, 42], are formed *in vivo* in significant amount reflect that 5-*exo* cyclization competes with the direct oxygen addition due to the low oxygen tension in human tissues. Since the rate constant for oxygen is diffusion controlled ( $k_D = 10^8$ - $10^9$  mol<sup>-1</sup>s<sup>-1</sup>) and the rate constant for the carbon-centered radical initiated 5-*exo* cyclization or intramolecular homolytic substitution ( $S_{Hi}$ ) as a first order reaction is around  $k_s = 10^5$  mol<sup>-1</sup>s<sup>-1</sup> [43, 44], micromolar or submillimolar concentration of oxygen in a biological system makes these two transformations competitive.

- d) *Homolytic cleavage of oxygen-oxygen bond of hydroperoxides*: The hydroperoxyl group of HpETEs is capable of spontaneous thermal decomposition which affords hydroxyl and the corresponding ETEO· radicals, as shown in (Fig. 12).

### Termination

- a) The chain reaction can be terminated by recombination of radicals. For example, the recombination of hydroxyl and ETEO radicals with surrounding radicals, e.g. with H radical, gives water and hydroxy-ETE (Eq. 6 and 7), respectively.



- b) If the molecular oxygen is present in high concentration, (±)-8-carbon radical is converted by  $S_{Hi}$  attack and the

subsequent homolytic cleavage of O-O bond into the corresponding alkoxy radical possessing an epoxide function at C-8 and -9. Its stabilization by  $S_{Hi}$  attack is followed by addition of oxygen and hydrogen radical that results in a diastereomeric mixture of (±)-bisepoxide possessing a hydroperoxyl group at C-15, as depicted in (Fig. 13). Its regioselective hydrolysis gives the corresponding 11,12-diol derivative, from which the diastereomeric mixture of (±)-tetrahydrofuran-containing compounds (rac-IsoFs) are formed by an intramolecular nucleophilic attack of the C-12 hydroxyl group on C-9 of the epoxide moiety, as depicted in (Fig. 13) [45].

- c) Starting from (±)-8-carbon radical, the diastereomeric mixture of monocyclic and bicyclic hydroperoxides are obtained in racemic form in one or three steps in the presence of molecular oxygen or a hydrogen donor such as LH, respectively, as shown in (Fig. 14) [46].
- d) As we have already mentioned in the discussion of free radical oxidation of AA, 5-,9-,11- and 15-OO-ETE possessing *E,Z*-unsaturated side chain are obtained in racemic form. The rac-11-OO-ETE can be transformed by cyclization, addition of molecular oxygen and acceptance of a hydrogen atom (bicyclization) into the 15-series of G<sub>2</sub>-IsoPs (Type IV), as depicted in (Fig. 15). Although the terminal peroxy radicals, rac-5- and 15-ETE-OO· cannot undergo direct bicyclization, their isomerization under thermodynamic control leads to rac-9 and -11 peroxy radicals whose cyclization provides the 8- and 15-series of G<sub>2</sub>-IsoPs (Type I and IV), respectively. The situation with 8- and 12-OO-ETEs is different from that of the mentioned regioisomers. Thus, from these compounds both Type II and Type III bicyclic endoperoxides can be formed (Fig. 15).

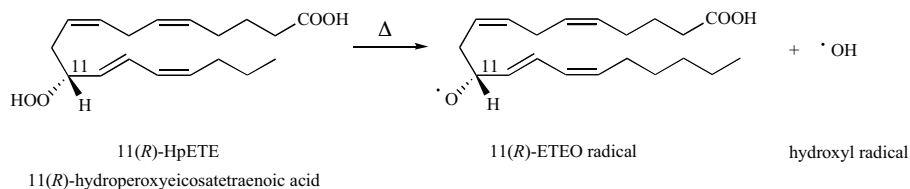


Fig. (12). Transformation of 11(R)-HpETE.

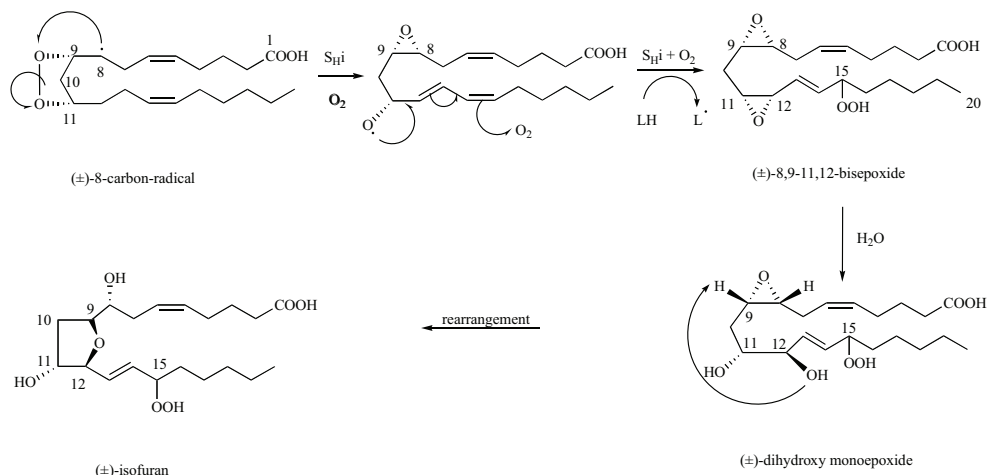
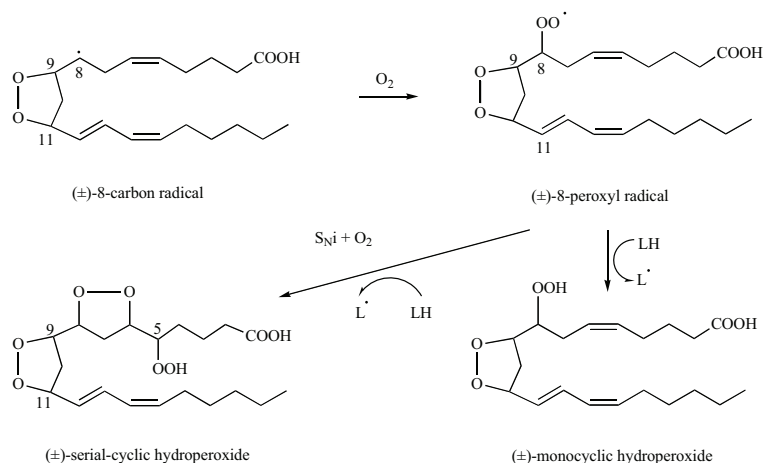
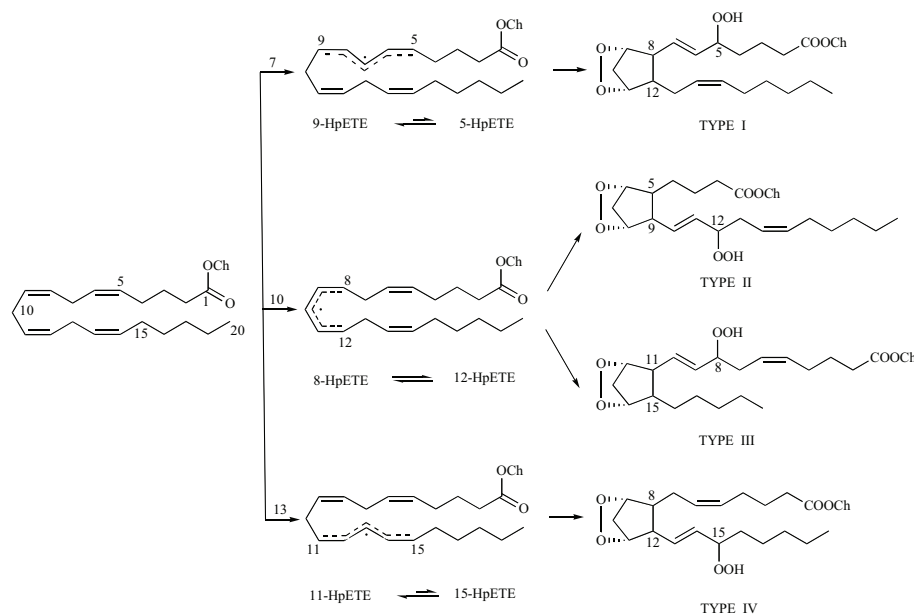


Fig. (13). Formation of (±)-isofurans.





**Fig. (14).** Transformation of 9(*R*\*), 11(*R*\*)-8-carbon radical into (±)-mono- and -serial-cyclic hydroperoxides.



**Fig. (15).** Formation of  $G_2$ -Isoprostans of type I-IV from AA by free radical oxidation.

### Epoxygenation of Arachidonic Acid

Oxidation of double bonds of PUFAs (e.g. AA) catalyzed by CYP *epoxygenase* enzyme gives relatively unstable epoxyeicosatrienoic acids (EETs) under regio- and enantioselective control, as shown in (Fig. 16) [47].

Intermolecular radical substitution of peroxide: addition of a peroxy free radical takes place smoothly at the carbon-carbon double bond of AA, and the corresponding racemic carbon radical is formed which can be stabilized by  $S_{Hi}$  to give an epoxide in racemic form, as shown in (Fig. 17) [48].

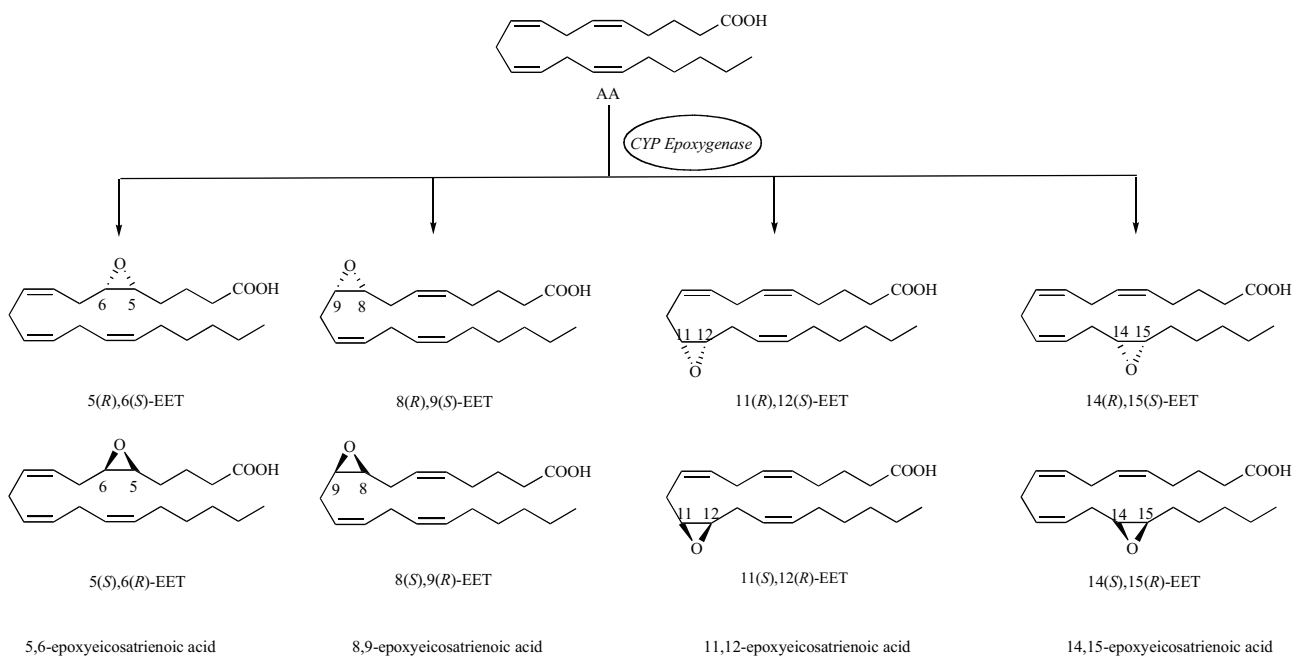
### Hydroxylation

a) Epoxides (EETs) can be transformed by soluble *epoxide hydrolase* (sEH) [49] into the corresponding vic-dihydroxy derivatives (dihydroxyeicosatrienoic acids [DHETs]) that are more stable (Fig. 18). Ring-opening of (5*R*,6*R*)-EET or its racemic form ([±]-5,6-EET) by water results in the formation of racemic 5,6-DHET.

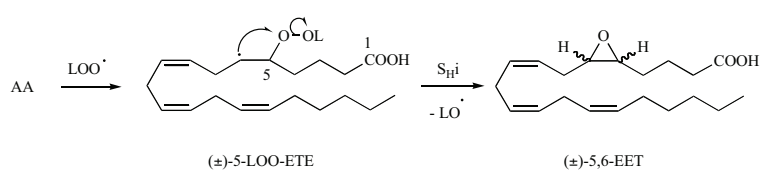
b) Monosubstitution of a hydrogen atom by a hydroxyl group at the  $\omega$ -1-4 carbon atom of PUFAs is mediated by  $\omega$ / $\omega$ -1 *hydroxylase* to give  $\omega$ -C1-4-hydroxyeicosatetraenoic acids (HETEs), as shown in (Fig. 19) [50].

