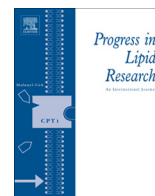




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## Review

## Evolutionary aspects of lipoxygenases and genetic diversity of human leukotriene signaling



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## ABSTRACT

Leukotrienes are pro-inflammatory lipid mediators, which are biosynthesized via the lipoxygenase pathway of the arachidonic acid cascade. Lipoxygenases form a family of lipid peroxidizing enzymes and human lipoxygenase isoforms have been implicated in the pathogenesis of inflammatory, hyperproliferative (cancer) and neurodegenerative diseases. Lipoxygenases are not restricted to humans but also occur in a large number of pro- and eucaryotic organisms. Lipoxygenase-like sequences have been identified in the three domains of life (bacteria, archaea, eucarya) but because of lacking functional data the occurrence of catalytically active lipoxygenases in archaea still remains an open question. Although the physiological and/or pathophysiological functions of various lipoxygenase isoforms have been studied throughout the last three decades there is no unifying concept for the biological importance of these enzymes. In this review we are summarizing the current knowledge on the distribution of lipoxygenases in living single and multicellular organisms with particular emphasis to higher vertebrates and will also focus on the genetic diversity of enzymes and receptors involved in human leukotriene signaling.

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Abbreviations: AA, arachidonic acid; EPA, 5,8,11,14,17-eicosapentaenoic acid; DHA, 4,7,10,13,16,19-docosahexaenoic acid; LOX, lipoxygenase; COX, cyclooxygenase; PG, prostaglandins; Tx, thromboxane; cPLA2, cytosolic phospholipase 2; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; GGT, gamma-glutamyltranspeptidase; DPEP, dipeptidase; Mya, million years ago; Bya, billion years ago; LX, lipoxins; RS, resolvins.

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## 1. Introduction

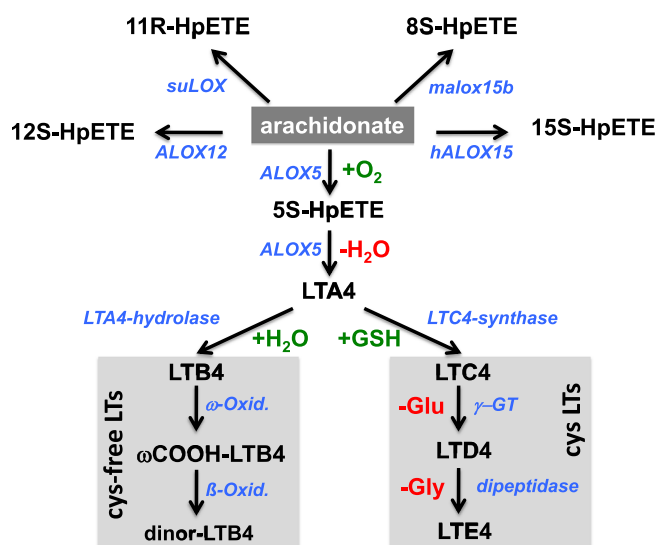
Polyunsaturated fatty acids (PUFAs) are important constituents of membrane lipids, which impact membrane functionality [1]. In addition, they also constitute substrates for the biosynthesis of signaling molecules such as prostaglandins [2], leukotrienes [3] or endocannabinoids [4]. Linoleic acid (C18:Δ2, n-6) and arachidonic acid (C20:Δ4, n-6) are abundant polyenoic fatty acids in the ester lipids of mammalian membranes but alpha- (C18:Δ3, n-6) and gamma-linolenic acid (C18:Δ3, n-3), eicosapentaenoic acid (C20:Δ5, n-3) and docosahexaenoic acid (C22:Δ6, n-3) also occur in lower abundance. However, the cellular concentrations of free polyenoic fatty acids are rather small. In order to be converted to prostaglandins, leukotrienes and related substances polyenoic fatty acids must be released from the membrane ester lipids and subsequently be oxygenated via two major alternative metabolic routes (Fig. 1). The cyclooxygenase (COX) pathway of the arachidonic acid cascade leads to the biosynthesis of classical prostaglandins (PGE2, PGF2, PGD2), thromboxanes (TxA2) and prostacyclin (PGI2). In contrast, leukotrienes, (LT), lipoxins (LX), resolvins (RS) and others are biosynthesized via the lipoxygenase (LOX) pathway [5]. Leukotrienes are the most extensively studied LOX products [6] and their biosynthesis involves a concerted action of several enzymes such as cytosolic phospholipase A2 (cPLA2), 5-LOX (ALOX5), Leukotriene A4 hydrolase (LTA4H), leukotriene C4 synthase (LTC4S), gamma-glutamyltranspeptidase (gGTs) and dipeptidases (DPEPs).

There are several current review articles [5–10], which together provide a detailed overview on chemistry, enzymology, biological roles and medical relevance of LOX and leukotriene signaling. However, to the best of our knowledge evolutionary aspects and genetic variability of LOX and of leukotriene signaling have never been reviewed before. This paper is aimed at filling this gap. After a short introduction into the field we will summarize our current knowledge on classification, biosynthesis and physiological importance of LOX (chapter 2) and leukotriene signaling (chapter 3). Then we will address evolutionary aspects of leukotriene signaling (chapter 4) and this part of the paper is focused on distribution of LOX isoforms and other enzymes of leukotriene biosynthesis within the three domains of life on this planet. The growing num-

ber of sequenced genomes of living and extinct organisms has led to identification of an increasing number of potential LOX and/or LOX-like sequences and it would exceed the frame of this review to refer to all of them. Instead, we selected frequently employed model organisms, which represent different stages in the evolution of life on earth and searched their genomes for putative LOX sequences. Since for most of these sequences no functional data are currently available, we used our knowledge on structural biology of these enzymes to draw functional conclusions. Finally, we will summarize the current knowledge on genetic variability of human leukotriene signaling (chapter 5). For this purpose we evaluated the public databases summarizing naturally occurring mutants of human LOX and other enzymes of leukotriene signaling. Here again, the databases are rapidly growing and it is not possible to consider all naturally occurring variants for this review. In this sense the review is somewhat selective and we are not aiming at completeness. However, we would like to apologize to those distinguished colleagues whose work we have not found space to describe and to reference.

## 2. Leukotrienes: classification, biosynthesis and physiological importance

Leukotrienes (LTs) are pro-inflammatory LOX products regulating the extent of the inflammatory reaction [6,10,11]. When formed in pathological quantities these mediators induce pain, fever, and inflammation. LTs are biosynthesized from arachidonic acid (AA) via the consecutive action of different enzymes (Fig. 1). The reaction cascade is initiated by the liberation of AA from the cellular ester lipids by cytosolic phospholipase A2 (cPLA2). There are two principle types of leukotrienes, which exhibit different bioactivities. (i) Cysteinyl-free leukotrienes (LTA4, LTB4) are biosynthesized from arachidonic acid via consecutive dioxygenation (formation of LTA4) and subsequent enzymatic epoxide hydrolysis. For these reactions the 5-lipoxygenase (ALOX5) [12] and leukotriene A4 hydrolase (LTA4H) [13] are responsible. As allylic epoxide LTA4 is very unstable and undergoes rapid hydrolysis. Thus, it may not exhibit systemic bioactivity. LTB4 functions as potent chemoattractant for inflammatory cells and induces chemokinesis and



**Fig. 1.** Leukotriene biosynthesis and classification. Free arachidonic acid is oxygenated by different ALOX isoforms to different hydroperoxy arachidonic acid isomers (upper part). ALOX5 converts arachidonic acid to 5S-HpETE and further to LTA<sub>4</sub>, which then gives rise to cysteinyl leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>) and the cysteinyl-free leukotrienes (LTB<sub>4</sub>). LTB<sub>4</sub> is further metabolized via peroxisomal ω-oxidation to ω-carboxy-LTB<sub>4</sub> and then by β-oxidation to 18-carboxy,19,20-dinor-LTB<sub>4</sub>. hALOX15, human ALOX15, mAlox15b, mouse Alox15b, sea urchin 11R-LOX (suLOX).

chemotaxis [14,15]. It binds to cell surface receptors (BLT1, BLT2) and induces different intracellular signaling cascades. ii) Cysteinyl leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>), which are also called peptido leukotrienes, are formed when LTA<sub>4</sub> is conjugated with reduced glutathione (GSH) to form LTC<sub>4</sub> [3]. This reaction is catalyzed by the LTC<sub>4</sub>-synthase (LTC<sub>4</sub>S), which constitutes a special type of GSH-S-transferase [16]. When glutamate is removed from LTC<sub>4</sub> by the catalytic activity of gamma-glutamyltranspeptidase (gamma GT) LTD<sub>4</sub> is formed and additional removal of glycine by a dipeptidase (DPEP) yields LTE<sub>4</sub>. The cysteinyl leukotrienes are constituents of the slow reacting substance of anaphylaxis and play a major role in the pathogenesis of hyperergic diseases such as allergic rhinitis and bronchial asthma [6]. They induce airway constriction, bronchoalveolar mucus production and mucosal edema [17,18]. Two major cysteinyl leukotriene receptors (cysLTR1, cysLTR2) have been identified and antagonists at cysLTR1 (LUKASTs) are available for prescription as anti-asthmatics [19]. In contrast, the role of cysLTR2 still remains a matter of discussion [20]. In addition to classical LT receptors (BLT1, BLT2, cysLTR1, cysLTR2), other deorphanized receptors have been shown to bind LTs but their clinical relevance remains unclear [6].

Most enzymes (cPLA<sub>2</sub>, ALOX5, LTA<sub>4</sub>H, LTA<sub>4</sub>S) involved in leukotriene biosynthesis have been well characterized with respect to their structural and functional properties and a recent review summarizes our current knowledge in this field [6]. The X-ray structures of cPLA<sub>2</sub>, ALOX5, LTA<sub>4</sub>H, and LTC<sub>4</sub>S provide a solid structural basis for a deeper understanding of the catalytic mechanisms and may serve as valuable tools for the development of specific inhibitors interfering with LT biosynthesis. ALOX5 is a key enzyme for LT biosynthesis because of several reasons: (i) It catalyzes two consecutive reactions (dioxygenation of AA to 5S-HpETE and epoxidation of 5S-HpETE to LTA<sub>4</sub>) in the biosynthetic cascade (Fig. 1). (ii) It competes with other AA metabolizing enzymes, such as other LOX- and COX-isoforms, for the fatty acid substrate and this competition is important for the equilibrium between of pro- vs. anti-inflammatory lipid mediators. (iii) It is the molecular target for zileuton (ZYLFO), a drug that is currently

used for treatment of bronchial asthma. (iv) It is also a target of different regulators of LT biosynthesis such as the ALOX5 activating protein [21].