### Biosynthesis of Prostanoids

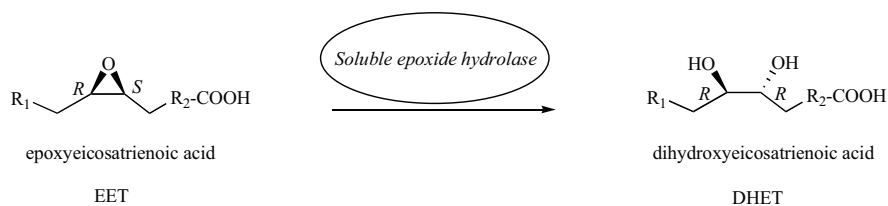
Biosynthesis of prostanoids (prostaglandins, prostacyclins and thromboxanes) is presumably the most extensively investigated metabolic pathways of AA. These cyclic compounds are formed in a stereocontrolled manner from AA *via* the bicyclic prostaglandin endoperoxides (PGG<sub>2</sub> and PGH<sub>2</sub>) by *prostaglandin endoperoxide H synthases* (PGHS-1 and PGHS-2). These enzymes are heme-containing *dioxygenases* (*cyclooxygenase and peroxidase*) with two different, but functionally active catalytic sites [51]. The COX incorporates two oxygen molecules into the AA to give PGG<sub>2</sub> endoperoxide [52]. The *peroxidase* (POX) reduces the 15-hydroperoxyl group of PGG<sub>2</sub> to a hydroxyl one resulting in PGH<sub>2</sub> endoperoxide, which is transformed by different



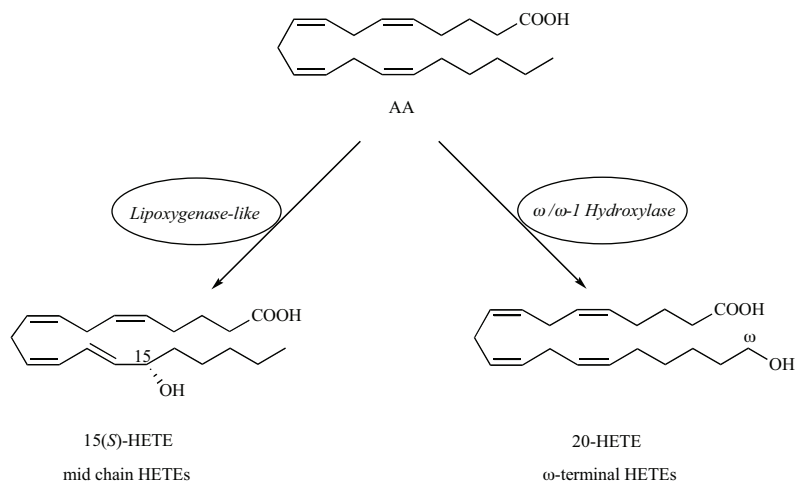
**Fig. (16).** Epoxydation of AA catalyzed by CYP *epoxygenase*.



**Fig. (17).** Epoxydation of AA by free radical oxidation.



**Fig. (18).** Stereoselective ring-opening of EETs by SHE.



**Fig. (19).** Hydroxylation of AA at ω or ω-(1-4) carbon atom.

cell-specific enzymes to the corresponding prostanoids, as shown in (Fig. 20).

The half-life of PGI<sub>2</sub>, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) and TXA<sub>2</sub> in the tissues is very low. Among these prostanoids, PGI<sub>2</sub> and TXA<sub>2</sub> can hydrolyze to more stable but biologically inactive derivatives such as 6-oxo-PGF<sub>2α</sub> and TXB<sub>2</sub>, respectively, while PGE<sub>2</sub> and PGD<sub>2</sub> can be transformed to PGA<sub>2</sub> and PGJ<sub>2</sub> by water elimination, respectively.

### Biosynthesis of Leukotrienes

By means of *5-lipoxygenase* enzyme activating protein (FLAP), the *5-lipoxygenase* enzyme (5-LOX) converts AA to 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which spontaneously loses water to give the highly reactive 7(*E*),9(*E*),11(*E*),14(*Z*)-5,6-epoxy-eicosatetraenoic acid (LTA<sub>4</sub>) in optically pure form, as depicted in (Fig. 21) [53].

The following step of the biosynthesis includes the transformation of LTA<sub>4</sub> into LTB<sub>4</sub> or LTC<sub>4</sub>. First LTB<sub>4</sub> is formed from LTA<sub>4</sub> by the LTA<sub>4</sub> hydrolase. Alternatively, LTA<sub>4</sub> is conjugated with glutathione catalyzed by *LCT<sub>4</sub> synthase and glutathione-S-transferase* in a regioselective manner to afford LTC<sub>4</sub> whose glutamate residue is cleaved by *γ-glutamyl transferase* (γGT1) resulting in LTD<sub>4</sub>. Finally, the glycine amino peptide of LTD<sub>4</sub> is removed by *dipeptidases* to obtain LTE<sub>4</sub> [53].

### Role of Eicosanoids In COPD

Several lines of evidence indicate that among mediators involved in COPD, AA metabolites such as LTs and PGs

may play an important role, particularly in the recruitment of inflammatory cells in the airways, the regulation of vascular and bronchial tone and the development of oxidative stress (Fig. 22) [54, 55].

Eicosanoids can be assessed usually quite easily in various respiratory samples including sputum and EBC by immunoassays. However, measurement of putative mediators in these body fluids, particularly in EBC may have considerable variability [56, 57]. The combination of gas chromatography (GC)- and/or liquid chromatography (LC) - mass spectrometry (MS) offers increased sensitivity for analysis, but these techniques are expensive and time consuming. Finally, cigarette smoking is often a confounding factor in eicosanoid measurements limiting thereby the clinical applicability of these assays.

Among lipid mediators, LTB<sub>4</sub> has been probably the most frequently investigated. LTB<sub>4</sub> acts as a potent neutrophil chemoattractant, both in stable disease [58] and during exacerbations [60, 61]. In addition to recruiting neutrophils and causing enhanced microvascular permeability [59, 62, 63], LTB<sub>4</sub> may stimulate lymphocyte migration in the lung [64] and constriction of pulmonary blood vessels *via* indirect mechanisms [65]. LTB<sub>4</sub> is produced predominantly by activated neutrophils, but also by alveolar macrophages in transcellular reactions involving other inflammatory cells and surrounding structural elements [66].

LTB<sub>4</sub> is increased in the sputum of stable patients with COPD [67, 68] and is further increased during exacerbations [60,61,69]. Plasma concentrations of LTB<sub>4</sub> have also been reported to be elevated in COPD patients [70]. Measurement of LTB<sub>4</sub> in EBC appears to be inconclusive, since in some

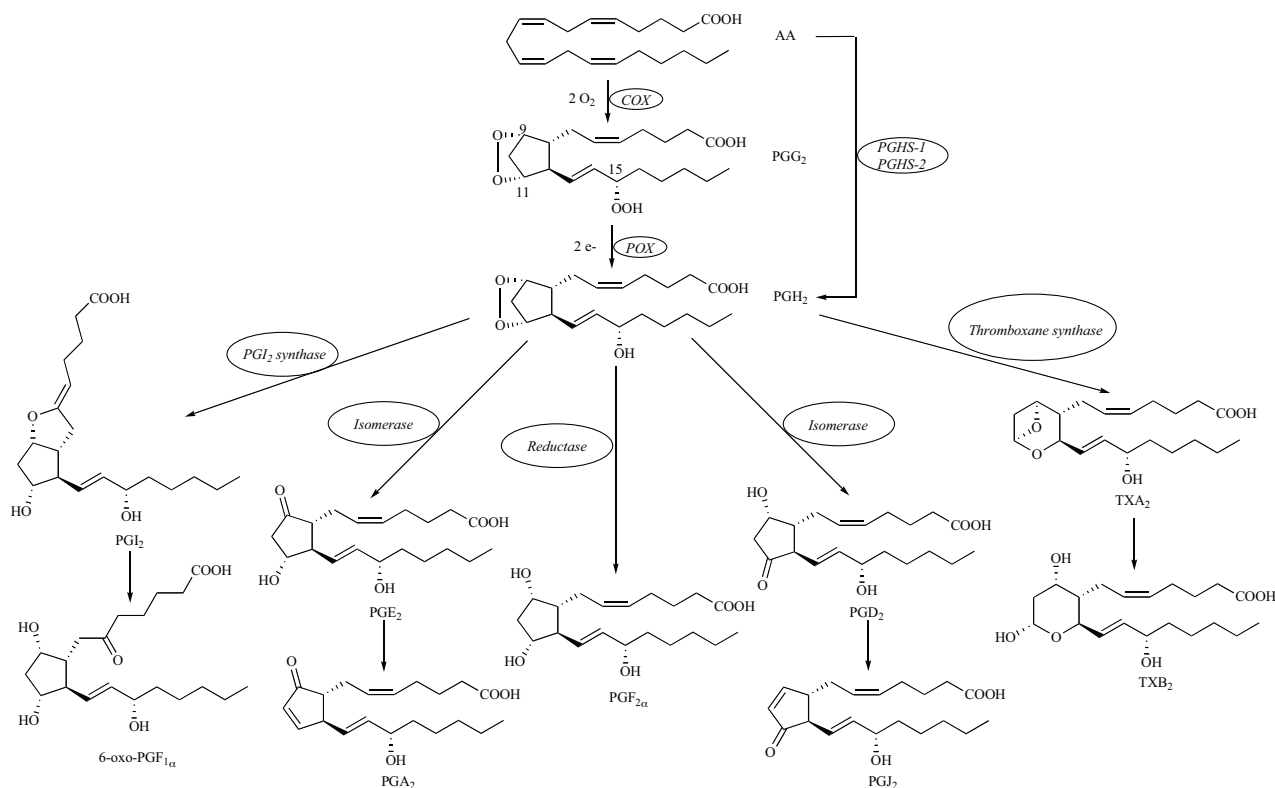
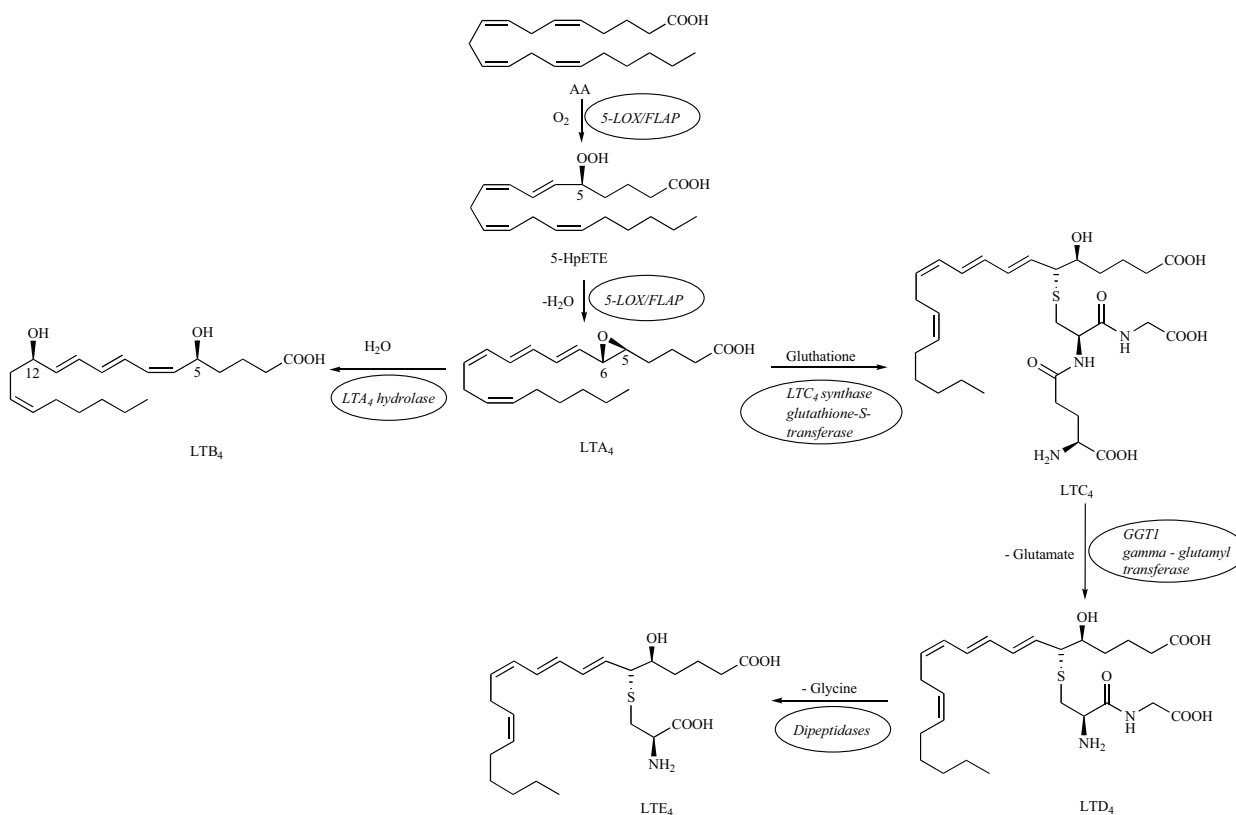
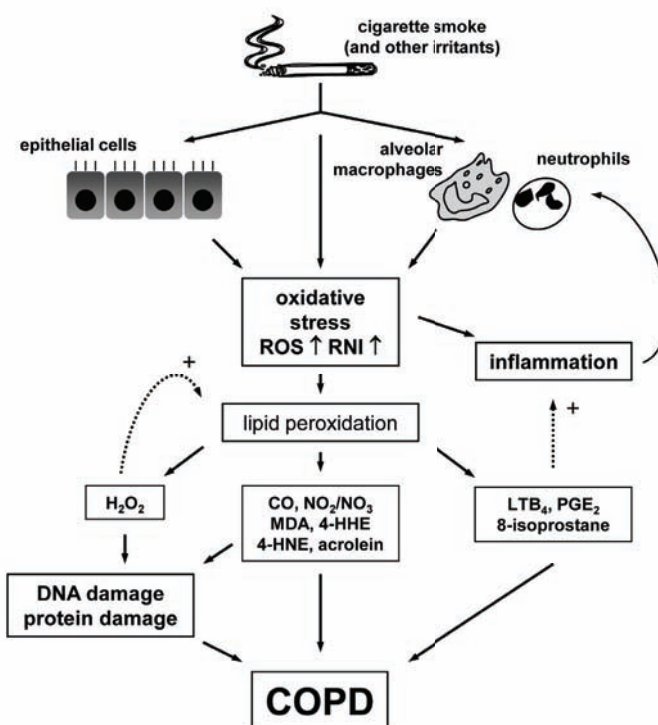


Fig. (20). Biosynthesis of prostanoids from AA.