### 3. Lipoxygenase isoforms

In humans two COX-isoforms [22] and six LOX-isoforms [23] have been described and the corresponding genes have all been sequenced. The ALOX5 gene is localized between 45.87 and 45.94 Mb on the long arm of chromosome 10. All other human LOX genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3) were mapped at different orientations to a joint gene cluster on the short arm of chromosome 17. In mice the lipoxygenase genes are located in syntenic regions on chromosome 6 (Alox5) and 11 (Alox15, Alox15b, Alox12, Alox12b, Alox3, Alox12). The biological roles of the different LOX-isoforms have been explored in the past but for some isoforms their functionalities are not clear. Since ALOX5 constitutes the key enzyme in leukotriene biosynthesis Alox5 deficient mice show reduced responsiveness for allergen-induced asthma [23]. In humans naturally occurring mutants in the ALOX5 gene were related to an increased risk for bronchial asthma [24] but the mechanistic details for the statistical association have not been explored. In addition, ALOX5 is expressed in diseased vessel walls [25] and in Alox5 deficient mice the formation of aortic aneurysms was markedly attenuated when the mice were bred into an apolipoprotein E-deficient background [26]. These data link the Alox5 pathway to inflammatory vessel wall diseases and to the pathogenesis of aortic aneurysms. Similarly, ALOX15B is expressed in human atherosclerotic lesions [27] and thus, this enzyme has also been related to the pathogenesis of atherosclerosis [28]. For other LOX-isoforms including ALOX15 only small amounts of the corresponding mRNA was detected [25]. ALOXE3 and ALOX12B have been implicated in epidermal differentiation. In fact, mice with targeted inactivation of these two genes die shortly after birth because of excessive epidermal dehydration [29,30]. In humans, loss-of-function mutations in the two genes have been implicated in the pathogenesis of ichthyosis, a rare skin disease [31]. ALOX12 [32] and ALOX15<sup>-/-</sup> [33] mice are viable and develop normally. However, blood platelets of ALOX12<sup>-/-</sup> mice show a defective aggregation behavior [32] and the skin of these animals has an increased water permeability [34]. ALOX15<sup>-/-</sup> mice are protected from diet induced vascular lipid deposition [35,36] suggesting a role of this LOX-isoform in atherosclerosis. ALOX15<sup>-/-</sup> mice develop more severe symptoms in different animal arthritis models [37] and corresponding data were obtained in a mouse glomerulonephritis model, in which expression of ALOX15 was enhanced by somatic gene transfer [38]. Moreover, ALOX15<sup>-/-</sup> mice have been suggested to suffer from myeloproliferative disorders [39] but this hypothesis has recently been challenged since symptoms do not consistently develop [40]. For ALOX15B<sup>-/-</sup> mice are not available but the expression profile of this enzyme in different cells and tissues suggests a role in normal epithelial differentiation and as a suppressor of prostate cancer development [41].

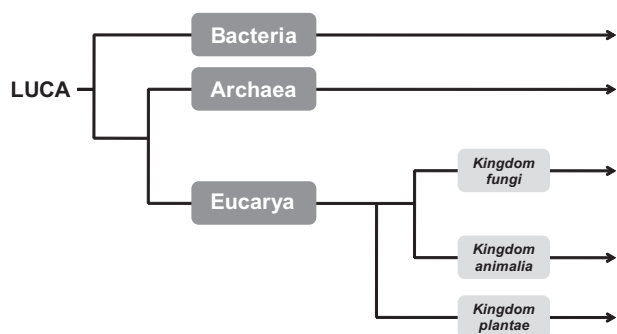
In biomedical research murine disease models are frequently employed to explore the pathomechanisms of human diseases and to test potential drugs. The basis for this strategy is that in many cases the human proteins and their corresponding murine orthologs share a high degree of structural and functional similarity. Unfortunately, in the LOX field there are pronounced species specific differences between mice and men: (i) There are six functional human LOX-isoforms but mice, rats and even some higher primates (chimpanzee, bonobo) have an additional LOX gene encoding for a seventh functional LOX-isoform (Alox12 in mice). In humans the ALOXE12 is corrupted to a functionless pseudogene so that no catalytically active ALOXE12 is expressed. (ii) Human

ALOX15 oxygenates arachidonic acid mainly to 15-HpETE and only small amounts of 12-HpETE are formed [42]. In contrast, mouse [33] and rat ALOX15 [43] orthologs are dominantly 12-lipoxygenating enzymes. The reaction specificity of LOX-isoforms is important for their bioactivity since a 15-lipoxygenating LOX-isoform is not capable of initiating biosynthesis of pro-inflammatory leukotrienes of the 5,6-series. The mechanistic basis for the different reaction specificities of ALOX15 orthologs has been explored (triad concept) and the critical amino acids have been determined [44]. It takes a single point mutation at the triad determinants (Ile418Ala or Phe353Ala) to convert the human 15-lipoxygenating ALOX15 into a 12-lipoxygenating enzyme. Applying this strategy human ALOX15 can be murinized and *vice versa* the murine enzyme can be humanized [44]. (iii) Human ALOX15B converts arachidonic acid almost exclusively to 15-HpETE [45]. In contrast, the mouse ortholog oxygenates the same fatty acid to 8S-HpETE. Mechanistic studies indicated that it simply requires the exchange of two critical amino acid residues to convert the 8-lipoxygenating mouse enzyme to a 15-lipoxygenating humanized isoform. The inverse mutagenesis strategy (murinizing the human enzyme) led to partial alteration of the reaction specificity [45].

## 4. Evolutionary aspects of lipoxygenases

### 4.1. Classification of living organisms

Life on earth is characterized by an enormous multiplicity and previous attempts to classify living organisms were mainly based on their phenotypic appearance. In the 18th century living organisms were classified either as vegetabilia or animalia but in the 19th century protista (microorganisms) were introduced as separate group. In 1925 an alternative concept was suggested which subclassified all living organisms into two alternative empires (prokarya, eucarya). The only classification criterion for this concept was the presence or absence of a morphologically clearly defined nucleus. In the early 1990s the three-domain concept of life was developed, which classifies all living species on earth mainly on the basis of the structure of their genes encoding for ribosomal RNA (rRNA) into three domains [46]: bacteria, archaea and eucarya (Fig. 2). Each of the three domains consists of several kingdoms. This classification system recognizes the fundamental divide between the two prokaryotic domains (archaea, bacteria) and also suggests that according to sequence comparisons archaea are more closely related to eucarya than to bacteria. Although the three-domain theory has been challenged, since sequence features and phylogenies of some highly conserved proteins are inconsistent with this concept [47,48], it is frequently used and widely accepted



**Fig. 2.** The three-domain concept of life. All living organisms on earth can be grouped according to the structure of their genes encoding for their ribosomal RNA in either of the three domains: bacteria, archaea and eucarya. Each of these domains consists of several kingdoms as shown for eucarya. However, there are alternative concepts for subclassification. LUCA, last universal common ancestor.

[49]. The eucarya domain [50] was later on subdivided into 6 different supergroups (Excavata, Amoebozoa, Opishokonta, Rhizaria Chromalveolata, Archaeplastida). This subclassification system deliberately did not use the formal taxonomic rank “kingdom”. However, there are alternative approaches to classify living organisms. In 1998 a six-kingdom model of living organisms [51,52] has been suggested and this concept was later on refined [50]. According to this classification living organisms should be grouped into 6 kingdoms (Bacteria, Protozoa, Chromista, Plantae, Fungi and Animalia) and this classification system does not accept the principle divide between bacteria and archaea. Here the kingdom Bacteria constitutes the sole kingdom of the prokaryotic empire and it consists of two subkingdoms, which are opposed according to their membrane topologies. The bimembranous–unimembranous transition has been suggested to be far more fundamental and the long branch of genetic distance between bacteria and archaea is viewed as having no particular biological relevance [53].

### 4.2. Lipoxygenases in viruses

Viruses are small infectious particles that replicate only inside living cells. On their own they are not viable and thus, they have not been considered for any of the classification concepts. However, some viruses persist outside living cells for long time periods. They infect all types of cells regardless whether the host cells originate from plants, animals, bacteria or archaea [54]. Viruses are found in almost every ecosystem on earth and they are the most abundant type of biological entity [55]. For this review we carried out a PubMed search with the key words “lipoxygenase and virus” and obtained some 180 hits. However, neither paper describes a true viral LOX. We next searched the genomes of important human pathogenic viruses (SARS corona virus, human immune deficiency virus, human hepatitis B virus, human hepatitis C virus, Herpes simplex virus type 1, small pocks virus, Polio virus, Dengue virus, Ebola virus, Lyssa virus, human papilloma virus, Influenza virus B, Mumps virus, Yellow fever virus, Vaccinia virus) for LOX genes but did not find any corresponding sequences. However, when we searched all viral genomes present in the NCBI database for LOX-like sequences we obtained five hits. Three of them were eliminated from further consideration since amino acid alignments with known LOXs from organisms of different evolutionary levels (*Pseudomonas aeruginosa*, *Plexaura homomalla*, *Danio rerio* and *Homo sapiens*) did not provide meaningful results suggesting that these sequences do not encode for functional LOX-isoforms. However, in the genomes of *Acanthamoeba Polyphaga Mimivirus* (YP\_003986553.1) and of *Hirudovirus* (strain Sangsue, AHA45815.1) LOX-like sequences were found. Next, we aligned on the protein level the *Acanthamoeba Polyphaga Mimivirus* (YP\_003986553.1) sequence with different well described LOXs such as human ALOX15 (NP\_001131), human ALOX5 (NP\_000689), zebrafish LOX (NP\_955912.1), *P. homomalla* 8R-LOX (AAC47283.1), *P. aeruginosa* LOX (AAL85880.2) and found a plausi-

#### iron cluster 1 (C1)

```

257 T L Y H L L L G H L M A E V I hALOX15
    T L Y H M R K T L A K T H P I Mimivirus
  
```

#### iron cluster 2 (C2)

```

537 T G Q H A S V H L G Q hALOX15
    S I R H S K N H I N V Mimivirus
  
```

**Fig. 3.** Partial amino acid alignment of the iron cluster regions of human ALOX15 and with the corresponding sequences of the *Acanthamoeba Polyphaga Mimivirus*. The one letter amino acid code is employed and the (potential) iron ligands are indicated in bold. Amino acid numbering for hALOX15.

ble amino acid line-up despite of low degrees of amino acid conservation (<20%). In the alignment with human ALOX15 four of the five proteinogenic iron ligands (His361, His540, H544, Ile663; numbering for human ALOX15) are strictly conserved (Fig. 3). His366 of human ALOX15 that constitutes the fifth proteinogenic iron ligand in the human enzyme, aligns with a Leu (no iron-coordination properties of the side chain) in the putative Mimivirus LOX. However, this Leu immediately trails a Thr and this amino acid with its free OH-group might function as iron ligand. Moreover, we found an additional His in iron cluster 1 of the Mimivirus sequence, which also might serve as iron ligand (Fig. 3). Thus, this viral sequence might indeed encode for a catalytically active LOX but to the best of our knowledge no functional data are currently available to prove this hypothesis. In a collaborative approach we recently cloned the cDNA of the putative LOX from the *Anthamoeba Polyphaga Mimivirus* and are now in the process of expressing and characterizing the corresponding enzyme.

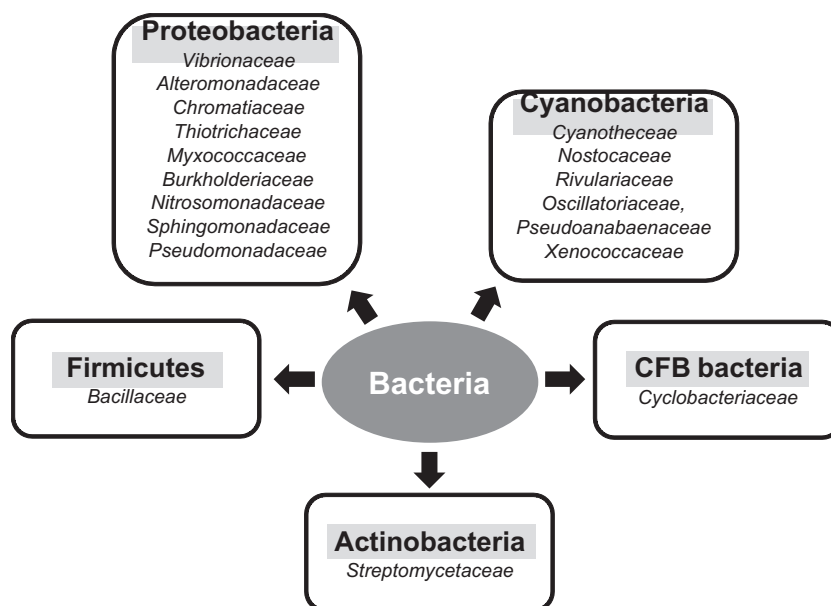
Virus infection impacts the LOX pathways of a number of host cells [56]. For instance, BS-C1 cells infected with cowpox or vaccinia virus exhibit enhanced 12- and 15-HETE production although the viruses do not contain any LOX genes. On the other hand, human parainfluenza type 3 or Herpes simplex virus type 1 infections did not impact the arachidonate metabolism. In other models of influenza infection lipidomic profiling demonstrated that ALOX5 metabolites correlated with the pathogenic phase of the infection, whereas ALOX15 metabolites were associated with the resolution phase [57]. Hydroxylated linoleic acid, specifically the ratio of 13- to 9-hydroxyoctadecadienoic acid, was identified as a potential biomarker for the immune status during an active infection and some of these findings were recapitulated when human nasopharyngeal lavage fluids obtained during the 2009–2011 influenza seasons were taken through the same protocol [57].

### 4.3. Lipoxygenases in bacteria

#### 4.3.1. Distribution of lipoxygenase sequences in bacteria

Bacteria form the most diverse domain of life on earth and based on genomic sequence comparisons a phylogeny-driven encyclopedia of bacteria has been established [58]. However, when it comes to LOXs this diversity is much less pronounced. In fact,

since the discovery of the first LOXs in plants [59] and mammals [60] it has been the common understanding for many years that LOXs do not occur in bacteria. This conclusion was supported by the observation that the genome of *Escherichia coli*, which serves as major bacterial model organism, does not contain LOX-like sequences. On the other hand, there were scattered reports on the expression of LOXs in certain bacteria [61,62] but the increasing availability of bacterial genomes put recently the search for bacterial LOXs on a more systematic basis. Today a number of catalytically active LOX-isoforms have been described in bacteria but the biological roles of them remain largely elusive [9,63–67]. To obtain more detailed information on the distribution of LOX-sequences in bacteria we searched several sequence databases (Integrated Microbial Genomes, National Center for Biotechnology Information, Uniprot, Ensembl and GenBank) for potential LOX sequences in bacteria. Unfortunately, there is no strongly conserved nucleotide sequence shared by all LOXs, and thus false positive and/or false negative hits are likely. When we searched the bacterial genomes sequenced so far (~13,000) some 60 LOX-like sequences were identified and these bacterial species include firmicutes, different types of proteobacteria, cyanobacteria, actinobacteria and representatives of the CFB group (Fig. 4). Although the vast majority of these potential LOX genes have not been characterized with respect to their functionality the observation that less than 0.5% of all bacterial genomes sequenced so far contain potential LOX genes suggest that these enzymes only sporadically occur in bacteria and thus, may not be essential for bacterial physiology. In fact, most human pathogenic bacteria (Table 1) do not carry LOX genes so that for many specific bacterial infections pathogen LOXs may not play a major role for disease development. However, host LOX may well contribute to disease progression or to the immune response. On the other hand, the opportunistic pathogen *P. aeruginosa* carries several well-defined LOX alleles and thus, the corresponding enzymes might be relevant for the pathogenicity of this microorganism [9,63–67]. In cyanobacteria LOX sequences occur with a similarly low frequency even though these organisms are capable of producing oxygen, which is required as second substrate for the LOX reaction substrate. Bacteria living in anaerobic environments do obviously not contain LOX sequences.



**Fig. 4.** Bacterial families carrying LOX-like sequences. Bacteria can be subdivided into four major subfamilies [cyanobacteria, proteobacteria, firmicutes, CFB group (Chlorobi, Fibrobacteres, Bacteroides)] and in each of the four subfamilies LOX positive species have been identified. Examples for LOX-positive bacteria are given in italic letters.

**Table 1**  
Selected human pathogenic bacteria that do not contain LOX-like genes.

Classification	Human pathogen	Infectious diseases
Gram-positive	<i>Staphylococcus aureus</i>	Skin infections, pneumonia, sepsis
	<i>Streptococcus pneumoniae</i>	Pneumonia, meningitis, sinusitis, otitis
	<i>Streptococcus pyogenes</i>	Skin infections, tonsillitis, scarlet fever
	<i>Enterococcus faecium</i>	Urinary tract infections (UTI), sepsis
	<i>Bacillus anthracis</i>	Anthrax
	<i>Bacillus cereus</i>	Food poisoning
	<i>Clostridium perfringens</i>	Gas gangrene
	<i>Clostridium tetani</i>	Tetanus
	<i>Corynebacterium diphtheriae</i>	Diphtheria
	<i>Listeria monocytogenes</i>	Listeriosis
Gram-negative	<i>Klebsiella pneumoniae</i>	Pneumonia, UTI, sepsis
	<i>Vibrio cholerae</i>	Severe diarrhea
	<i>Salmonella typhi</i>	Typhus
	<i>Haemophilus influenzae</i>	Bronchitis, sinusitis, meningitis
	<i>Neisseria gonorrhoeae</i>	Urethritis ("gonorrhoea")
	<i>Brucella melitensis/abortus</i>	Brucellosis
	<i>Bordetella pertussis</i>	Pertussis
	<i>Francisella tularensis</i>	Tularemia
	<i>Helicobacter pylori</i>	Gastric ulcer
	<i>Legionella pneumophila</i>	Legionnaires' disease
Spirochetes	<i>Treponema pallidum</i>	Syphilis
	<i>Borrelia burgdorferi</i>	Lyme disease
	<i>Leptospira interrogans</i>	Weil's disease
Others	<i>Mycobacterium tuberculosis</i>	Tuberculosis
	<i>Mycobacterium leprae</i>	Lepra
	<i>Mycoplasma pneumoniae</i>	Pneumonia

#### 4.3.2. Properties, structures and biological function of bacterial lipoxygenases

Most bacterial LOXs characterized so far are arachidonate 15-lipoxygenating enzymes [9]. However, the LOX from the cyanobacteria *Nostoc sp. PCC 7120* [68] oxygenates linoleic acid preferentially to 9R-HpODE whereas the enzyme from *Anabaena sp.* oxygenates different fatty acids with variable specificity [69]. Linoleic acid is converted by the secretable *P. aeruginosa* LOX [64] almost exclusively to 13S-HpODE [70] and a similar product specificity was reported for two LOX-isoforms from *Nostoc punctiforme* [65]. LOXs in lower organisms occasionally occur as fusion proteins combining different catalytic activities within one protein. For instance, the cyanobacterium *Acaryochloris marina* expresses a fusion protein consisting of an N-terminal heme domain with sequence homology to catalase and a C-terminal LOX domain [71]. When the LOX-domain was separately expressed in *E. coli* it oxygenated omega-3 fatty acids at the n-7 position with R stereospecificity.  $\alpha$ -Linolenic and (C18:9,12,15 $\Delta$  $\omega$ 3) and stearidonic acid (C18:6,9,12,15 $\Delta$ : $\omega$ 3), are converted to the corresponding 12R-hydroperoxides, eicosapentaenoic acid (C20:5,8,11,14,17 $\Delta$ : $\omega$ 3) to its 14R-hydroperoxide and docosahexaenoic acid (C22:4,7,10,13,13,19  $\Delta$ : $\omega$ 3) to its 16R-hydroperoxide. In contrast, omega-6 polyunsaturated fatty acids were oxygenated at the n-10 position [72]. Metabolic transformation of stearidonic acid (C18:6,9,12,15 $\Delta$ : $\omega$ 3) by the full length fusion protein leads to the formation of an allene epoxide and the catalase-domain appears to be responsible for the secondary transformation of the primary 12R-oxygenation product [71].