**Fig. (21).** Biosynthesis of leukotrienes from AA.



**Fig. (22).** Role oxidative and nitrosative stress in the pathophysiology of COPD.

series an increase in LTB<sub>4</sub> concentrations were reported in COPD patients [69, 71], while Gaber and co-workers were unable to consistently detect LTB<sub>4</sub> in EBC samples [72]. Furthermore, there is a wide variation in absolute levels of

EBC LTB<sub>4</sub> between studies despite the use of the same type of condenser and/or methodology [56, 73]. The potential methodological pitfalls include oral salivary contamination, variations in the rate of aerosolization of airway surface liq-

uid during sample collection (dilution), differences in sampling methodology and lack of assay sensitivity.

In our laboratory LTB<sub>4</sub> could not be found in EBC either [74]. By contrast, LTB<sub>4</sub> was detectable in the sputum [75]. Utilizing this specimen, we demonstrated that COPD patients with exacerbations have higher LTB<sub>4</sub> concentrations than stable subjects which confirms the results of some previous studies. The significant correlation between sputum neutrophils and LTB<sub>4</sub> levels observed in our study is also consistent with the general view that LTB<sub>4</sub> is involved in neutrophil recruitment in COPD.

In COPD the role of other eicosanoids such as cysteinyl-LTs (cys-LTs) and PGE<sub>2</sub> is less clear. Cys-LTs are major eicosanoid products of activated eosinophils, mast cells, basophils and monocytes. In asthma it has been extensively documented that cys-LTs including LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> induce bronchoconstriction, increase mucus production and oedema and promote airway remodeling [76], while PGE<sub>2</sub> is an airway smooth muscle relaxant and it is likely to have bronchoprotective and anti-inflammatory actions [77]. Cys-LTs may also be important mediators of exercise-induced bronchoconstriction [78] and eosinophil bronchitis [79].

Few studies have investigated cys-LTs in patients with COPD. There is some evidence that cys-LTs levels are increased in EBC of COPD patients, both in stable state and in exacerbation [80]. Moreover, a prominent cys-LT1 receptor expression in bronchial mucosa has also been recently demonstrated during the course of an exacerbation [81]. These data raise the possibility that antileukotriene treatment might be beneficial in patients with COPD when additional anti-inflammatory activity is needed. Nevertheless, studies utilizing montelukast<sup>®</sup>, a well-known selective antagonist of cys-LT1 receptor, indicated only modest clinical benefit in COPD patients [82]. Our data do not support a major role for cys-LTs in COPD exacerbations either [75].

With regard to PGE<sub>2</sub>, Montuschi *et al.* reported an increased PGE<sub>2</sub> concentration in EBC of stable COPD patients and argued that, like in asthma, this might represent a protective mechanism for counteracting airway inflammation [71]. The same authors have also demonstrated that ibuprofen, a non-selective COX inhibitor, significantly reduced PGE<sub>2</sub> in EBC compared with placebo in COPD patients, whereas rofecoxib<sup>®</sup>, a selective COX-2 inhibitor, did not have such an effect, suggesting that this biomarker can sensitively differentiate between selective and non-selective COX inhibition in COPD patients [83].

Our findings suggest that PGE<sub>2</sub> levels in the sputum are sharply increased at the onset of an exacerbation, while treatment of the condition resulted in a marked decrease of PGE<sub>2</sub> concentrations [75]. On one hand, it is possible that this is merely a secondary protective regulatory response, as it has been suggested in asthma. However, it is also conceivable that PGE<sub>2</sub> is involved in cigarette smoke-induced neutrophilic recruitment into the airways, since there is evidence that PGE<sub>2</sub> can enhance the adhesion of neutrophils to bronchial epithelial cells *via* a COX-2-dependent mechanism in COPD patients [84]. Indeed, PGE<sub>2</sub> is increased in healthy smokers and ex-smoker COPD patients [71,84]. Finally, it is also possible that PGE<sub>2</sub> influences airway inflammation and

parenchymal destruction through the regulation of matrix metalloproteinases (MMP), since some studies suggest a relationship between the production of PGE<sub>2</sub> and MMP-2 in the airways of COPD patients [85]. Thus, PGE<sub>2</sub> may play diverse and even opposing effects in respiratory diseases.

### Isoprostanes

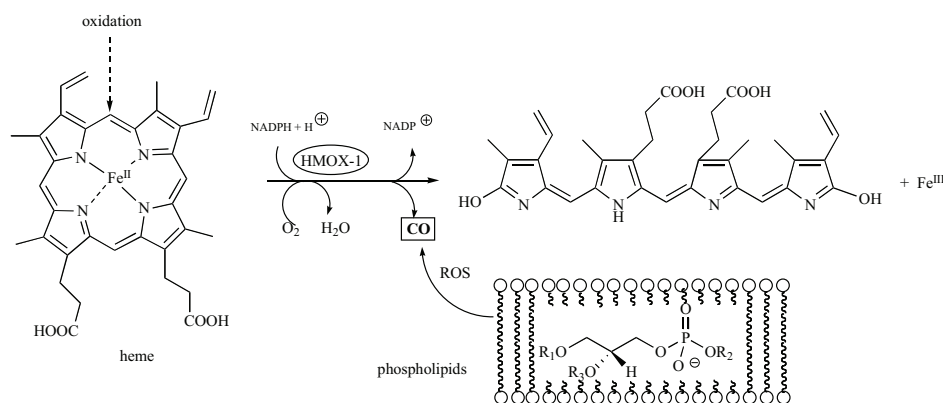
One diastereomer of (±)-PGF<sub>2α</sub> is (±)-8-epi-prostaglandin F<sub>2α</sub> which is called in the literature as 8-isoprostane (Fig. 11). According to the observation of Morrow and co-workers [26, 35], it is produced by free-radical catalyzed peroxidation of AA in similar manner as PGF<sub>2α</sub>. Since it has a side chain at C-8 and -12 of *cis* relative configuration, it is thermodynamically more stable than PGF<sub>2α</sub> possessing its side chain at C-8 and C-12 of *trans* relative configuration. Accordingly, it has been suggested to be one of the most reliable biomarker of oxidative stress *in vivo* [86-88].

Several studies showed higher 8-isoprostane levels in the EBC and/or the sputum of stable COPD patients compared to healthy controls, although mean values have varied across studies substantially despite the use of identical methods [89-91]. This may be attributed to differences in condenser coating or assay-sensitivity issues discussed already in case of LTB<sub>4</sub>. Also the length of sample storage and the way of sample collection could serve as confounding factors. In COPD exacerbations, 8-isoprostane levels could be further increased, as documented by our [75] and other laboratories [69, 91]. These findings are in agreement with those of other trials showing increased oxidative stress in COPD patients with exacerbations using other markers such as exhaled H<sub>2</sub>O<sub>2</sub> [92] or alpha-1 antitrypsin [93]. Interestingly, our observations also indicated that a successful hospital treatment resulting in clinical and functional recovery of the patient does not abolish the increased oxidative stress observed in COPD exacerbations by the time of the patient's discharge from the hospital [75]. Nonetheless, delayed resolution of inflammatory response during recovery from exacerbation has been observed by other investigators as well [94].

### Exhaled Carbon Monoxide

One of the simple gases present in the exhaled breath that has been suggested to reflect ongoing oxidative stress and/or airway inflammation is CO [95]. Exhaled CO (eCO) originates from the inspiration of ambient CO and from endogenous metabolic sources that include heme metabolism catalyzed HO enzymes upon oxidative stress (Fig. 23). Beside heme metabolism peroxidative degradation of the membrane lipids may also act as an important source of eCO [96].

Measurement of eCO is also a brief, non-invasive procedure that provides immediate results, and is most commonly measured with electrochemical selective sensors. Several recent studies have reported elevated levels of eCO in COPD patients [97]. However, the signal is usually small. Moreover, eCO levels are also elevated in healthy smokers due to the high CO content of cigarette smoke [98, 99]. Nevertheless, eCO concentrations also increased in ex-smokers with COPD that may be the result of enhanced oxidative stress in the lungs [97].



**Fig. (23).** Proposed routes of the formation of CO in human tissues by oxidative stress.

Levels of eCO further increase during COPD exacerbations; however, again, smoking outweighs this effect [100]. Thus, eCO may be involved in smoking-associated inflammatory processes in the airways, but its monitoring in COPD patients is of limited clinical value. The test may rather serve as a convenient method to monitor smoking cessation [101].

### Hydrocarbons of Low Molecular Weight

Beside CO exhaled hydrocarbons including ethane and pentane are also reliable biomarkers to estimate the extent of oxidative stress-related tissue injury *in vivo* [102]. Ethane is a specific product of the interaction between free radicals and the  $\omega$ -3 PUFAs such as ALA, whereas pentane derives from the peroxidation of  $\omega$ -6 PUFAs such as AA.

Several pathways have been postulated for the formation of ethane starting from ALA, the simplest route is depicted in (Fig. 24). In the first step, a hydrogen abstraction at C-14 by ROS such as HO $\cdot$  results in the corresponding bisallylic radical, whose reaction with molecular oxygen at its carbon atom (C-16) is followed by hydrogen abstraction to give 16-hydroperoxyl-9(Z),12(Z),14(E)-octadecatrienoic acid *via* the corresponding 16-peroxyl-ALA derivative. In the next step, a spontaneous thermal decomposition of the 16-hydroperoxyl group takes place, and the corresponding 16-alkoxy and hydroxyl radicals are formed. Subsequently the  $\beta$ -cleavage leads to the corresponding  $\alpha,\beta$ -unsaturated C<sub>16</sub> aldehyde derivative (Hock reaction) and ethyl radical whose recombination with hydrogen radical obtained from hydrogen donors such as lipids affords ethane.

Habib and co-workers have demonstrated that levels of ethane in the exhaled breath of smokers are significantly higher than those of ex-smokers and non-smokers [103]. Paredi and co-workers have also suggested that concentrations of exhaled ethane are elevated in patients with COPD and correlate with disease severity (FEV<sub>1</sub>) [104]. Importantly, steroid treatment reduced exhaled ethane levels indicating that steroids reduce lipid peroxidation, at least to some extent. Nevertheless, measurement of ethane or pentane by GC/MS is time-consuming and expensive; thus, measurements are unlikely to be useful in clinical trials or clinical practice.

### Malondialdehyde, n-alkanals, $\alpha,\beta$ -unsaturated Aldehydes and Their Toxic Transformation Products

Oxidation of cell membrane phospholipids results in the formation of various lipid hydroperoxides and aldehydic products. Among these molecules malondialdehyde (MDA), C<sub>6</sub>-, C<sub>7</sub>- and C<sub>9</sub>-alkanals, 4-oxo- and 4-hydroxy-2(E)-nonenals have been studied as markers of oxidative stress in COPD. According to published data MDA appears to be the most promising marker, but even this molecule should be further validated in clinical settings.

Several routes have been proposed for the formation of MDA. Based on the pathway suggested by Pryor and Stanley in 1975 [105], thermal degradation of prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) results in the formation of MDA and 12-hydroxyheptadeca-5(Z),8(Z),10(E)-trienoic acid (12-hydroxy-HDTA) (Fig. 25). The homolytic cleavage of the oxygen-oxygen bond of PGH<sub>2</sub> results in the corresponding 9,11-bisalkoxy radical whose stabilization takes place subsequently by  $\beta$ -cleavage to give MDA and 12-hydroxy-HDTA.

Recently, Esterbauer and co-workers have suggested two other routes for the formation of MDA from PUFAs with more than two allylic double bonds [106] (Fig. 26). Both pathways involve two successive  $\beta$ -cleavages which gives the corresponding  $\alpha,\beta$ -unsaturated C<sub>16</sub> and C<sub>15</sub> aldehyde derivatives from 5- and 15-peroxyl-ETEs, respectively. Peroxidation of these aldehydes results in the corresponding 4-hydroperoxylaldehydes whose decomposition is followed by another  $\beta$ -cleavage (second degradation) resulting in acrolein radical. Finally, the reaction of acrolein radical with a hydroxyl radical gives MDA *via* its enol-form.

In certain tissues, MDA can also be formed by an enzymatic process. For example, MDA, thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and the corresponding C<sub>17</sub> compound can be formed in 1:1:1 ratio by the *thromboxane synthase* from AA *via* PGH<sub>2</sub> in human platelets, as proposed by two different research groups [107, 108] (Fig. 27).

Methods used for measuring MDA in aqueous matrices [109] can be subdivided into derivatization-based and label-free methodologies. The most frequently applied derivatization-based method is the thiobarbituric acid (TBA) assay in which condensation of two molecules of TBA with one molecule of MDA gives a colored reaction product possessing a very high absorption maximum at 532 nm

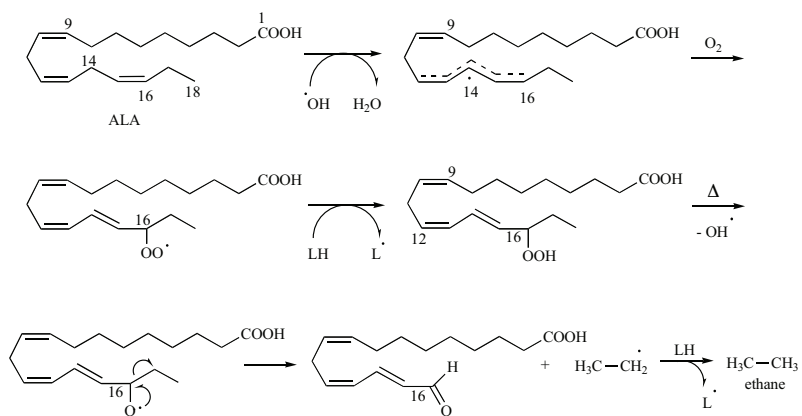


Fig. (24). Biosynthetic pathway for the formation of ethane from ALA.