LOXs of higher plants [73–75] and humans [12,76–79] consist of two domains. The C-terminal domain, which is mainly composed of  $\alpha$ -helices, harbors the substrate-binding pocket with catalytic non-heme iron ion. The N-terminal  $\beta$ -barrel domain may be

of regulatory importance [44]. Truncation studies on various LOX-isoforms suggested that the N-terminal  $\beta$ -barrel domains may not be essential for the catalytic activity of plant [80] and mammalian LOXs [81]. Corresponding experiments were also carried out with selected bacterial LOXs. The mini-LOX from *Anabaena sp.* [66], the mini-9R-LOX from *Nostoc sp. PCC7120* [68] and the mini-LOX from *Cyanotheca sp.* [82] exhibit full catalytic functionality. Beside from these data the structural biology of bacterial LOXs is not well advanced. The only crystal structure solved for a bacterial LOXs is that for the secretable *P. aeruginosa* LOX [67]. There is no two-domain structure for this enzyme but a cluster of helices might function as lid for the entrance of the substrate into the active center. The mobility of this lid and the structural flexibility of the entire N-terminal region appear to allow substrate penetration into the active site [67].

Although our knowledge on the enzymology of bacterial LOXs is currently increasing little is known about their biological function. Polyenoic fatty acids are the natural substrates of LOXs, which can be converted to lipid mediators such as eicosanoids [83] or jasmonic acid [84] as it is the case in mammals and plants. Similar roles might be postulated for bacteria containing such lipids. However, some LOX-containing bacteria such as *P. aeruginosa* are devoid of polyenoic fatty acids. For these bacteria it was proposed that their LOXs might modulate host defense and inflammation during bacterial infection [64,67]. Another interesting hypothesis for the biological role of bacterial LOXs suggests that the plasticity observed in bacteria harboring LOXs may have provided a selective advantage for adaptation to different ecological niches [9] but there is little experimental evidence for this hypothesis. Occurrence of LOX genes in photosynthesizing cyanobacteria is of particular evolutionary interest. Ancient cyanobacteria largely contributed to converting the reducing ancient atmosphere of our planet into an oxidizing environment by producing oxygen gas as by-product of photosynthesis. The enrichment of oxygen gas in the atmosphere, which started 650 million years ago (Mya), was of dual consequence: (i) It allowed oxygenation reactions, in which atmospheric oxygen is used as electron acceptor. (ii) It enabled oxidative energy metabolism, which is by at least one order of magnitude more efficient than reductive ATP biosynthesis. The oxidative energy metabolism has been suggested as major driving force of Cambrian species explosion, which dramatically increased the diversity of life on earth but also led to near-extinction of anaerobic organisms [85]. According to the endosymbiotic theory the chloroplasts found in photosynthesizing plants and algae have evolved from cyanobacterial ancestors via endosymbiosis [86]. LOXs in cyanobacteria have been implicated in the metabolism of chloroplasts, the major photosynthesizing organelles [65]. Indeed, chloroplast membranes are rich in polyenoic fatty acids making them susceptible for LOX attack. Unfortunately, there is a lack of experimental confirmation of this hypothesis.

#### 4.3.3. Evolution of bacterial lipoxygenases

The increasing number of completely sequenced bacterial genomes allows comparative studies to explore the evolutionary relatedness of bacterial LOX-isoforms between different species [9]. Employing parsimony and distance methodology algorithms phylogenetic trees of LOX evolution can be constructed. The major disadvantage of such comparative studies is the limited number of bacterial LOX sequences, which have been confirmed to encode for functional LOXs. As indicated above only 0.5% of today's sequenced bacterial genomes contain LOX-like sequences and for only a few of them functional data are currently available. However, comparison of true pro- and eucaryotic LOX sequences suggested that red algae LOXs were more closely related to human isoforms [9]. Moreover, these sequence comparisons suggested

that introduction of LOX into *P. aeruginosa*, *Sorangium cellulosum* and *N. punctiforme* were not the results of a continuous evolution but rather of horizontal gene transfer events [9,63,65]. However, it remains unclear why these bacterial species, but not others, were susceptible for horizontal LOX gene transfer and why the transferred sequences were retained during further development. Is there any evolutionary advantage LOX expressing strains of *P. aeruginosa* have gained over strains, which do not carry LOX genes? On the other hand, it may well be that other bacterial species might also have picked up LOX sequences but since these enzymes did not confer an evolutionary advantage the sequences were gradually disrupted during further development. Since LOX genes from *N. punctiforme* share a high degree of homology with the *P. aeruginosa* enzymes and were closely related to LOX-like genes of nitrifying bacteria a role of these enzymes in nitrogen metabolism has been suggested [65]. However, it remains unclear whether these LOX-like sequences really encode for functional LOX-isoforms and how oxygen-metabolizing enzymes might be involved in nitrogen metabolism.

All attempts to portray a complete picture of bacterial LOX evolution suffer from two major difficulties: (i) there is only scattered sequence information on functionally well-characterized bacterial LOX-isoforms. For most genomic LOX-like sequences it remains to be shown whether or not they encode for true LOX enzymes. (ii) The methodology (neighbor joining) used for defining the evolutionary relatedness of LOX-isoforms has clear limitations. Since evolution of bacterial LOXs appears to be a complex interplay of horizontal gene transfer and lineage-specific loss amongst different bacterial families, combining the outcome of the neighbor joining methodology with other sequence- and structure-based approaches may yield a more realistic picture. Nevertheless, on the basis of the currently available sequence data bacterial LOXs are an ancient entity, which has been constantly influenced by different kinds of purifying selection pressures [9,87]. Although, they form a joint enzyme family [9] three subfamilies (group 1: cyanobacteria LOXs, group 2A: proteobacteria LOXs, subgroup A, group 2B: proteobacteria LOXs, subgroup B) can be differentiated according to their phylogenetic relatedness. This classification underlines the fact that cyanobacterial LOXs are structurally somewhat different when compared with other bacterial LOXs.

#### 4.4. Lipoxygenases in archaea

As bacteria archaea are prokaryotes that have unique properties separating them from the representatives of other domains of life [88]. Despite the visual similarity to bacteria, archaea possess metabolic pathways that are more closely related to eucarya [89] and both domains appear to share a common ancestor (Fig. 2). Archaea are capable of employing a large diversity of energy sources including sugars, ammonia and hydrogen gas and some archaea are even capable of employing sunlight for energy metabolism. Originally, archaea were viewed as extremophiles living in harsh environments but they have also been found in more moderate biotopes including the human gastro-intestinal tract [88]. Archaea are widely distributed in the oceans and are considered to be one of the most abundant groups of organisms on the planet. Unfortunately, little is known about the occurrence of LOX in archaea.

When we searched the genomic databases of archaea we found LOX-like sequences for *Methanococcus jannaschii* (*M. jannaschii*), *Methanococcus voltae* (*M. voltae*) and *Halorubrum kocurii* (*H. kocurii*). *M. voltae* [90] is a methanogenic microorganism capable of reducing carbon dioxide to methane and employing this process as energy source. Methanogenes are widely distributed in nature and occur in wetlands (marsh gas production), in hot springs and hydrothermal vents. They are also found in the digestive tract of ruminants and humans where they are responsible for

the methane formation. When we attempted to carry out amino acid alignments of the *M. voltae* LOX-like sequence with plant, mammalian and bacterial LOXs we did not obtain meaningful data. In particular, the iron ligands (His, Asn, Ile) of the well characterized LOXs were not conserved in the *M. voltae* sequence. Moreover, *M. voltae* lives under anaerobic conditions and thus, it remains unclear why it should have an oxygen consuming enzyme. *M. jannaschii* was discovered in the East Pacific at a white smoker. It was the first archaea species whose genome was completed and the sequence data revealed eucaryotic elements. *H. kocurii* are rod-shaped gram-negative, halophilic archae discovered in salt lakes. Some strains are able to grow at 25–55 °C but required at least 2.5 M NaCl for growth. Hypotonic treatment with less than 2 M NaCl caused cell lysis [91]. Here again, when we performed multiple amino acid alignments no meaningful information could be obtained and LOX iron ligands were not conserved. Taken together, these data suggest that archaea do not have true LOX genes but this conclusion might turn out wrong in the future when more archaea genomes become available.

#### 4.5. Lipoxygenases in eucarya

##### 4.5.1. Lipoxygenases in algae

Algae form a large and diverse group of simple organisms, which occur in unicellular or multicellular forms. Since algae do not include a common ancestor they do not constitute a homogeneous phylogenetic group and there is no generally accepted definition of algae [92]. They exhibit a high degree of morphological diversity and a wide range of reproductive strategies ranging from simple asexual cell division to complex forms of sexual reproduction. Most algae are phototroph but some of them became heterotroph. Fossilized algae have been dated back to 1.6–1.7 billion years ago (Bya). Algae are considered as primary producers of polyunsaturated fatty acids [93]. Because of this metabolic property the occurrence of polyenoic fatty acid metabolizing enzymes such as LOX is likely. In fact, there is active oxylipin biosynthesis in algae [94,95] but for a long time little was known about the presence of true LOX sequences in these organisms. This was somewhat surprising since functional LOXs have been described in cyanobacteria (see Section 4.3), which are commonly considered as prokaryotic algae. In 1994 the first LOX sequences has been detected in red algae [96] and these data indicated that LOX in principle occur in these organisms. When we searched the NCBI databases for putative LOX sequences in algae we got five hits for *Rhodophyta* (AFQ59981.1, AAA61791.1, XP\_005718273.1, AGN54275.1, AFQ59981.19), four hits for *Chlorophyta* (GI:159464351, XP\_005643943.1, XP\_005643190.1, XP\_005853347.1), four hits for *Phaeophyta* (CBN79157.1, CBN79156.1, CBJ33123.1, CBJ33122.1) and one hit for *Diatoms* (DQ658366.1). However, multiple amino acid alignments suggested a low degree of homology with functional LOXs from other species and in most of these sequences the iron ligands were not conserved. Thus, although a true LOX has been detected in algae [96] these enzymes do not generally occur in these lower organisms.

##### 4.5.2. Lipoxygenases in yeast and other fungi

Yeasts are single-cell microorganisms that are classified, along with molds and mushrooms, as members of the fungi kingdom (Fig. 2). They are evolutionary diverse and classified on the basis of their cellular characteristics as well as their cellular physiology into two separate phyla [97]: *Ascomycota* (sac fungi) and *Basidiomycota* (higher fungi). Budding yeasts, which are also referred to as “true yeasts”, are members of the phylum *Ascomycota*. The most famous yeast species is *Saccharomyces cerevisiae*. For thousands of years it has been employed for baking and alcohol production [98]. It has been selected as eucaryotic model organism in today's cell



biology research. Other yeast species as *Candida albicans* are opportunistic pathogens and can cause infections in immunocompromised humans [99].