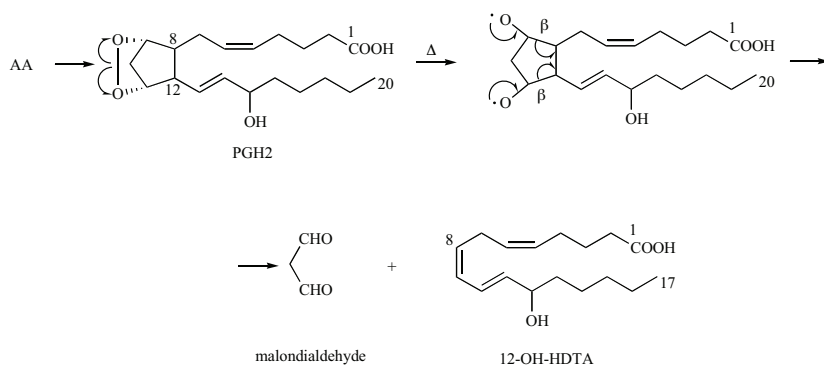


Fig. (25). Possible mechanism of formation of MDA and 12-hydroxy-HDTA.

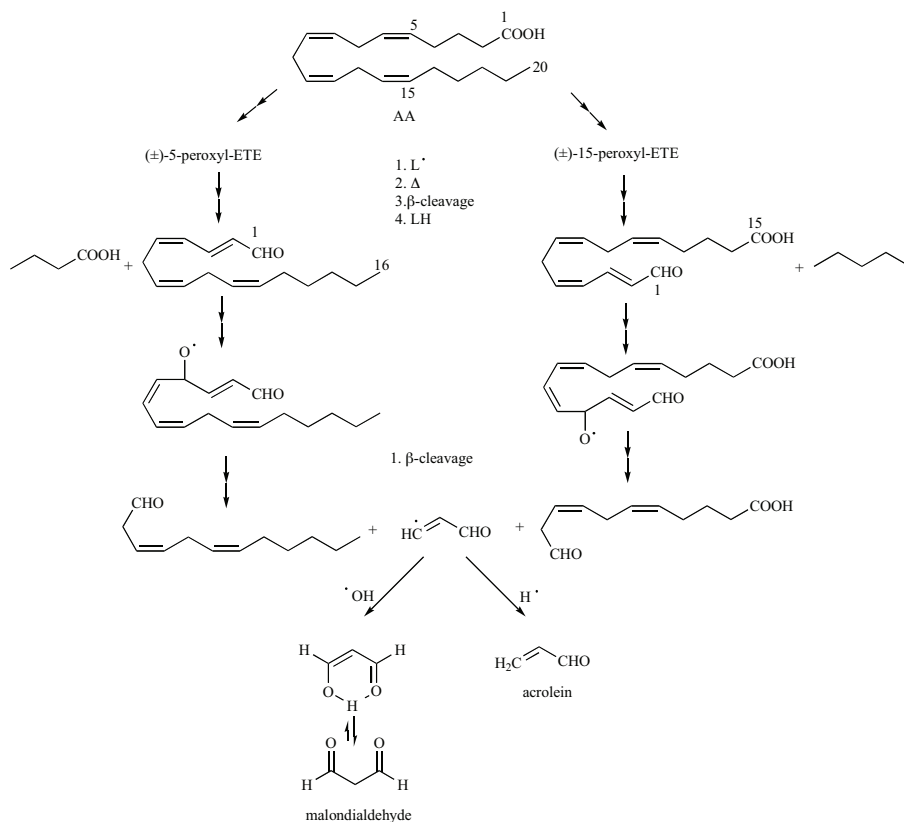
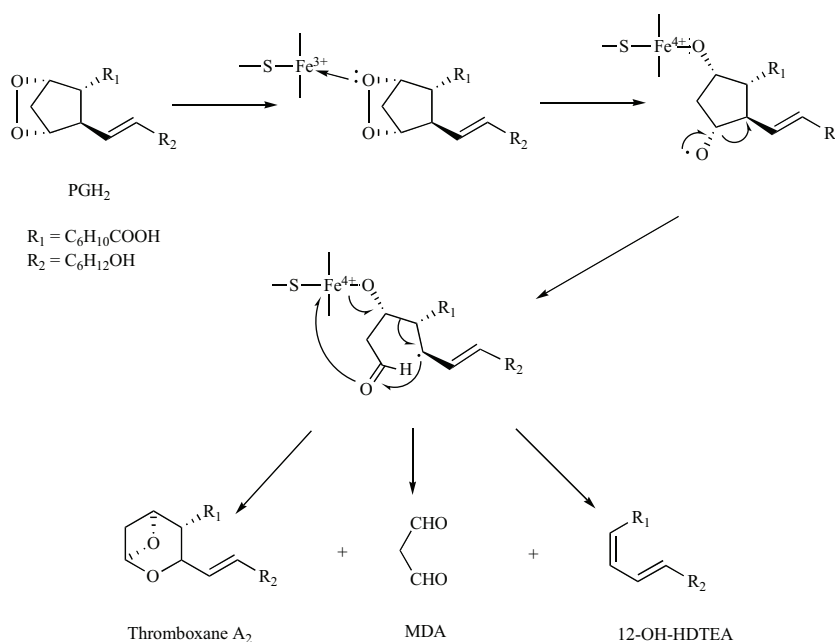


Fig. (26). Possible mechanisms of the formation of MDA and acrolein from AA via rac-5- or -15-peroxyl-ETE.



**Fig. (27).** Formation of MDA suggested by Hecker and Ullrich.

( $\epsilon = 153.000$ ). This can be easily measured spectrophotometrically or by fluorescence detection.

Nevertheless, this method lacks specificity. High performance liquid chromatography (HPLC) or liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (LC/APCI-MS/MS) are considered to be more advanced and sophisticated techniques [110, 111].

Using these methods it was recently found that MDA levels in EBC [112, 113] and/or induced sputum [114] are elevated in stable COPD patients compared with those of healthy controls. Additionally, we demonstrated that MDA concentrations in sputum, but not in EBC are further increased in acute exacerbation of COPD requiring hospitalization [115]. Treatment of exacerbation led to a decrease in sputum MDA levels, primarily in those patients who had more pronounced improvement in airflow limitation post-treatment. Measurement of MDA has good reproducibility in sputum, and thus, we concluded that MDA could be a useful marker for monitoring exacerbation-associated oxidative stress in patients with COPD.

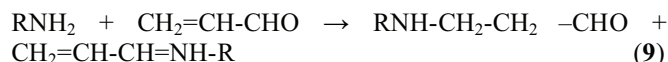
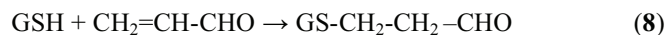
It noteworthy that the concentration of MDA in EBC appears to be always lower compared to that formed from PGH<sub>2</sub> or AA *via* optically pure and *rac*-, 5- and 15-peroxy-ETE. Thus, it has been suggested that its reaction with the free amino group of lysine residues of proteins [116-119] or the deoxy-guanosine of DNA and RNA [120, 121] reduces its concentration, which contributes to the development of COPD (Fig. 22 and 28).

Besides MDA, hexanal, heptanal and nonanal appear to be also detectable in EBC using HPLC-MS methodology [111, 112]. Levels of hexanal and heptanal are increased in stable COPD patients compared to healthy non-smoking controls. However, these aldehyde concentrations are also elevated in healthy smokers indicating that smoking is an important confounding factor in these measurements.

The main process leading to aldehydes is likely to be the so-called Hock-reaction of lipid alkoxy radicals derived from lipid hydroperoxides. For example, hexanal arises by  $\beta$ -cleavage of 15-O-ETE obtained from AA *via* 15-HpETE (Fig. 29).

Besides these aldehydes, acrolein, 4-hydroxyhexenal (4-HHE) and 4-hydroxynonanal (4-HNE) have also been detected in EBC of humans [114]. The possible biosynthetic pathway of these compounds starting from AA and decosa-hexaenoic acid (DHA) was recently reviewed by Esterbauer and co-workers, as shown in (Fig. 30) [106].

Among all  $\alpha,\beta$ -unsaturated aldehydes including 4-hydroxy-2-alkenals (*rac*-4-HNE and -HHE) acrolein is far the strongest electrophile with high reactivity towards nucleophiles such as aliphatic thiols or amines. Esterbauer and co-workers and other research groups have observed that in case of acrolein the Michael-addition of glutathione (GSH) takes place regioselectively (1,4-addition) about 110 to 150 times faster than with primary amines (RNH<sub>2</sub>) [122, 123] (Eq. 8 and 9).



Moreover, this transformation leads to a mixture of 1,2- and 1,4-adducts and the formation of Schiff's base (1,2-adduct, CH<sub>2</sub>=CH-CH=N-R) is preferred. Therefore, in proteins, acrolein preferentially attacks free SH groups of cysteine residues,  $\epsilon$ -amino groups of lysine residues and one of the nitrogens of histidine residues, but it does not react with the amino group of other amino acid residues, as shown in (Fig. 31) [124].

<sup>6</sup> The formation of *N*<sup>ε</sup>-(3-formyl-3,4-dihydropiperidino) lysine (FDP-lysine) is the result of addition of two molecules of acrolein to the  $\epsilon$ -amino group of lysine residue followed by condensation (-H<sub>2</sub>O).



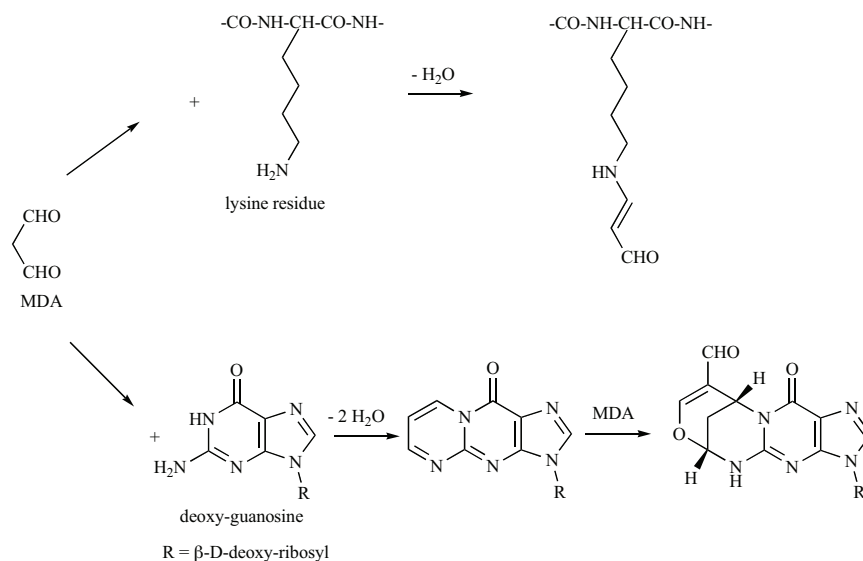


Fig. (28). Reaction of MDA with lysine residue of proteins and deoxyguanosine.

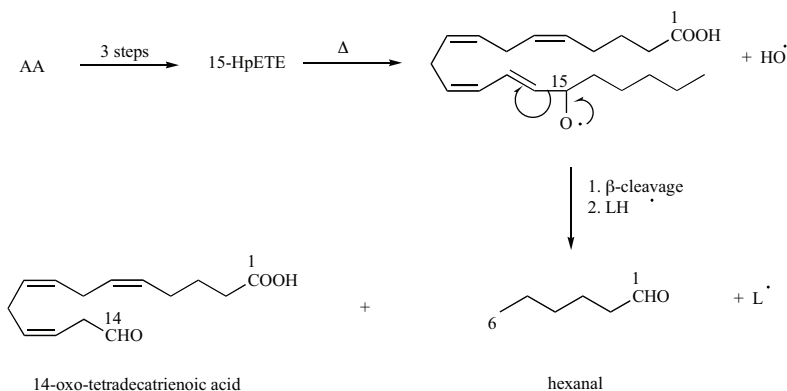


Fig. (29). Formation of hexanal from AA via 15-HpETE.

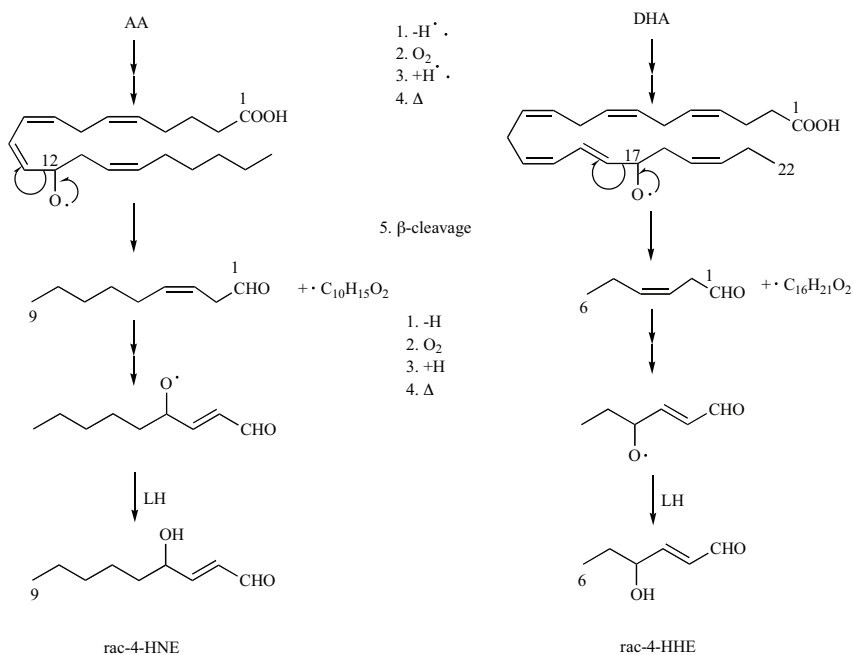
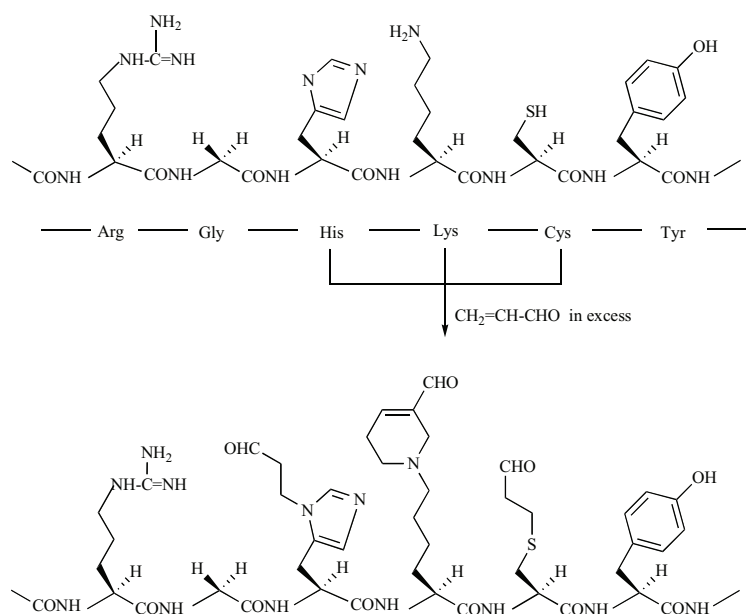


Fig. (30). Possible formation of racemic 4-hydroxy-nonal and racemic 4-hydroxyhexenal.



**Fig. (31).** Modified structure of a protein by acrolein.

Acrolein and its related compounds are also able to modify the structure of nucleic acid bases at neutral pH. For example, deoxy-guanosine forms two regioisomeric adducts (A and B) with acrolein in a ratio of 1:1 [125], as shown in (Fig. 32). Similarly,  $\alpha,\beta$ -unsaturated aldehydes are also highly reactive bifunctional electrophiles and can modify the structures of proteins and DNA, which may significantly contribute to cellular injury in the course of COPD (Fig. 22).

### Hydrogen Peroxide

Any system that produces superoxide anion will produce  $H_2O_2$  as a result of the dismutase reaction [126] (Eq. 10).

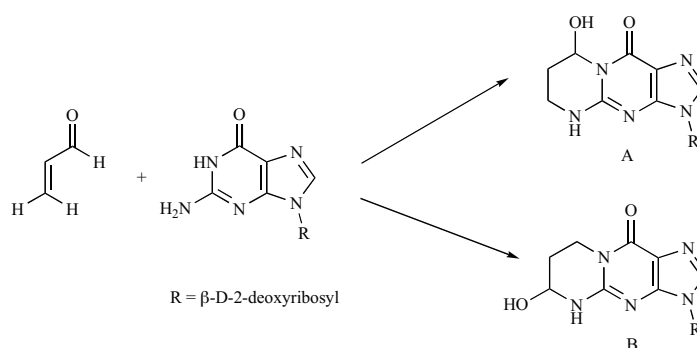


Moreover, many enzymes such as *glucose oxidase*, *MPO catalase* and  *$\alpha$ -amino acid dehydrogenase ( $\alpha$ -AADH)* produce  $H_2O_2$  directly by transferring two electrons to molecular oxygen, as shown in (Fig. 33) [127]. Therefore, increased concentrations of  $H_2O_2$  in human tissues indicate acceleration of lipid peroxidation (Fig. 22).

There is some moderate differentiation between EBC  $H_2O_2$  levels observed in disease and those observed in healthy subjects, as well as between healthy smokers and

COPD patients [92, 128, 129]. Moreover, increased  $H_2O_2$  levels have been detected during COPD exacerbations indicating enhanced oxidative stress [92, 128]. There is also some evidence that  $H_2O_2$  can be used as a biomarker in clinical trials. In a double-blind, randomized, placebo-controlled study the effect of N-acetyl-cysteine (NAC), an antioxidant mucolytic, could be successfully monitored during long-term treatment of COPD patients simply by measuring  $H_2O_2$  in EBC [130].

However, it has been also reported that  $H_2O_2$  concentration in EBC depends on the expiratory flow rate [131]. Furthermore, even under strictly controlled conditions, there is a high degree of variability of  $H_2O_2$  values when measurements are repeated on two consecutive days. Recently, we have observed that the concentration of  $H_2O_2$  in EBC depends on the breathing pattern during sample collection, as well [132]. In this study  $H_2O_2$  was measured on-line using an EcoCheck biosensor system, which circumvents the common problem that  $H_2O_2$  can be degraded during storage of samples at  $-80^\circ C$ . Nevertheless, due to the effect of the breathing type on exhaled  $H_2O_2$  concentrations, the usefulness of the test is limited as a biomarker of oxidative stress at present and highlights the need of standardization of the collection techniques.



**Fig. (32).** Reaction of acrolein with deoxyguanosine.

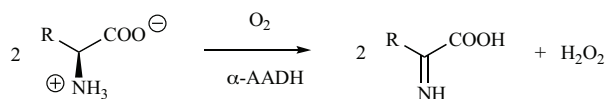


Fig. (33). Formation of hydrogen peroxide.

### Nitrogen Oxides and Nitrotyrosine

In contrast to nitrous oxide (N<sub>2</sub>O), nitric oxide (NO·) contains odd number of electrons, and is therefore a highly reactive free radical that is stabilized ultimately as nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) or in biological complexes with thiols to form nitrosothiols (RS-NO). In the presence of oxidative stress the reaction between NO· and O<sub>2</sub><sup>-</sup> yields ONOO<sup>-</sup>, which in turn exerts various harmful effects in the lungs [133]. ONOO<sup>-</sup> is a powerful oxidant, which is able to enhance the formation of the highly reactive HO· radical via different reactions (Eq. 11 and 12).



Additionally, ONOO<sup>-</sup> reacts with a wide variety of compounds including DNA, cellular lipids and sulfhydryl groups of proteins that promotes nitrosative stress [134]. There is some evidence that levels of NO<sub>2</sub> in EBC are elevated in stable COPD patients compared to healthy non-smoker or smokers [135]. Nevertheless, the change is non-specific, since asthmatic subjects have also elevated NO<sub>2</sub> levels.

The amino acid tyrosine appears to be particularly susceptible to nitration, which leads to the formation of 3-nitrotyrosine (3-NT) (Fig. 34). Nitration of tyrosine residues inactivates numerous enzymes and prevents kinase substrate phosphorylation suggesting that tyrosine nitration does not only give rise to inactive “footprints” of nitrosative stress, but may also have a functional relationship with the pathogenesis of COPD (Fig. 22).

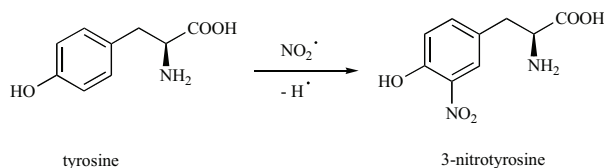


Fig. (34). Formation of 3-nitrotyrosine.

Few studies have investigated levels of 3-NT in the airways of COPD patients so far. In one report higher number of 3-NT positive cells was found in the submucosa of severe COPD patients compared to patients with mild/moderate COPD, smokers with normal lung function or non-smokers [136]. 3-NT concentration appears to be increased in the sputum supernatant of patients with COPD, as well [137]. Nonetheless, the clinical utility of 3-NT measurement for monitoring oxidative stress in stable patients and in those with exacerbations remains to be established.

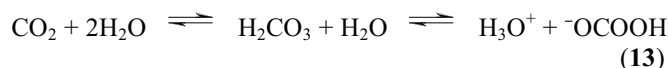
### EBC pH As a Pulmonary Biomarker

Since EBC is believed to reflect mediator content of the airway surface liquid, the pH of EBC, as a pulmonary biomarker, may provide important information on the acid-base balance of this fluid. Acidification of the airways may have

important pathophysiological effects, such as bronchoconstriction [138] and decreased ciliary beat frequency [139].

Nevertheless, EBC is an extremely diluted fluid, with a water content of 99%. It has a very weak buffer capacity, and contains a large number of volatile (carbon dioxide [CO<sub>2</sub>], ammonia [NH<sub>3</sub>]) and non-volatile molecules (organic and nonorganic ions, acids and bases, etc.) that could influence its acidity. While these molecules are difficult to measure in EBC due to their very low concentration, assessment of pH in EBC is a simple and inexpensive technique which does not require sophisticated laboratory infrastructure.

One of the major volatile components of EBC is CO<sub>2</sub>. It is transformed by water into carbonic acid which reacts reversibly with water to form hydroxonium cation (H<sub>3</sub>O<sup>+</sup>) and bicarbonate anion (HCO<sub>3</sub><sup>-</sup>) (Eq. 13).



Nonetheless, the effect of the other major volatile component of EBC such as ammonia must also be considered based on the above reaction (Eq. 14).



These two processes are not independent from each other, and the pH of EBC is determined mainly by buffering capacity of ammonium hydrocarbonate (NH<sub>4</sub><sup>+</sup>HCOO<sup>-</sup>).

In recent years several investigators have postulated that acidification of EBC may be a surrogate marker of airway inflammation and oxidative stress. In line with this view, a decrease in EBC pH has been detected in several inflammatory airway diseases such as asthma [140, 141], COPD [142] and cystic fibrosis (CF) [143]. Some reports even suggested that EBC pH may be suitable for monitoring airway inflammation during treatment of an acute exacerbation [144].

However, some authors failed to observe significant changes in EBC pH in these disorders [145-147]. Several factors may contribute to these inconsistent findings. First, it should be emphasized that measurement of EBC pH is not yet standardized, and methodological differences between various measurement techniques influence pH readings. For example, it is well recognized that EBC pH is profoundly affected by its CO<sub>2</sub> content [148]. EBC samples are commonly de-aerated with a CO<sub>2</sub>-free gas (e.g. argon) to eliminate the effect of CO<sub>2</sub>, but even after this procedure, samples contain an unpredictable amount of CO<sub>2</sub> which influences pH readings. We recently developed a method for EBC pH determination that circumvents the effects of CO<sub>2</sub> found in EBC samples [149]. The essence of the so-called CO<sub>2</sub> loading protocol is the short, repeated perfusion of samples with CO<sub>2</sub>-gas coupled with parallel measurement of pH and partial pressure of carbon dioxide (PaCO<sub>2</sub>) at each interval. We calculate the pH at the PaCO<sub>2</sub> of 5.33 kPa based on the near-perfect logarithmic correlation between pH and PaCO<sub>2</sub> in EBC. The CO<sub>2</sub> gas standardization method has better reproducibility compared to all other techniques, and thus, can be used to detect even very small changes in EBC pH.

Using this method we recently demonstrated that, in contrast to some earlier studies, EBC pH does not differentiate between healthy subjects and patients with COPD [150], CF [151] or lung cancer [152], while it does detect acidification

in asthmatics [150]. Similarly, development of bronchiolitis obliterans syndrome in lung transplant recipients was not associated with acidification of EBC either [153]. We also demonstrated that EBC pH is influenced by a number of other factors including the type of the condenser [74], the condensing temperature [74], smoking [150] and drinking [154].

As mentioned before, the other major volatile component that could affect EBC pH is ammonia. However, the relationship between ammonia and EBC pH appears to be contradictory. Accordingly, for example, Effros and colleagues proposed that ammonia derived from the lower airways or the oral cavity may be an important confounding factor in all EBC pH assays [155], while experimental data from other laboratories argue against this theory [156].

Overall, different study outcomes regarding the pH of EBC appears to be primarily attributed to different pH measurement techniques used and the various technical factors during sample collection and analysis. The clinical utility of EBC pH measurements with more standardized techniques should be investigated further to clarify its role in management of patients with COPD.

## CONCLUSIONS

While the biosynthesis of eicosanoids is an enzyme-mediated stereoselective process resulting in optically pure compounds with diverse, biological important activities, the uncontrolled, free radical-initiated lipid peroxidation pro-

duces a mixture of several racemic compounds that may play a crucial role in the development of COPD. Many of them exert cytotoxic effects in addition to having relevant biological activities as cellular regulators and signaling messengers.

Among these molecules 8-isoprostane can be quite easily measured in sputum and/or EBC of patients with COPD and could be a useful biomarkers of oxidative stress. Beside 8-isoprostane, a large number of other oxidative stress markers including CO, ethane, pentane, MDA, 4-HHE, 4-HNE, acrolein, H<sub>2</sub>O<sub>2</sub>, nitrogen oxides and 3-NT have been detected in the airways of COPD in recent years. Other AA metabolites include LTB<sub>4</sub> and PGE<sub>2</sub> that may play an important role in the recruitment of inflammatory cells, the regulation of vascular and bronchial tone and the development of oxidative stress in the airways. Acidification of EBC may also be a surrogate marker of airway inflammation and oxidative stress, but results can be influenced by several technical factors during sample collection and analysis. The most commonly detected oxidative markers in COPD are summarized in (Table 1).