On the metabolite level hydroxylated polyenoic fatty acids are present in many fungi [100] and this finding suggested a wide distribution of LOXs. However, since oxylipins can be biosynthesized via different pathways, such as LOXs, fatty acid dioxygenases, diol synthases, peroxygenases, and cytochrome P450 hydroxylases etc., the presence of oxylipins does not prove the existence of LOXs. When we searched the databases for LOX-like sequences in fungi we obtained some 40 hits, which include LOX sequences in *Aspergillus fumigatus* (XP\_746463.1), *Aspergillus oryzae* (XP\_001822165.2), *Colletotrichum orbiculare* (ENH86155.1), *Magnaporthe oryzae* (XP\_003716006.1), *Gibberella moniliformis* (AY699875.1) and *Neurospora crassa* (EAA28941.2). Unfortunately, for most of these sequences no functional data are currently available and although some of these sequences look promising more detailed studies are required to test whether these genes encode for functional LOXs. Interestingly, the genomes of *S. cerevisiae* and *C. albicans* do not involve any LOX-like sequences.

Although LOXs also occur in several fungi their biological roles are not well understood. They have been implicated in the formation of oxylipins but the bioactivity of these lipid mediators is rather diverse and varies from species to species [101,102]. They have been implicated in sporulation, regulate the biosynthesis of mycotoxins and aflatoxins and in the formation of sclerotia [103]. In the 1970s a heme containing fatty acid dioxygenase was characterized in *Fusarium oxysporum* [104,105]. Although this enzyme catalyzes the oxygenation of polyunsaturated fatty acids to the corresponding hydroperoxides it may not qualify as true LOX because of its structural characteristics. On the other hand, LOX activities have been detected in other fungal species, such as *Saprolegnia parasitica* [106], *Thermomyces lanuginosus* [107], *Pleurotus ostreatus* [108], *Terfezia clavaryi* [109] and *Aspergillus flavus* [110]. Recently, a non-heme iron containing LOX has been characterized in *F. oxysporum* [111]. The best-characterized fungal LOX is the manganese-containing LOX from *Gaeumannomyces graminis*. This enzyme is distinct from most other LOX isoforms since it contains catalytically active manganese instead of iron [112,113]. The product patterns of Mn- and Fe-containing LOXs are somewhat different and so is the oxygenation mechanism [113]. For iron containing LOXs an antarafacial relation between hydrogen abstraction and oxygen insertion was observed whereas a suprafacial relation was shown for the manganese enzymes [112]. As indicated above the LOX pathway is not the only metabolic route leading to the formation of oxylipins in fungi. There are alternative enzymes such as linoleate diol synthases [114,115], which contribute to oxylipin biosynthesis via different reaction mechanisms [101,102].

#### 4.5.3. Lipoxygenases in mosses and higher plants

In contrast to mammals, in which LOXs are categorized according to their reaction specificity with arachidonic acid, a linoleic acid based classification scheme has been suggested for the plant enzymes [116,117]. The oxylipins formed via the LOX pathway function as signaling molecules and play important roles in a number of biological processes such as stress response [118], growth and development [119], germination [120], tuber formation [121], sex determination [122], senescence [123], fruit ripening [124], and aroma production [125].

Mosses (bryophyta) are small non-vascular plants. They absorb water and nutrients mainly through their leaves and produce organic material from carbon dioxide and water via photosynthesis. They differ from vascular plants in lacking water-bearing vessels. Mosses reproduce using spores (no seeds) and have no flowers. Little is known about LOX pathways in mosses. However,

after the genome of *Physcomitrella patens* (*P. patens*) was published and this species became a model organism for mosses [126,127] more detailed information on LOXs in mosses became available. The *P. patens* genome involves several LOX-like sequences and true LOX enzymes have been characterized in more detail [128,129]. This data indicate that mosses have both C20 and C18 based oxylipin biosynthesizing pathways. Meanwhile the genomes of other mosses have been sequenced and the NCBI protein database involves six entries for moss LOX-like sequences. However, no functional data are currently available for these putative LOX isoforms.

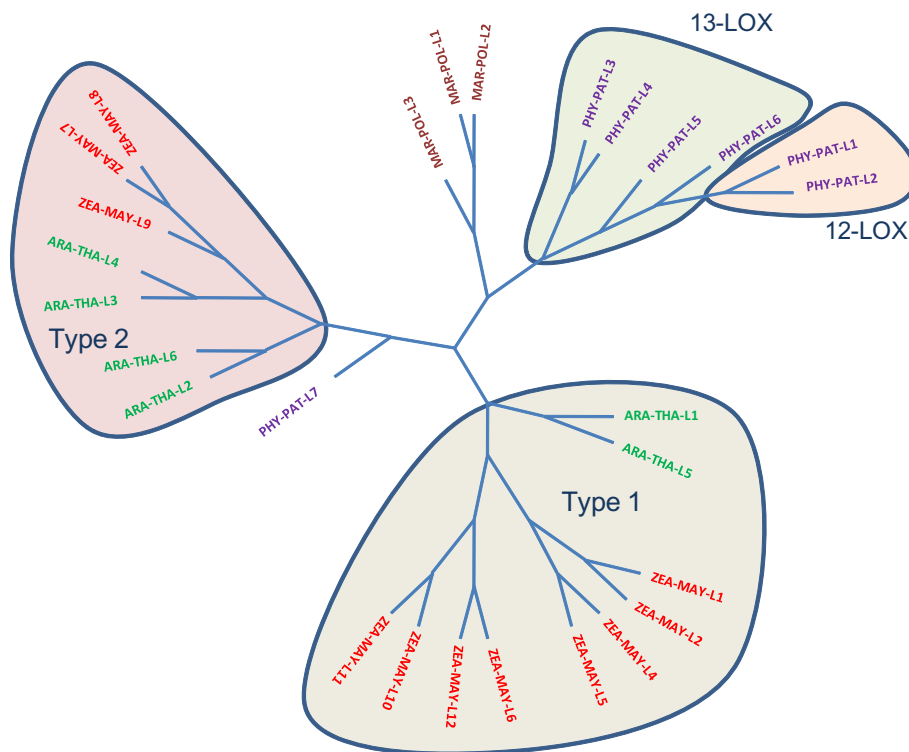
In higher plants LOXs are widely distributed [116,117,130]. Because of the availability of the complete genome sequences of *Arabidopsis thaliana*, soybean, rice, wheat, maize and others the isoform pattern and the biological functions of LOX pathways in plants have extensively been studied. Soybean LOX1 was the first LOX described in the mid-thirties of the past century [59] and since then an increasing number of LOX isoforms have been identified. In general, plants show remarkable heterogeneities of their LOX families. In humans six functional LOX genes have been described which encode for six different LOX isoforms [23]. In contrast, many plant genomes contain much more LOX sequences. For instance, completion of the cucumber genome identified more than 20 candidate LOX genes [131]. Polyploidy and frequent gene duplications have contributed to the expansion of plant LOX genes. More detailed analysis of the LOX sequences found in the genomes of *Glycine max* and *Medicago truncatula* indicated 34 LOX genes, which were localized in six different gene clusters [132]. Structural comparison of these sequences suggested that the soybean LOX gene family expanded by ancient polyploidy prior to taxon divergence, followed by soybean-specific duplication. Because of the high degree of heterogeneity of plant LOX sequences classification of the corresponding enzymes is rather difficult. Nevertheless, based on selected structural parameters plant LOXs have been categorized into type-1 and type-2 enzymes [132]. Type-1 LOX share a high degree of amino acid sequence similarity (>75%) and do not have a plastidic transit peptide. Type-2 LOX have a putative chloroplast transit peptide sequence and show only moderate overall amino acid sequence similarities (~35%).

For this review we performed *in silico* comparison of LOX sequences from plants representing different evolutionary stages (liverworts, mosses, monocots and dicots). This phylogenetic analysis (Fig. 5) indicated evolutionary divergence of LOXs from higher and lower plants. In lower plants, the LOXs from liverworts and mosses formed distinct classes. In mosses, 12-LOX evolved from the 13-LOX suggesting a common ancestor. Higher plant LOXs were grouped into two distinct clusters representing the type-1 and type-2 classes. This data suggest that type 1 and type 2 LOX from monocots and dicots evolved separately confirming the previously suggested LOX classification in higher plants [133,134].

#### 4.5.4. Lipoxygenases in animals

Protozoa and metazoa form the animal kingdom (Fig. 2) of life. This simple binary split of animal classification does not follow the rules of scientific taxonomy, which categorizes living species in a hierarchical order into kingdoms, phyla, classes, orders, families, species and subspecies. However, this binary split is simple and rather useful when it comes to explore the distribution of LOXs in the animal kingdom.