Theoretically, biomarkers could be useful in monitoring the severity and/or the progression of the disease, defining different phenotypes of the disease and evaluating the treatment responsiveness of the patients. Although some of these approaches are encouraging, it should be emphasized that there are a number of technical and methodological issues concerning sample collection, validation, assay repeatability and reproducibility, which may limit the clinical application of these biomarker measurements at present. Some meas-

**Table 1. Oxidative stress markers in COPD.**

Sample	Biomarker	Advantages	Disadvantages	Ref.
Exhaled air	CO	non-invasive simple inexpensive on-line measurement	signal is small smoking is a confounding factor	[97-101]
	ethane pentane	non-invasive	expensive requires advanced laboratory infrastructure	[102-104]
EBC	hexanal, heptanal, nonanal, MDA, 4-HHE, 4-HNE, acrolein, NO <sub>2</sub> , NO <sub>3</sub> , H <sub>2</sub> O <sub>2</sub> , nitrotyrosine, LTB <sub>4</sub> , PGE <sub>2</sub> , cys-LT, 8-isoprostane, pH	non-invasive no effect on underlying disease process repeatable on-line measurement <sup>§</sup> simple and inexpensive <sup>§</sup>	high variability poor reproducibility lack of standardization requires laboratory infrastructure smoking is often a confounding factor	[56-59, 68, 69, 71-74, 78, 80, 83, 89, 91-93, 111-115, 128-132, 135, 140-156]
Sputum*	hexanal, heptanal, nonanal, MDA, 4-HHE, 4-HNE, acrolein, LTB <sub>4</sub> , PGE <sub>2</sub> , cys-LT, 8-isoprostane	semi-invasive informative inflammatory cell profile	can cause inflammation/ bronchoconstriction <sup>#</sup> high variability (sputum cells) requires laboratory infrastructure smoking is often a confounding factor	[55, 60, 61, 67, 68, 75, 79, 84, 85, 90, 114, 115, 137]

EBC: exhaled breath condensate, CO: carbon monoxide, H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide, MDA: malondialdehyde, 4-HHE: 4-hydroxyhexenal, 4-HNE: 4-hydroxynonenal, NO<sub>2</sub>: nitrite, NO<sub>3</sub>: nitrate, LTB<sub>4</sub>: leukotriene B<sub>4</sub>, PGE<sub>2</sub>: prostaglandin E<sub>2</sub>, cys-LT: cystinyl-leukotrienes, <sup>§</sup>for H<sub>2</sub>O<sub>2</sub> measurement, <sup>#</sup>when sputum is obtained by induction, \*spontaneous or induced, <sup>§</sup>valid for pH measurement only.

measurements are very expensive and require sophisticated laboratory infrastructure, and thus, they are unlikely to be useful in clinical studies, especially when they are multicenter studies involving large number of patients. Moreover, some measurements may be confounded by the effect of smoking. Finally, assessment of oxidative markers in the sputum supernatant is probably a more reliable approach compared to breath condensate analysis to study the underlying pathophysiology and to identify pulmonary biomarkers of potential clinical utility in the management of the disease. Overall, additional work is required to clarify the exact role of these markers and mediators in COPD pathophysiology and to validate these findings in clinical trials.

### CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

### ACKNOWLEDGEMENTS

The work was supported by the Hungarian National Scientific Foundation (OTKA 83338). The authors thank Professor Dr. H. Kalasz (Semmelweis University, Department of Pharmacology and Pharmacotherapy) for critical reading the manuscript. Dr. B. Antus is a recipient of Bolyai Janos Scholarship of the Hungarian Academy of Science.

### REFERENCES

- Mannino, D.M.; Buist, A.S. Global burden of COPD: risk factors, prevalence, and future trends. *Lancet*, **2007**, *370*, 765-773.
- Mathers, C.D.; Loncar, D. Projections of global mortality and burden of disease from 2002 to 2030. *Plos. Med.*, **2006**, *3*, e442.
- Kirkham, P.A.; Barnes, P.J. Oxidative stress in COPD. *Chest*, **2013**, *144*, 266-273.
- Tuder, R.M.; Petrache, I. Pathogenesis of chronic obstructive pulmonary disease. *J. Clin. Invest.*, **2012**, *122*, 2749-2755.
- Barnes, P.J. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J. Allergy. Clin. Immunol.*, **2013**, *131*, 636-645.
- Barnes, P.J.; Chowdhury, B.; Kharitonov, S.A.; Magnussen, H.; Page, C.P.; Postma, D.; Saetta, M. Pulmonary biomarkers in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **2006**, *174*, 6-14.
- Loukides, S.; Bakakos, P.; Kostikas, K. Oxidative stress in patients with COPD. *Curr. Drug Targets*, **2011**, *12*, 469-477.
- Koutsokera, A.; Kostikas, K.; Nicod, L.P.; Fitting, J.W. Pulmonary biomarkers in COPD exacerbations: a systematic review. *Respir. Res.*, **2013**, *14*, 111.
- Malerba, M.; Montuschi, P. Non-invasive biomarkers of lung inflammation in smoking subjects. *Curr. Med. Chem.*, **2012**, *19*, 187-196.
- Biswas, S.; Hwang, J.W.; Kirkham, P.A.; Rahman I. Pharmacological and dietary antioxidant therapies for chronic obstructive pulmonary disease. *Curr. Med. Chem.*, **2013**, *20*, 1496-1530.
- Niki, E. Lipid peroxidation: Physiological levels and dual biological effects. *Free Radical Biol. Chem.*, **2009**, *47*, 469-484.
- Yin, H.; Xu, L.; Porter, N.A. Free radical lipid peroxidation: mechanisms and analysis. *Chem. Rev.*, **2011**, *111*, 5944-5972.
- Antus, B. Pharmacotherapy of chronic obstructive pulmonary disease a clinical review. *ISRN Pulmonol.* **2013**, 2013, ID582807.
- Kehrer, J.P. The Haber-Weiss reaction and mechanism of toxicity. *Toxicology*, **2000**, *149*, 43-50.
- Fenton, H.J.R. Catalytic decomposition of hydrogen peroxide presence of iron. *J. Chem. Soc.*, **1894**, *65*, 890-910.
- Moncada, S.; Higgs, E.A. Endogenous nitric oxide: Physiology, pathology and clinical relevance. *Eur. J. Clin. Invest.*, **1991**, *21*, 361-374.
- Church, D.F.; Pryor, W.A. Free radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.*, **1985**, *64*, 111-126.
- Rahman, I.; MacNee, W. Antioxidant pharmacological therapies for COPD. *Curr. Opin. Pharmacol.*, **2012**, *12*, 256-265.
- Blair, I.A. Lipid-hydroperoxide-mediated DNA damage. *Exp. Gerontol.*, **2004**, *36*, 1473-1481.
- Tappel, A.L. Lipid peroxidation damage to cell components. *Fed. Proc.*, **1973**, *32*, 1870-1874.
- He, J.; Turino, G.M.; Lin, Y.Y. Characterization of peptide fragments from lung elastin degradation in chronic obstructive pulmonary disease. *Exp. Lung Res.*, **2010**, *36*, 548-557.
- Cao, Y.; Cao, Y.; Zhao, M. Biotechnological production of eicosapentaenoic acid: From a metabolic engineering point of view. *Process Biochem.*, **2012**, *47*, 1320-1326.
- Bogatcheva, N.V.; Seergeva, M.G.; Dudek, S.M.; Verin, A.D. Arachidonic acid cascade in endothelial pathobiology. *Microvascu. Res.*, **2005**, *69*, 107-127.
- Milne, G.L.; Yin, H.; Morrow, J.D. Human biochemistry of the isoprostanes pathway. *J. Biol. Chem.*, **2008**, *283*, 15533-15537.
- Arreaza, G.; Devane, W.A.; Omeir, R.I.; Sajjani, G.; Kunz, J.; Cravatt B.F.; Deutsch, D.G. The cloned rat hydrolytic enzyme responsible for the breakdown of anandamide also catalyzes its formation via the condensation of arachidonic acid and ethanolamide. *Neurosci. Lett.*, **1977**, *234*, 59-62.
- Morrow, J.D.; Hill, K.E.; Burk, R.F.; Nammour, T.M.; Badr, K.F.; Roberts, L.J. A series of prostaglandin F<sub>2</sub>-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA*, **1990**, *87*, 9383-9387.
- Morrow, J.D.; Award, J.A.; Boss, H.J.; Blair, I.A.; Roberts, L.J. Non-cyclooxygenase-derived prostanoids (F<sub>2</sub>-isoprostanes) are formed *in situ* on phospholipids. *Proc. Natl. Acad. Sci. USA*, **1992**, *89*, 10721-10725.
- Ingold, K.U. Peroxy Radicals. *Acc. Chem. Res.*, **1969**, *2*, 1-9.
- Barclay, L.R.C.; Ingold, K.U. Autoxidation of a model membrane. A comparison of the autoxidation of egg lecithin phosphatidylcholine in water and in chlorobenzene. *J. Am. Chem. Soc.*, **1980**, *102*, 7792-7794.
- Barclay, L.R.C.; Baskin, K.A.; Dakin, K.A.; Locke, S.J. Vinqvist, M.R. The antioxidant activities of phenolic antioxidants in free radical peroxidation of phospholipids membranes. *Can. J. Chem.*, **1990**, *68*, 2258-2269.
- Pratt, D.A.; Mills, J.H.; Porter, N.A. Theoretical calculations of carbon-oxygen bond dissociation enthalpies of peroxy radicals formed in the autoxidation of lipids. *J. Am. Chem. Soc.*, **2003**, *125*, 5801-5810.
- Howard, J.A. In *Advances in Free Radical Chemistry*. G. H. Williams, Ed.; Logos Press London Vol. IV, **1972**, p.49.
- Eliel, E.L. Recent advances in stereochemical nomenclature. *J. Chem. Educ.*, **1971**, *48*, 163-167.
- Davies, K.J. Oxidative stress, antioxidant defenses, and damage; Removal, repair, and replacement systems. *IUBMB. Life*, **2000**, *50*, 279-289.
- Morrow, J.D.; Harris, T.M.; Roberts Jr., J.L. Non-cyclooxygenase oxidative formation of series of novel prostaglandins-analytical ramifications for measurement of eicosanoids. *Anal. Biochem.*, **1990**, *184*, 1-10.
- Morrow, J.D.; Roberts, L.J. 2nd. The isoprostanes. Current knowledge and directions for future research. *Biochem. Pharmacol.*, **1996**, *51*, 1-9.
- Beckwith, A.L.J.; Schiesser, C.H. Regio- and stereo-selectivity of alkenyl radical ring closure: A theoretical study. *Tetrahedron*, **1985**, *41*, 3925-3941.
- Spellmeyer, D.; Houck, K. Force-field model for intramolecular radical additions. *J. Org. Chem.*, **1987**, *52*, 959-974.
- O'Connor, D.E.; Mihelich, E.D.; Coleman, M.C. Stereochemical course of the autoxidative cyclization of lipid hydroperoxides to prostaglandin-like bicyclic endoperoxides. *J. Am. Chem. Soc.*, **1984**, *106*, 3577-3584.
- Corey, E.J.; Wang, Z. Conversion of arachidonic acid to the prostaglandin endoperoxide PGG<sub>2</sub>, a chemical analog of the biosynthetic pathway. *Tetrahedron Lett.*, **1994**, *35*, 539-542.
- Niki, E.; Yoshida, Y.; Saito, V.; Noguchi, N. Lipid peroxidation: Mechanisms, inhibition and biological effects. *Biochem. Biophys. Res. Commun.*, **2005**, *338*, 668-676.