**4.5.4.1. Lipoxygenases in protozoa.** Protozoa are eucaryotic organisms containing non-filamentous structures and mainly occur in aquatic habitats. There are an estimated 30,000 protozoan species on earth and many of them live as symbionts or parasites. Most protozoa are motile since they carry moving organelles such as flagella or cilia. Alternatively, they are capable of moving by the for-



**Fig. 5.** Phylogenetic tree of plant lipoxygenases. For this analysis LOX sequences from four plant species with different evolutionary levels were selected: Eudicots – *Arabidopsis thaliana* (ARA-THA), Monocots – *Zea mays* (ZEA-MAY), mosses – *Physcomitrella patens* (PHY-POL), club-mosses *Selaginella moellendorffii* – SEL-MOE. The phylogenetic tree was constructed by the neighbor-joining method in MEGA 4.0 with bootstrap values estimated based on 5000 replications.

mation of pseudopodia. According to their way of movement protozoa can be subclassified into *Sporozoa* (no active movement), *Ciliata*, (cilia as moving organelles), *Flagellata* (flagella as moving organelles) and *Rhizopoda* (formation of pseudopodia). When we searched the NCBI database for LOX-like sequences we did not get any hits for Sporozoa. For Ciliata we extracted two candidate sequences for *Oxytricha* (EJY76823, EJY73499). Next, we screened these sequences for additional structural parameters to increase the probability that they encode for functional LOX isoforms. For this purpose we employed a three step filtering strategy: (i) we excluded all sequences, which encode for proteins consisting of <300 amino acids. Since the two sequences encoded for 813 and 825 amino acid proteins this filtering step did not exclude either of the proteins. (ii) We searched the two sequences for the presence of the iron coordinating amino acids and excluded those sequences lacking one or both iron ligand clusters. Most functional LOX carry two iron ligand clusters [C1, **His**-w-x-y-z-**His**; C2, **His**-x-y-z-**Asn** (**His**, **Ser**, **Thr**)] and each cluster contains two coordinating amino acids (bold face) for the catalytic nonheme iron. In the two *Oxytricha* sequences both iron coordinating clusters (C1, C2) were found, which did not eliminate either sequence. (iii) For most well-characterized LOX isoforms the distance of the two iron clusters varies between 180 and 200 amino acids. Longer and shorter distances decrease the probability that the corresponding sequences encode functional LOX isoforms. For the two *Oxytricha* sequences a distance of 200 amino acids was calculated. Summarizing these data one may conclude that these two sequences might encode for functional LOXs. Searching the *Flagellata* database we obtained two putative LOX sequences but both of them were excluded from further consideration because of their small size (142 and 100 amino acids). Thus, there is probably no real LOX in *Flagellata*. In the *Rhizopoda* database we found a promising sequence (ETO32332, *Foraminifera*) encoding for a 393 amino acid protein. In this sequence we detected the two iron coordinating

clusters C1 and C2 but they were only 95 amino acids apart. This small distance and the low molecular weight suggested that there are no real LOXs in *Rhizopoda*.

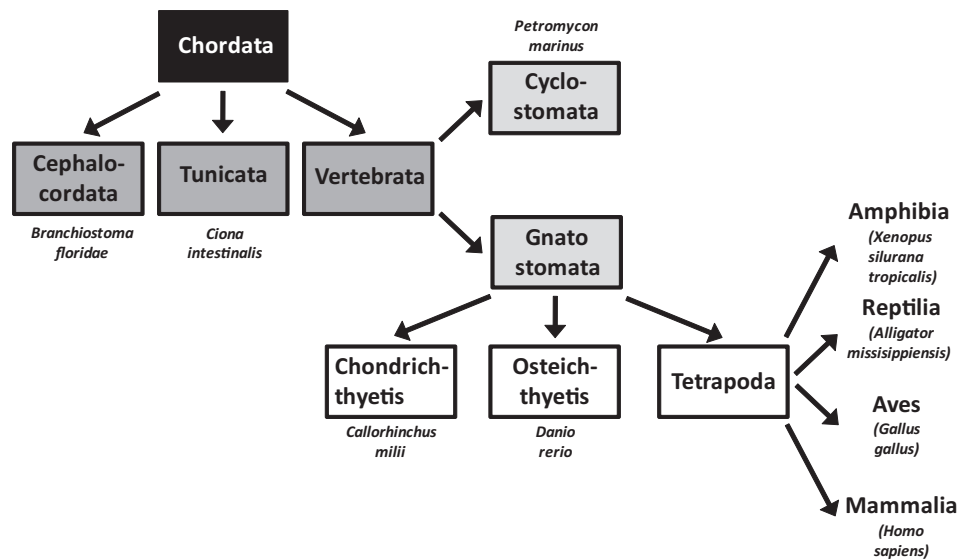
Taken together one may conclude that for the time being there is no compelling evidence for the occurrence of true LOXs in protozoa. Even if future will reveal that the two *Oxytricha* sequences encode for true LOXs these enzymes are not widely distributed in Protozoa.

**4.5.4.2. LOX in invertebrates.** Metazoa can be subclassified as vertebrates or invertebrates according to the presence or absence of a vertebral column. This bilateral classification is rather descriptive and does not consider evolutionary relatedness. However, it is straightforward and very useful when searching for the distribution of certain proteins in different species. The overwhelming majority (95–98%) of animal species on earth are invertebrates and many individual invertebrate subcategories have a greater number of species than all vertebrates together. Morphologically, invertebrates are extremely diverse and they do not have a bony skeleton. Many of them (jelly fish, worms) have soft hydrostatic skeletons but others have hard exoskeletons. Invertebrates include multiple groups of living organisms such as insect, crab, lobster, snail, clam, octopus, starfish, sea urchin, worm and sponge. Functional LOX isoforms from various invertebrates have been characterized as fusion or nonfusion proteins in different corals [135–140], in starfish oocytes [141], in the eggs of a sea urchin [142] and in the ovaries and oocytes of different mussels (*Mytilus edulis*, *Spisula solidissima*) [143,144]. Recently the distribution of LOX isoforms in 14 different invertebrate species was explored [145] and the authors found putative LOX-like sequences in 10 of them (*Ciona intestinalis*, *Saccoglossus kowalevski*, *Strongylocentrotus purpuratus*, *Crassostrea gigas*, *Lottia gigantea*, *Capitella teleta*, *Helobdella robusta*, *Acropora digitifera*, *Nematostella vectensis*, *Trichoplax adhaerens*). Moreover, there is evidence for functional LOXs in *Daphnia* subspecies [146]

**Table 2**

Lipoxygenase-like sequences obtained with our two-step searching procedure for metazoa and lower invertebrates. The size of the predicted proteins is given by the number of amino acids. Clusters 1 and 2 represent the iron ligand clusters that are explained in the text.  $\Delta(C1-C2)$  quantifies the distance of the two iron ligand clusters counting the number of amino acids. In the LOX column we estimated the probability that the corresponding sequence encodes for a functional LOX (+, high probability; –, low probability). The criteria of our estimates are the following: + (size > 600 amino acids, presence of C1 and C2 in correct order,  $180 < \Delta C1-C2 < 200$ ), – (absence of either C1, C2 or both or presence of C1 and C2 but  $\Delta C1-C2 < 180$ ). \*Not reported in Yuan et al. (2014).

Species	Acc. number	size	Cluster1	Cluster2	$\Delta(C1-C2)$	LOX
<i>Lottia gigantea</i>	ESP02741	671	+	+	193	+
<i>Helobdella robusta</i>	ESN98770	665	+	+	193	+
<i>Nematostella vectensis</i>	XP_001634357	642	+	+	188	+
<i>Amphimedon queenslandica</i> *	XP_003383419	762	+	+	192	+
<i>Hydra vulgaris</i> *	XP_002157326	623	+	+	184	+
<i>Strongylocentrotus purpuratus</i>	XP_782245	691	+	+	195	+
<i>Ixodes scapularis</i> *	XP_002407642	358	+	+	97	–
<i>Saccoglossus kowalevskii</i>	XP_006820345	664	+	+	193	+
<i>Crassostrea gigas</i>	EKC38941	663	+	+	196	+
<i>Echinococcus granulosus</i> *	CDJ23913	745	–	+		–
<i>Schistosoma mansoni</i> *	CCD75342	463	+	–		–
<i>Plexaura homomalla</i> *	AAC47283	715	+	+	187	+
<i>Gersemia fruticosa</i> *	AA98506	679	+	+	189	+
<i>Trichoplax adhaerens</i>	EDV27172	645	+	+	192	+
<i>Capitella teleta</i>	ELT89495	659	+	+	192	+



**Fig. 6.** Schematic view of subclassification of Chordata. Model organisms, which are referred to in this study are indicated in italics.

but no LOX-like sequences were found in *Drosophila melanogaster*, *Caenorhabditis elegans* which constitute frequently employed model organisms for invertebrates. When we searched ENSEMBL (<http://metazoa.ensembl.org>) and the NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) protein databases for Placozoa, Porifera, Coelenterata, Platyhelminthes, Nematoda, Mollusca, Annelida, Echinodermata, Chelicerata, Cephalochordata, Tunicata with key word “lipoxygenase” we confirmed the occurrence of LOX-like sequences in these invertebrates but got additional hits. Filtering the obtained sequences with the three-step strategy described for protozoa (search for iron coordinating clusters) we reduced the multiplicity of hits. The results of this screening strategy are shown in Table 2 and can be summarized as follows: (i) Functional LOX isoforms occur in selected invertebrates and this conclusion is consistent with previous characterization of several enzymes in corals, mussels, sea urchins and others. In many of these organisms several putative LOX sequences can be identified but for Table 2 only one example was selected. (ii) Most invertebrates do not have functional LOX genes. Of the estimated more than one million invertebrate species on earth less than 1% carries LOX-like sequences. Of course, only for a minor share of all invertebrates complete genome data are currently available and it

is rather likely that more invertebrate LOX-like sequences will be identified with increasing numbers of completely sequenced invertebrate genomes.

**4.5.4.3. Lipoxygenases in Chordata.** Chordates are animals possessing a notochord, which is a hollow dorsal neural organ. Taxonomically, chordates include vertebrata, tunicata and lancelets (Fig. 6). Primitive chordates are known from at least as early as the Cambrian explosion. Today almost 60,000 different chordate species are living on this planet and the world’s largest (blue whale) and fastest (peregrine falcon) animals belong to this phylum. Almost half of the chordate species are bony fish.

**4.5.4.3.1. Lipoxygenases in Cephalochordata and Tunicata.** Lancelets (*Branchiostoma floridae*, *Branchiostoma belcheri*) are model organisms for Cephalochordata and they have separated from the vertebrate lineage about 520 Mya [147]. Analysis of the genome of *B. belcheri* indicated the presence of putative LOX sequences (BbtC2LOX1 and BbtLH2LOX2; NCBI accession numbers KJ196391 and KJ196392, respectively). However, the iron ligand clusters of mammalian LOXs are not conserved in the BbtC2LOX1 sequence suggesting that the corresponding protein may not constitute a

functional LOX isoform. Searching the *B. floridae* genome we also found LOX-like genes (XP002601197.1, XP002606132.1), in which the iron ligands of mammalian enzymes are conserved. Phylogenetic analysis suggested a close evolutionary relation to the allene oxide synthase-lipoxygenase fusion proteins of lower marine organisms [136]. Unfortunately, to the best of our knowledge there are no functional data available demonstrating the LOX activity of these enzymes.

*C. intestinalis* (sea squirt) serves as a model organism for *Tunicata* and seven LOX-like sequences have recently been identified in this animal [145]. When we searched the NCBI and the ENSEMBL protein databases for LOX-like sequences we found three entries (XP\_002123813, XP\_002123202, XP\_002123695). When we took these sequences through our filtering protocol we found that in the XP\_002123813 sequence only the C1 iron cluster was present. Since we did not find a C2 cluster the functionality of this putative arachidonate 5-lipoxygenase-like protein is questionable. The XP\_002123202 (predicted allene oxide synthase-lipoxygenase fusion protein, 766 amino acids) and XP\_002123695 (arachidonate 5-lipoxygenase-like protein, 694 amino acids) sequences involve both iron clusters and the distances between them ( $\Delta$ C1-C2) were 186 and 188 amino acids, respectively. Considering these structural characteristics it may well be, that these sequences encode for functional LOX isoforms.

**4.5.4.3.2. Lipoxygenases in primitive Vertebrata.** Vertebrata can be subclassified into *Cyclostomata* and *Gnathostomata* (Fig. 6) and their evolution started about 550 Mya during the Cambrian species explosion [148–152]. The most primitive vertebrates (hagfish, lampreys) separated from other vertebrates 550 Mya and have hardly changed since then [148–152]. *Petromyzon marinus* (sea lamprey) serves as model organism for *Cyclostomata*. It is considered a living fossil and has been well-characterized with respect to its morphological and metabolic properties. Its complete genome was recently sequenced [153,154] and a search of the Uniprot database yielded one LOX-like sequence (S4RFK5). Phylogenetic analysis suggested that this sequence shared a high degree of amino acid similarity with human ALOX5 and corresponding orthologs from other mammals. This 454 amino acid fragment involved the two iron clusters C1 and C2 from the human 5-LOX (His368, His373, His551, Asn555) in a distance of 183 amino acids. As fifth iron ligand serves the C-terminal Val with its free carboxylic group. Although there are no functional data confirming the catalytic activity of the sea lamprey LOX these structural characteristics suggest a catalytically active enzyme. For the time being there are no genomic data available for hagfish and thus, it remains unclear whether these species have functional LOXs.

**4.5.4.3.3. Lipoxygenases in cartilaginous and bony fish.** Cartilaginous and bony fish first appeared around 420–400 Mya during the transition from the Silurian to the Devonian period [155,156]. Sharks are representatives of cartilaginous fish and the genome of the elephant shark (*Callorhynchus milii*) has recently been published [157]. The genome contains several LOX-like sequences and amino acid

alignments suggested that two of them (XP\_007902723.1, XP\_007910024.1) share a high degree of sequence similarity with ALOX5 isoforms of higher vertebrates. Other sequences (AFO96333.1, AFP03230.1) are more closely related to epidermal LOX isoforms. Three of them (XP\_007902723.1, XP\_007910024.1, AFO96333.1) have both iron coordination clusters C1 and C2, but in one of them (AFP03230.1) only cluster C1 is conserved. In addition, the elephant shark genome also contains a number of sequences which are important for leukotriene signaling, especially the leukotriene receptors BLT1 and CYSLTR1/2 (XP004945706.1, XP007884788.1, XP007890412). This is an interesting observation since in a previous study it was reported that the genome of the thornback ray (*Raja clavata*) does not contain a functional LTA4-hydrolase (LTA4H) gene and thus, it was concluded that cartilaginous fish may not have functional leukotriene signaling [158]. In the absence of more detailed experimental data such conclusion may be somewhat risky since biosynthesis of cysteinyl leukotrienes does not require a functional LTA4H and it remains unclear whether genes encoding for other epoxide hydrolases are present in the genome. Moreover, the genome involves at least one LTB4 receptor gene (XP004945706.1) suggesting functional LTB4 signaling.

Bony fish separated into ray-finned (e.g., *D. rerio*) and lobe-finned fish (e.g., *Latimeria chalumnae*) about 400 Mya and the genomes of these two bony fish species have recently been sequenced [159,160]. When we searched the *D. rerio* sequence databases for potential LOX sequences we detected several LOX cDNAs (Table 3), which originate from different genes localized on different chromosomes [161]. Comparison of the amino acid sequences of zbfLOX1 and zbfLOX2 with the six functional human ALOX isoforms indicated that zbfLOX1 shared a similar degree (40–45%) of amino acid identity with all human ALOX isoforms. Thus, on the basis of the available sequence data it was impossible to sort out for which particular human LOX isoenzyme the zbfLOX1 was the *D. rerio* ortholog. Functional inactivation of zbfLOX1 causes severe impairment of embryonic development with deformation in brain, eyes and the tail region [162]. Since functional inactivation of Alox12b [29] and Alox3 [30] in mice also leads to structural defects in epithelial differentiation the authors concluded that zbfLOX1 might constitute the zebrafish ortholog for mouse Alox12b and/or Alox3 despite the low degree of amino acid identity. More detailed functional characterization of the recombinant enzyme indicated [161] that this LOX does neither follow the triad [163] nor the Ala-vs-Gly-concept [164] explaining the reaction specificity of different mammalian LOXs. For zbfLOX2 functional assignment to human ALOX isoform was less problematic since this enzyme shares a 74% of amino acid identity with the human ALOX5. Comparison with the other human ALOX isoforms revealed much lower identity degrees (37–41%). Thus, on the basis of the amino acid sequence zbfLOX2 was classified as arachidonic acid 5-LOX. Recently this LOX-isoform was expressed as recombinant His-tag fusion protein in *E. coli* and activity assays confirmed its

**Table 3**  
Lipoxygenase sequences in the zebrafish genome.

Database number	Chromosome	Possible orthology based on sequence homology
ENSDARP00000091900	7	High homology to 12/15-LOX or platelet-type 12-LOX (zbfLOX1),
ENSDARP00000074335	13	Among zebrafish LOXs highest homology to human ALOX5 (zbfLOX2)
ENSDARP00000063256	15	Alternative 5-LOX gene, lower homology to human ALOX5 than ENSDARP00000074335
ENSDARP00000093033	15	Alternative 5-LOX gene, lower homology to human ALOX5 than ENSDARP00000074335
ENSDARP00000063263	15	Alternative 5-LOX gene, lower homology to human ALOX5 than ENSDARP00000074335
ENSDARP00000106795	21	Long transcript, highest homology to human epidermal LOX isoforms (ALOX15B, ALOX12B)
ENSDARP00000023401	21	short transcript, 100% sequence identity to ENSDARP00000106795
ENSDARP00000113298	21	Short transcript, possibly an epidermal LOX isoform (ALOX15B)
ENSDARP00000095620	21	Long transcript, 99% sequence identity with ENSDARP00000113298.

reaction specificity of arachidonic acid oxygenation [165]. Unfortunately, for the other zebrafish LOX isoforms no functional data are currently available. However, the existence of a functional ALOX5 gene in the genome of *D. rerio*, the presence of a corresponding sequence in the *L. chalumnae* genome and the occurrence of sequences encoding for BLT1/2 and CYSLTR1/2 receptors suggest functional leukotriene signaling in bony fish. This conclusion is consistent with previous reports indicating the presence of LOX metabolites including LTB4 bony fish macrophages and thrombocytes [166,167]. On the other hand, to confirm this prediction additional experimental data on the functionality of the identified sequences are required.

**4.5.4.3.4. Lipoxygenase in amphibia.** The earliest amphibian-like animals, *Ichthyostega* and *Eusthenopteron*, appeared about 370 Mya [168]. So far, a number of amphibian genomes have been published but for this review we will focus on the Western clawed frog (*Xenopus Silurana tropicalis*) [169]. Its genome involves at least three putative LOX genes and different sequences encoding for BLT1/2 and the CYSLTR1/2 receptors. Sequence comparison suggested that one of the putative LOX genes (XP\_004815982.1) shares a high degree of amino acid similarity with human ALOX5. Both iron coordination clusters C1 and C2 are conserved suggesting a functional enzyme. This finding together with our observation that the *Xenopus* genome contains BLT- and CYSLT-receptor-like sequences suggests functional leukotriene signaling in this amphibian species. This conclusion is consistent with the previously reported formation of ALOX5 products (5-HETE, LTB4, LTC4) by frog leukocytes [170]. Recently, the LTA4H of the African claw toad (*Xenopus laevis*) was cloned and the enzyme was shown to convert LTA4 to LTB4 and  $\Delta(6)$ -*trans*- $\Delta(8)$ -*cis*-LTB4 [171]. This data suggest that amphibia apparently had a functional leukotriene B4 synthesizing cascade. Moreover, leukotriene biosynthesis was observed in bullfrog erythrocytes and thrombocytes [172,173]. In 2000, a 12-lipoxygenase was partially purified from bullfrog erythrocytes but unfortunately, no sequence data are currently available for this enzyme. Its MW of 77.7 kDa was in a normal range for LOXs [174]. Although there is no convincing evidence for the existence of an ALOX15 ortholog in frogs, 15(S)-HETE was isolated as arachidonic acid oxygenation product when frog leukocytes or oocytes were treated with exogenous arachidonic acid [170,175]. Because of the limited experimental data on the LOX pathway of amphibians little is known about the biological function of these enzymes. Involvement of these enzymes in amphibian regeneration, wound healing [176] and oocyte maturation [177] has been suggested.

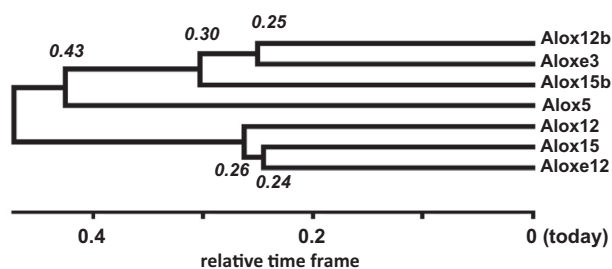
**4.5.4.3.5. Lipoxygenases in reptilia and aveae.** Reptiles appeared on earth 320–310 Mya in the late Carboniferous period and *Cassineria* is one of the oldest known amniotes [178,179]. The genome of the American alligator (*Alligator mississippiensis*) was published in 2012 [180] and we considered this vertebrate as model organism for reptiles. Searching the corresponding genome database we identified three LOX-like sequences. One of them (XP\_006263756.1) shared a high degree of amino acid conservation with human ALOX5 while the others (XP\_006264318.1, XP\_006272458.1) were more closely related to human epidermal LOX isoforms. So far, there is no functional data for reptile ALOX isoforms but snake erythrocytes apparently contain ALOX5 metabolites [181].