- [42] Tang, D.G.; La, E.; Kehrer, J.P. Fatty acid oxidation and signaling in apoptosis. *Biol. Chem.*, **2002**, *383*, 425-442.
- [43] Porter, N.A.; Nixon, J.R. Stereochemistry of free-radical substitution on the peroxide bond. *J. Am. Chem. Soc.*, **1978**, *100*, 7116-7117.
- [44] Chatgililoglu, C.; Ingold, K.U.; Scaiano, C.J. Rate constants and Arrhenius parameters for the reactions of primary, secondary, and tertiary alkyl radicals with tri-n-butyltin hydride. *J. Am. Chem. Soc.*, **1981**, *103*, 7739-7742.
- [45] Fessel, J.P.; Jackson Roberts, L. Isofurans: Novel products of lipid peroxidation that define the occurrence of oxidant injury in settings of elevated oxygen tension. *Antioxid. Redox Signal.*, **2005**, *7*, 202-209.
- [46] Yin, H.; Havrilla, C.M.; Morrow, J.D.; Porter, N.A. Formation of isoprostane bicyclic endoperoxides from the autoxidation of cholesterol arachidonate. *J. Am. Chem. Soc.*, **2002**, *124*, 7745-7754.
- [47] Spector, A.A. Arachidonic acid *cytochrome P450 epoxygenase* pathway. *J. Lipid Res.*, **2009**, *50*, S52-S56.
- [48] Liu, W.; Porter, N.A.; Schneider, C.; Brash, A.R.; Yin, H. Formation of 4-hydroxynonenal from cardiolipin oxidation: Intramolecular peroxy radical addition and decomposition. *Free Radical Biol. Med.*, **2011**, *50*, 166-178.
- [49] Yu, Z.; Xu, F.; Huse, L. M.; Morisseau, C.; Draper, A.J.; Newman, J.W.; Parker, C.; Graham, L.; Engler, M.M.; Hammock, B.D.; Zeldin, D.C.; Kroetz, D.L. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. *Circ. Res.*, **2000**, *87*, 992-998.
- [50] Capdevila, J.H.; Falck, J.R. Biochemical and molecular properties of the *cytochrome P450* arachidonic acid *monooxygenases*. *Prostaglandins Other Lipid Mediat.*, **2002**, *68-69*, 325-344.
- [51] Smith, W.L.; Urade, Y.; Jakobsson, P.J. Enzymes of the cyclooxygenase pathway of prostanoid biosynthesis. *Chem. Rev.*, **2011**, *111*, 5821-5865.
- [52] Rouzer, C.A.; Marnett, L.J. *Cyclooxygenase*: structural and functional insights. *J. Lipid Res.*, **2009**, *50*, S29-S34.
- [53] Samuelsson, B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science*, **1983**, *220*, 568-575.
- [54] Barnes, P.J. Mediators of chronic obstructive pulmonary disease. *Pharmacol. Rev.*, **2004**, *56*, 515-548.
- [55] Lundström, S.L.; Balgoma, D.; Wheelock, Å.M.; Haeggström, J.Z.; Dahlén, S.E.; Wheelock, C.E. Lipid mediator profiling in pulmonary disease. *Curr. Pharm. Biotechnol.*, **2011**, *12*, 1026-1052.
- [56] Borrill, Z.L.; Starkey, R.C.; Singh, S.D. Variability of exhaled breath condensate leukotriene B4 and 8-isoprostane in COPD patients. *Int. J. Chron. Obstruct. Pulmon. Dis.*, **2007**, *2*, 71-76.
- [57] Franklin, P.; Moeller, A.; Hall, G.L.; Horak, F. Jr.; Patterson, H.; Stick, S.M. Variability of nitric oxide metabolites in exhaled breath condensate. *Respir. Med.*, **2006**, *100*, 123-129.
- [58] Corhay, J.L.; Henket, M.; Nguyen, D.; Duysinx, B.; Sele, J.; Louis, R. Leukotriene B4 contributes to exhaled breath condensate and sputum neutrophil chemotaxis in COPD. *Chest*, **2009**, *136*, 1047-1054.
- [59] Warwick, G.; Thomas, P.S.; Yates, D.H. Non-invasive biomarkers in exacerbation of obstructive lung disease. *Respirology*, **2013**, *18*, 874-884.
- [60] Bathoorn, E.; Liesker, J.J.; Postma, D.S.; Koëter, G.H.; van der Toorn, M.; van der Heide, S.; Ross, H.A.; van Oosterhout, A.J.; Kerstjens, H.A. Change in inflammation in out-patient COPD patients from stable phase to a subsequent exacerbation. *Int. J. Chron. Obstruct. Pulmon. Dis.*, **2009**, *4*, 101-109.
- [61] Crooks, S.W.; Bayley, D.L.; Hill, S.L.; Stockley, R.A. Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4. *Eur. Respir. J.*, **2000**, *15*, 274-280.
- [62] Björk, J.; Hedqvist, P.; Arfors, K.E. Increase in vascular permeability induced by leukotriene B4 and the role of polymorphonuclear leukocytes. *Inflammation*, **1982**, *6*, 189-200.
- [63] Dahlén, S.E.; Björk, J.; Hedqvist, P.; Arfors, K.E.; Hammarström, S.; Lindgren, J.A.; Samuelsson, B. Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: *in vivo* effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. USA.*, **1981**, *78*, 3887-3891.
- [64] Ott, V.L.; Cambier, J.C.; Kappler, J.; Marrack, P.; Swanson, B.J. Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. *Nat. Immunol.*, **2003**, *4*, 974-981.
- [65] Sakata, K.; Dahlén, S.E.; Bäck, M. The contractile action of leukotriene B4 in the guinea-pig lung involves a vascular component. *Br. J. Pharmacol.*, **2004**, *141*, 449-456.
- [66] Busse, W.W. Leukotrienes and inflammation. *Am. J. Respir. Crit. Care Med.*, **1998**, *157*, S210-S213.
- [67] Profita, M.; Giorgi, R.D.; Sala, A.; Bonanno, A.; Riccobono, L.; Mirabella, F.; Gjomarkaj, M.; Bonsignore, G.; Bousquet, J.; Vignola, A.M. Muscarinic receptors, leukotriene B4 production and neutrophilic inflammation in COPD patients. *Allergy*, **2005**, *60*, 1361-1369.
- [68] Kostikas, K.; Gaga, M.; Papatheodorou, G.; Karamanis, T.; Orphanidou, D.; Loukides, S. Leukotriene B4 in exhaled breath condensate and sputum supernatant in patients with COPD and asthma. *Chest*, **2005**, *127*, 1553-1559.
- [69] Biernacki, W.A.; Kharitonov, S.A.; Barnes, P.J. Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax*, **2003**, *58*, 294-298.
- [70] Seggev, J.S.; Thornton, W.H. Jr.; Edes, T.E. Serum leukotriene B4 levels in patients with obstructive pulmonary disease. *Chest*, **1991**, *99*, 289-291.
- [71] Montuschi, P.; Kharitonov, S.A.; Ciabattini, G.; Barnes, P.J. Exhaled leukotrienes and prostaglandins in COPD. *Thorax*, **2003**, *58*, 585-588.
- [72] Gaber, F.; Acevedo, F.; Delin, I.; Sundblad, B.M.; Palmberg, L.; Larsson, K.; Kumlin, M.; Dahlén, S.E. Saliva is one likely source of leukotriene B4 in exhaled breath condensate. *Eur. Respir. J.*, **2006**, *28*, 1229-1235.
- [73] Borrill, Z.L.; Roy, K.; Singh, D. Exhaled breath condensate biomarkers in COPD. *Eur. Respir. J.*, **2008**, *32*, 472-486.
- [74] Czebe, K.; Barta, I.; Antus, B.; Valyon, M.; Horváth, I.; Kullmann, T. Influence of condensing equipment and temperature on exhaled breath condensate pH, total protein and leukotriene concentrations. *Respir. Med.*, **2008**, *102*, 720-725.
- [75] Drozdovszky, O.; Barta, I.; Antus, B. Sputum eicosanoid profiling in exacerbations of chronic obstructive pulmonary disease. *Respiration*, **2014**, *87*, 408-415.
- [76] Laidlaw, T.M.; Boyce, J.A. Cysteinyl leukotriene receptors, old and new; implications for asthma. *Clin. Exp. Allergy.*, **2012**, *42*, 1313-1320.
- [77] Pavord, I.D.; Tattersfield, A.E. Bronchoprotective role for endogenous prostaglandin E2. *Lancet*, **1995**, *345*, 436-438.
- [78] Bikov, A.; Gajdócsi, B.; Huszár, É.; Szili, B.; Lázár, Z.; Antus, B.; Losonczy, G.; Horváth, I. Exercise increases exhaled breath condensate cysteinyl leukotriene concentration in asthmatic patients. *J. Asthma*, **2010**, *47*, 1057-1062.
- [79] Birring, S.S.; Parker, D.; Brightling, C.E.; Bradding, P.; Wardlaw, A.J.; Pavord, I.D. Induced sputum inflammatory mediator concentrations in chronic cough. *Am. J. Respir. Crit. Care Med.*, **2004**, *169*, 15-19.
- [80] Antczak, A.; Ciebiada, M.; Pietras, T.; Piotrowski, W.J.; Kurmanowska, Z.; Górski, P. Exhaled eicosanoids and biomarkers of oxidative stress in exacerbation of chronic obstructive pulmonary disease. *Arch. Med. Sci.*, **2012**, *8*, 277-285.
- [81] Zhu, J.; Bandi, V.; Qiu, S.; Figueroa, D.J.; Evans, J.F.; Barnes, N.; Guntupalli, K.K.; Jeffery, P.K. Cysteinyl leukotriene 1 receptor expression associated with bronchial inflammation in severe exacerbations of COPD. *Chest*, **2012**, *142*, 347-357.
- [82] Drakatos, P.; Lykouras, D.; Sampsonas, F.; Karkoulas, K.; Spiropoulos, K. Targeting leukotrienes for the treatment of COPD? *Inflamm. Allergy. Drug. Targets.*, **2009**, *8*, 297-306.
- [83] Montuschi, P.; Macagno, F.; Parente, P.; Valente, S.; Lauriola, L.; Ciappi, G.; Kharitonov, S.A.; Barnes, P.J.; Ciabattini, G. Effects of cyclooxygenase inhibition on exhaled eicosanoids in patients with COPD. *Thorax*, **2005**, *60*, 827-833.
- [84] Profita, M.; Sala, A.; Bonanno, A.; Riccobono, L.; Ferraro, M.; La Grutta, S.; Albano, G.D.; Montalbano, A.M.; Gjomarkaj, M. Chronic obstructive pulmonary disease and neutrophil infiltration: role of cigarette smoke and cyclooxygenase products. *Am. J. Physiol. Lung. Cell. Mol. Physiol.*, **2010**, *298*, L261-L269.
- [85] Chen, Y.; Chen, P.; Hanaoka, M.; Droma, Y.; Kubo, K. Enhanced levels of prostaglandin E2 and matrix metalloproteinase-2 correlate with the severity of airflow limitation in stable COPD. *Respirology*, **2008**, *13*, 1014-1021.
- [86] Louhelainen, N.; Myllärmiemi, M.; Rahman, I.; Kinnula, V.L. Airway biomarkers of the oxidant burden in asthma and chronic ob-

- structive pulmonary disease: current and future perspectives. *Int. J. Chron. Obstruct. Pulmon. Dis.*, **2008**, *3*, 585-603.
- [87] Kubáň, P.; Foret, F. Exhaled breath condensate: determination of non-volatile compounds and their potential for clinical diagnosis and monitoring. A review. *Anal. Chim. Acta.*, **2013**, *805*, 1-18.
- [88] Wood, L.G.; Gibson, P.G.; Garg, M.L. Biomarkers of lipid peroxidation, airway inflammation and asthma. *Eur. Respir. J.*, **2003**, *21*, 177-186.
- [89] Montuschi, P.; Collins, J.V.; Ciabattini, G.; Lazzeri, N.; Corradi, M.; Kharitonov, S.A.; Barnes, P.J. Exhaled 8-isoprostane as an *in vivo* biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am. J. Respir. Crit. Care Med.*, **2000**, *162*, 1175-1177.
- [90] Kinnula, V.L.; Ilumets, H.; Myllärniemi, M.; Sovijärvi, A.; Ryttilä, P. 8-Isoprostane as a marker of oxidative stress in nonsymptomatic cigarette smokers and COPD. *Eur. Respir. J.*, **2007**, *29*, 51-55.
- [91] Carpagnano, G.E.; Resta, O.; Foschino-Barbaro, M.P.; Spanevello, A.; Stefano, A.; Di Gioia, G.; Serviddio, G.; Gramiccioni, E. Exhaled Interleukine-6 and 8-isoprostane in chronic obstructive pulmonary disease: effect of carbocysteine lysine salt monohydrate (SCMC-Lys). *Eur. J. Pharmacol.*, **2004**, *505*, 169-175.
- [92] Dekhuijzen, P.N.; Aben, K.K.; Dekker, I.; Aarts, L.P.; Wielders, P.L.; van Herwaarden, C.L.; Bast, A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **1996**, *154*, 813-816.
- [93] Koczulla, A.R.; Noeske, S.; Herr, C.; Koepke, J.; Jörres, R.A.; Nell, C.; Schmid, S.; Vogelmeier, C.; Bals, R. Alpha-1 antitrypsin is elevated in exhaled breath condensate and serum in exacerbated COPD patients. *Respir. Med.*, **2012**, *106*, 120-126.
- [94] Mohan, A.; Prasad, D.; Sharma, A.; Arora, S.; Guleria, R.; Sharma, S.K.; Pandey, R.M. Delayed resolution of inflammatory response compared with clinical recovery in patients with acute exacerbations of chronic obstructive pulmonary disease. *Respirology*, **2012**, *17*, 1080-1085.
- [95] Antus, B.; Horváth, I. Exhaled nitric oxide and carbon monoxide in respiratory diseases. *J. Breath Res.*, **2007**, *1*, 024002.
- [96] Wolff, D.G.; Bidlack, W.R. The formation of carbon monoxide during peroxidation of microsomal lipids. *Biochem. Biophys. Res. Commun.*, **1976**, *4*, 850-857.
- [97] Montuschi, P.; Kharitonov, S.A.; Barnes, P.J. Exhaled carbon monoxide and nitric oxide in COPD. *Chest*, **2001**, *120*, 496-501.
- [98] Carpagnano, G.E.; Kharitonov, S.A.; Foschino-Barbaro, M.P.; Resta, O.; Gramiccioni, E.; Barnes, P.J. Increased inflammatory markers in the exhaled breath condensate of cigarette smokers. *Eur. Respir. J.*, **2003**, *21*, 589-593.
- [99] Deveci, S.E.; Deveci, F.; Açik, Y.; Ozan, A.T. The measurement of exhaled carbon monoxide in healthy smokers and non-smokers. *Respir. Med.*, **2004**, *98*, 551-556.
- [100] Barta, I.; Drozdovszky, O.; Antus, B. Monitoring exhaled carbon monoxide in COPD exacerbations. *Eur. Respir. J.*, **2014**, *44*, Suppl. 58, 999.
- [101] Ryter, S.W.; Choi, A.M. Carbon monoxide in exhaled breath testing and therapeutics. *J. Breath Res.* **2013**, *7*, 0017111.
- [102] Zarling, E.J.; Clapper, M. Technique for gas-chromatographic measurement of volatile alkanes from single-breath samples. *Clin. Chem.*, **1987**, *33*, 140-141.
- [103] Habib, M.P.; Clements, N.C.; Garewal, H.S. Cigarette smoking and ethane exhalation in humans. *Am. J. Respir. Crit. Care Med.*, **1995**, *151*, 1368-1372.
- [104] Paredi, P.; Kharitonov, S.A.; Leak, D.; Ward, S.; Cramer, D.; Barnes, P.J. Exhaled ethane, a marker of lipid peroxidation, is elevated in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **2000**, *162*, 369-373.
- [105] Pryor, W.A.; Stanley, J.P. A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. *J. Org. Chem.*, **1975**, *40*, 3615-3617.
- [106] Esterbauer, H.; Schaur, R.J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.*, **1991**, *11*, 81-128.
- [107] McMillan, R.M.; MacIntyre, D.E.; Booth, A.; Gordon, J.L. Malonaldehyde formation in intact platelets catalysed by thromboxane synthase. *Biochem. J.*, **1978**, *176*, 596-598.
- [108] Hecker, M.; Ullrich, V. On the mechanism of prostacyclin and thromboxane A2 biosynthesis. *J. Biol. Chem.*, **1989**, *264*, 141-150.
- [109] Giera, M.; Lingeman, H.; Niessen, W.M. Recent advancements in the LC- and GC-based analysis of malondialdehyde (MDA): A brief overview. *Chromatographia*, **2012**, *75*, 433-440.
- [110] Lärstad, M.; Ljungkvist, G.; Olin, A.C.; Torén, K. Determination of malondialdehyde in breath condensate by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.*, **2002**, *766*, 107-114.
- [111] Andreoli, R.; Manini, P.; Corradi, M.; Mutti, A.; Niessen, W.M. Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Commun. Mass. Spectrom.*, **2003**, *17*, 637-645.
- [112] Corradi, M.; Rubinstein, I.; Andreoli, R.; Manini, P.; Caglieri, A.; Poli, D.; Alinovi, R.; Mutti, A. Aldehydes in exhaled breath condensate of patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.*, **2003**, *167*, 1380-1386.
- [113] Bartoli, M.L.; Novelli, F.; Costa, F.; Malagrino, L.; Melosini, L.; Bacci, E.; Cianchetti, S.; Dente, F.L.; Di Franco, A.; Vagaggini, B.; Paggiaro, P.L. Malondialdehyde in exhaled breath condensate as a marker of oxidative stress in different pulmonary diseases. *Mediators Inflamm.*, **2011**, *2011*, 891752.
- [114] Corradi, M.; Pignatti, P.; Manini, P.; Andreoli, R.; Goldoni, M.; Poppa, M.; Moscato, G.; Balbi, B.; Mutti, A. Comparison between exhaled and sputum oxidative stress biomarkers in chronic airway inflammation. *Eur. Respir. J.*, **2004**, *24*, 1011-1017.
- [115] Antus, B.; Harnasi, G.; Drozdovszky, O.; Barta, I. Monitoring oxidative stress during COPD exacerbations using malondialdehyde. *Respirology*, **2014**, *19*, 74-79.
- [116] Crawford, D.L.; Yu, T.C.; Sinnbauer, R.O. Reaction of malondialdehyde with glycine. *J. Agric. Food Chem.*, **1966**, *14*, 182-184.
- [117] Kikugawa, K.; Takayanagi, K.; Watanabe, S. Polylysines modified with malondialdehyde, hydroperoxylinoleic acid and monofunctional aldehyde. *Chem. Pharm. Bull.*, **1985**, *33*, 5437-5444.
- [118] Draper, H.H.; Hadley, M.; Lissemore, L.; Liang, N.M.; Cole, P.D. Identification of *N*-epsilon-(2-propenal)lysine as a major urinary metabolite of malondialdehyde. *Lipids*, **1988**, *23*, 626-628.
- [119] Chio, K.S.; Tappel, A.L. Synthesis and characterization of fluorescent products derived from malonaldehyde and amino acids. Products of malonaldehyde and amino acids. *Biochemistry*, **1969**, *8*, 2821-2832.
- [120] Seto, H.; Okuda, T.; Takesue, T.; Ikemura, T. Reaction of malondialdehyde with nucleic acid I. Formation of fluorescent pyrimidino(1,2-a)purin(10(3H)-one nucleoside. *Bull. Chem. Soc. Jap.*, **1983**, *56*, 1799-1802.
- [121] Marnett, J.L.; Basu, A.K.; O'Hara, S.M.; Weller, P.E.; Rahman, A.F.M.M.; Oliver, J.P. Reaction of malondialdehyde with guanine nucleosides. Formation of adducts containing oxadiazabicyclononene residues in the base pairing region. *J. Am. Chem. Soc.*, **1986**, *108*, 1348-1350.
- [122] Esterbauer, H.; Zollner, H.; Scholz, N. Reaction of glutathione with conjugated carbonyls. *Z. Naturforsch.*, **1975**, *30*, 466-473.
- [123] Witz, G. Biological interactions of alpha, beta-unsaturated aldehyde. *Free Radic. Biol. Med.*, **1989**, *7*, 333-349.
- [124] Gan, J.C.; Ansari, G.A.S. Plausible mechanism of inactivation of plasma alpha-1-proteinase inhibitor by acrolein. *Res. Comm. Chem. Pathol. Pharmacol.*, **1987**, *55*, 419-422.
- [125] Chung, F.L.; Young, R.; Hecht, S.S. Formation of cyclic 1,N2-propanodeoxyquanosine adducts in DNA upon with acrolein or crotonaldehyde. *Cancer Res.* **1984**, *44*, 990-995.
- [126] Gutteridge, J.C.M. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin. Chem.*, **1996**, *41*, 1819-1828.
- [127] Bovens, A.; Oshino, N.; Chance, B. The cellular production of hydrogen peroxide. *Biochem. J.*, **1972**, *128*, 612-630.
- [128] Oudijk, E.J.; Gerritsen, W.B.; Nijhuis, E.H.; Kanters, D.; Maesen, B.L.; Lammers, J.W.; Koenderman, L. Expression of priming-associated cellular markers on neutrophils during an exacerbation of COPD. *Respir. Med.*, **2006**, *100*, 1791-1799.
- [129] Nowak, D.; Kasielski, M.; Antczak, A.; Pietras, T.; Bialasiewicz, P. Increased content of thiobarbituric acid-reactive substances and hydrogen peroxide in the expired breath condensate of patients with stable chronic obstructive pulmonary disease: no significant effect of cigarette smoking. *Respir. Med.*, **1999**, *93*, 389-396.

- [130] Kasielski, M.; Nowak, D. Long-term administration of N-acetylcysteine decreases hydrogen peroxide exhalation in subjects with chronic obstructive pulmonary disease. *Respir. Med.*, **2001**, *95*, 448-456.
- [131] Schleiss, M.B.; Holz, O.; Behnke, M.; Richter, K.; Magnussen, H.; Jörres, R.A. The concentration of hydrogen peroxide in exhaled air depends on expiratory flow rate. *Eur. Respir. J.*, **2000**, *16*, 1115-1118.
- [132] Gajdocsi, R.; Bikov, A.; Antus, B.; Horvath, I.; Barnes, P.J.; Kharitonov, S.A. Assessment of reproducibility of exhaled hydrogen peroxide concentration and the effect of breathing pattern in healthy subjects. *J. Aerosol. Med. Pulm. Drug Deliv.*, **2011**, *24*, 271-275.
- [133] Ricciardolo, F.L.; Di Stefano, A.; Sabatini, F.; Folkerts, G. Reactive nitrogen species in the respiratory tract. *Eur. J. Pharmacol.*, **2006**, *533*, 240-252.
- [134] Gaston, B.; Drazen, J.M.; Loscalzo, J.; Stamler, J.S. The biology of nitrogen oxides in the airways. *Am. J. Respir. Crit. Care Med.*, **1994**, *149*, 538-551.
- [135] Corradi, M.; Montuschi, P.; Donnelly, L.E.; Pesci, A.; Kharitonov, S.A.; Barnes, P.J. Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases. *Am. J. Respir. Crit. Care Med.*, **2001**, *163*, 854-858.
- [136] Ricciardolo, F.L.; Caramori, G.; Ito, K.; Capelli, A.; Brun, P.; Abatangelo, G.; Papi, A.; Chung, K.F.; Adcock, I.; Barnes, P.J.; Donner, C.F.; Rossi, A.; Di Stefano, A. Nitrosative stress in the bronchial mucosa of severe chronic obstructive pulmonary disease. *J. Allergy Clin. Immunol.*, **2005**, *116*, 1028-1035.
- [137] Sugiura, H.; Ichinose, M.; Tomaki, M.; Ogawa, H.; Koarai, A.; Kitamura, T.; Komaki, Y.; Akita, T.; Nishino, H.; Okamoto, S.; Akaike, T.; Hattori, T. Quantitative assessment of protein-bound tyrosine nitration in airway secretions from patients with inflammatory airway disease. *Free. Radic. Res.*, **2004**, *38*, 49-57.
- [138] Ricciardolo, F.L.; Rado, V.; Fabbri, L.M.; Sterk, P.J.; Di Maria, G.U.; Geppetti, P. Bronchoconstriction induced by citric acid inhalation in guinea pigs: role of tachykinins, bradykinin, and nitric oxide. *Am. J. Respir. Crit. Care Med.*, **1999**, *159*, 557-562.
- [139] Clary-Meinesz, C.; Mouroux, J.; Cosson, J.; Huitorel, P.; Blaive, B. Influence of external pH on ciliary beat frequency in human bronchi and bronchioles. *Eur. Respir. J.*, **1998**, *11*, 330-333.
- [140] Hunt, J.F.; Fang, K.; Malik, R.; Snyder, A.; Malhotra, N.; Platts-Mills, T.A.; Gaston, B. Endogenous airway acidification. Implications for asthma pathophysiology. *Am. J. Respir. Crit. Care Med.*, **2000**, *161*, 694-699.
- [141] Kostikas, K.; Papatheodorou, G.; Ganas, K.; Psathakis, K.; Panagou, P.; Loukides, S. pH in expired breath condensate of patients with inflammatory airway diseases. *Am. J. Respir. Crit. Care Med.*, **2002**, *165*, 1364-1370.
- [142] Borrill, Z.; Starkey, C.; Vestbo, J.; Singh, D. Reproducibility of exhaled breath condensate pH in chronic obstructive pulmonary disease. *Eur. Respir. J.*, **2005**, *25*, 269-274.
- [143] Tate, S.; MacGregor, G.; Davis, M.; Innes, J.A.; Greening, A.P. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax*, **2002**, *57*, 926-929.
- [144] Ojoo, J.C.; Mulrennan, S.A.; Kastelik, J.A.; Morice, A.H.; Redington, A.E. Exhaled breath condensate pH and exhaled nitric oxide in allergic asthma and in cystic fibrosis. *Thorax*, **2005**, *60*, 22-26.
- [145] MacGregor, G.; Ellis, S.; Andrews, J.; Imrie, M.; Innes, A.; Greening, A.P.; Cunningham, S. Breath condensate ammonium is lower in children with chronic asthma. *Eur. Respir. J.*, **2005**, *26*, 271-276.
- [146] Koczulla, R.; Dragonieri, S.; Schot, R.; Bals, R.; Gauw, S.A.; Vogelmeier, C.; Rabe, K.F.; Sterk, P.J.; Hiemstra, P.S. Comparison of exhaled breath condensate pH using two commercially available devices in healthy controls, asthma and COPD patients. *Respir. Res.*, **2009**, *10*, 78.
- [147] Nicolaou, N.C.; Lowe, L.A.; Murray, C.S.; Woodcock, A.; Simpson, A.; Custovic, A. Exhaled breath condensate pH and childhood asthma: unselected birth cohort study. *Am. J. Respir. Crit. Care Med.*, **2006**, *174*, 254-259.
- [148] Vaughan, J.; Ngamtrakulpanit, L.; Pajewski, T.N.; Turner, R.; Nguyen, T.A.; Smith, A.; Urban, P.; Hom, S.; Gaston, B.; Hunt, J. Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. *Eur. Respir. J.*, **2003**, *22*, 889-894.
- [149] Kullmann, T.; Barta, I.; Lazar, Z.; Szili, B.; Barat, E.; Vallyon, M.; Horvath, I. Exhaled breath condensate pH standardised for CO<sub>2</sub> partial pressure. *Eur. Respir. J.*, **2007**, *29*, 496-501.
- [150] Antus, B.; Barta, I.; Kullmann, T.; Lazar, Z.; Vallyon, M.; Horvath, I.; Csiszer, E. Assessment of exhaled breath condensate pH in exacerbations of asthma and chronic obstructive pulmonary disease: A longitudinal study. *Am. J. Respir. Crit. Care Med.*, **2010**, *182*, 1492-1497.
- [151] Antus, B.; Barta, I.; Csiszer, E.; Kelemen, K. Exhaled breath condensate pH in patients with cystic fibrosis. *Inflamm. Res.*, **2012**, *61*, 1141-1147.
- [152] Antus, B.; Barta, I. Exhaled breath condensate pH in patients with lung cancer. *Lung Cancer*, **2012**, *75*, 178-180.
- [153] Soter, S.; Kelemen, K.; Barta, I.; Vallyon, M.; Csiszer, E.; Antus, B. Exhaled breath condensate pH in lung transplant recipients with bronchiolitis obliterans syndrome. *Transplantation*, **2011**, *91*, 793-797.
- [154] Kullmann, T.; Barta, I.; Antus, B.; Horvath, I. Drinking influences exhaled breath condensate acidity. *Lung*, **2008**, *186*, 263-268.
- [155] Effros, R.M.; Casaburi, R.; Su, J.; Dunning, M.; Torday, J.; Biller, J.; Shaker, R. The effects of volatile salivary acids and bases on exhaled breath condensate pH. *Am. J. Respir. Crit. Care Med.*, **2006**, *173*, 386-392.
- [156] Wells, K.; Vaughan, J.; Pajewski, T.N.; Hom, S.; Ngamtrakulpanit, L.; Smith, A.; Nguyen, A.; Turner, R.; Hunt, J. Exhaled breath condensate pH assays are not influenced by oral ammonia. *Thorax*, **2005**, *60*, 27-31.