Avians have emerged from feathered theropod dinosaurs during the Jurassic period [182]. We employed chicken (*Gallus gallus*) as model organism for birds and searching the chicken genome database [183] suggested the presence of at least two LOX-like sequences. Sequence comparison demonstrated that one of these sequences (XP\_003641560.1) shares a high degree of amino acid similarity with human ALOX5 while the other (XP\_42376.4) seem to be more closely related to the epidermis-type LOX isoforms. Leukotriene signaling appears to exist in birds since LTB4 receptors have been identified in avian osteoclasts [184]. Although to the

best of our knowledge no bird LOX has been expressed as recombinant protein and characterized with respect to their enzymatic properties LOX metabolites have been detected in different chicken and turkey cells and tissues [185–187].

**4.5.4.3.6. Lipoxygenases in mammalia.** Mammals were first introduced in evolution during the mid-Permian period about 270 Mya. They evolved from the mammal-like dinosaur lineage called synapsids (later theropsids), and species like *Dryolestes* were closely related to nowadays placentals and marsupials whereas species like *Ambondro* were similar to monotremes. Later on, the eutherian and metatherian lineages separated. Eutherians are the ancestors of today's living placentals whereas the metatherian developed to marsupials [188,189]. The earliest known eutherian species (*Juramaia sinensis*) lived 160 Mya in the late Jurassic period and at that time-point the three existing mammalian subgroups, the monotremes, the marsupials and the placentals, have separated from each other [190]. For our search for mammalian LOX sequences we employed the platypus (*Ornithorhynchus anatinus*) and the Tasmanian devil (*Sarcophilus harrisii*) as model organisms for the monotremes and marsupials, respectively. The genomes of the two species have been published [191,192] and each of them contains a number of LOX-like sequences, which share a high degree of amino acid conservation with human LOX-isoforms. Interestingly, we did not find any sequence in the Tasmanian devil exhibiting a high degree of sequence similarity with human ALOX15. This finding is rather surprising since the evolutionary more distant platypus has an ALOX15-like sequence. Many putative LOX sequences of primitive mammalian species are present as truncated versions and in the absence of direct functional data it is difficult to conclude whether the corresponding genes encode for active LOX isoforms. Placentals have developed from eutherians 160 Mya, and the genomes of many rodents and primates are currently available to search for LOX-like sequences. Rodents and primates evolved from a common ancestor called *Euarchontoglires* [193]. Analysis of the mouse (*Mus musculus*) and rat (*Rattus norvegicus*) genomes [194,195] revealed seven functional LOX genes (*Alox15*, *Alox12*, *Alox5*, *Aloxe3*, *Alox15b*, *Alox12b*, and *Aloxe12*) in these two rodents. Since the *Alox15b* gene in mice encodes an 8-lipoxygenating enzyme it is sometimes referred to as *Alox8* but this nomenclature is really confusing. Leukotriene signaling is functional in these mammals since ALOX5 and leukotriene receptors are present [196–200]. Although the mouse *Alox15* and *Alox15b* share a high degree of amino acid sequence identity with the corresponding human orthologs they differ from the human enzymes with respect to their reaction specificity of arachidonic acid oxygenation. The structural basis for these differences will be discussed in the following chapters. The epidermal LOXs (*Alox12b*, *Aloxe3*), which have been well-characterized with respect to their enzymatic properties [201,202], have been implicated in epidermal differentiation [29,30] and in the formation of the epidermal water barrier [203]. Modern humans (*H. sapiens*) have only six functional LOX genes since the ALOX12 gene encoding for an additional epidermis-type LOX is present in humans as corrupted pseudogene [204]. The mouse enzyme was discovered in 1995 and later on characterized in more detail [205,206].

**Lipoxygenase orthologs in mammalian evolution:** As indicated above there are seven different LOX isoforms in mice and knockout studies suggested different biological roles for most of them [23]. *Alox5* has been implicated in leukotriene biosynthesis [207], *Alox12b*, *Aloxe3* and *Alox12* are involved in skin development [29,30,34] and *Alox12* might also play a role in platelet function [32]. For *Alox15* and *Alox15b* the biological roles are still unclear. The structural multiplicity of mammalian LOXs within a single species is frequently considered a sign of biological redundancy among scientists not working in the LOX field. Consequently, inactivation of one ALOX gene might be compensated for by



**Fig. 7.** Gene duplication of mouse LOX isoforms. For this analysis the amino acid sequences of the seven known LOX isoforms from mouse (*Mus musculus*) were selected and compared with each other: Alox5 (NP\_033791.1), Alox12 (NP\_031466.2), Alox15 (NP\_033790.3), Alox12e (NP\_663717.1), Alox12b (NP\_033789.1), Alox3 (NP\_035916.2), and Alox15b (NP\_033791.1). The amino acid sequences were aligned with MEGA 6.0 using the ClustalW package with default settings. The phylogenetic tree was constructed by the neighbor-joining method in MEGA 6.0 with bootstrap values estimated based on 5000 replications. The relative time-points for gene duplication events were calculated with MEGA 6.0 using default settings for calculation.

upregulation of the expression of other LOX isoforms. However, if one compares the enzymatic properties of the different human or mouse LOX such compensation mechanisms become questionable because of several reasons. (i) ALOX5 is the key enzyme in the biosynthesizing cascade of the 5,6-series of leukotrienes and no other LOX-isoform is capable of initiating the biosynthesis of these mediators. ALOX15 orthologs may catalyze the formation of 14,15-leukotrienes [208] but the biological role of these mediators has not been well characterized. (ii) ALOX12B has been implicated in epidermal differentiation and selective functional inactivation of the corresponding gene leads to dramatic alterations in skin development. If there is a compensatory upregulation of another LOX-isoform it appears to be insufficient to protect the animals from these defects. (iii) When we compared the expression of the mRNAs of various LOX isoforms (Alox12, Alox15b, Alox5) in different tissues of wildtype and Alox15 knockout mice we did not observe consistent indications for a major upregulation of other LOX isoforms as regulatory response towards defective Alox15 expression (Kuhn, unpublished data). These data suggest that expression regulation of these LOX isoforms may not be coupled to Alox15, which makes biological redundancy rather unlikely. Consequently, each LOX isoform is likely to have its own function (functional multiplicity of the LOX family). Unfortunately, the driving forces and the molecular mechanisms leading to the functional multiplicity remain largely elusive.

The three murine epidermal LOXs (Alox15b, Alox12b, Alox3) might have been evolved from a common precursor enzyme by at least two steps of gene duplications. The other murine LOX

isoforms (Alox15, Alox12) should have evolved independently from another common ancestor by at least two additional steps of gene duplications to form Alox12, Alox12e and Alox15 (Fig. 7). The Alox5 evolved independently and this conclusion is consistent with a previous suggestion [209]. Although the mechanisms and the driving forces for the duplication events have not been explored in detail it is well-known that the region of chromosome 17, where all but the ALOX5 gene are localized, is a hotspot for gene duplications [210].

According to their genomic sequence data all three mammalian subgroups (monotremes, marsupials, placentals) have the genetic basis (all genes for biosynthesizing enzymes and receptors) for functional leukotriene signaling. Thus, it may be concluded that the LOX signaling cascade has been completed during transition from therapsid dinosaurs to early mammal-like animals and has been conserved since then.

*Evolution of specificity determinants of mammalian ALOX15 orthologs:* Human ALOX15 converts arachidonic acid to 12S- and 15S-hydroperoxy arachidonic (12S- and 15S-HpETE) acid in a ratio of about 1:10 [42]. A similar reaction specificity was observed for the ALOX15 orthologs of rabbits [211] and orangutans [163]. On the other hand, the mouse ortholog [212] forms an inverse product pattern (12-HpETE vs. 15-HpETE ratio of about 10:1) and similar results were obtained for the ortholog enzymes from rats [43], pigs [213], cattle [214] and rhesus monkeys [163]. The molecular basis for the variable positional specificity of ALOX15 orthologs has previously been studied and the triad concept has been developed [44,215]. In 12-lipoxygenating enzymes the sequence determinants are occupied by small amino acids whereas more space filling residues are present in the 15-lipoxygenating isoforms (Table 4). Although the positional specificity of ALOX15 orthologs of chimpanzee, gorilla, gibbon and baboon remains to be determined experimentally 15-lipoxygenation might be predicted for the enzymes of chimpanzee and gorilla. In contrast, 12-lipoxygenation may be predicted for baboon ALOX15. For gibbon ALOX15 prediction of the reaction specificity is somewhat problematic but preliminary data from our lab suggest almost equal amounts of 12- and 15-HpETE (Adel et al., unpublished data). Taken together, these data suggest that in lower mammals (mice, rats, pigs, baboon, rhesus monkey) 12-lipoxygenating ALOX15 enzymes are expressed. In contrast, in higher mammals (orangutan, human) 15-lipoxygenating ALOX15 isoforms are present [216] and gibbons may constitute a transition species. The only exception from this rule is the rabbit but here 12- and 15-lipoxygenating ALOX15 species are expressed in a tissue specific manner [217]. Unfortunately, the biological relevance of this evolutionary switch in the reaction specificity still remains unclear.

**Table 4**

Triad determinants of ALOX15 orthologs and positional specificity of arachidonic acid oxygenation. The one letter code of amino acid nomenclature was used and the triad determinants are indicated in bold face. In human ALOX15 amino acid numbering is as follows: Borngräber 1 determinant (F353), Sloane determinants (I418, M419), Borngräber 2 determinants (I593, T594). n.d., not yet determined experimentally.

Species	Borngräber 1 determinant	Sloane determinant	Borngräber 2 determinant	Major product (predicted)	Major product (experimental)
Human ( <i>Homo sapiens</i> )	<b>DFQ</b>	<b>QIMS</b>	<b>SITW</b>	15-HpETE	15-HpETE
Chimpanzee ( <i>Pan troglodytes</i> )	<b>DFQ</b>	<b>QIMS</b>	<b>SITW</b>	15-HpETE	n.d.
Gorilla ( <i>Gorilla gorilla</i> )	<b>DFG</b>	<b>QIMS</b>	<b>SITW</b>	15-HpETE	n.d.
Orangutan ( <i>Pongo pygmaeus/ abelii</i> )	<b>DFQ</b>	<b>QIMS</b>	<b>SITW</b>	15-HpETE	15-HpETE
Gibbon ( <i>Nomascus leucogenys</i> )	<b>DFQ</b>	<b>QITS</b>	<b>SIVW</b>	12- + 15-HpETE	n.d.
Rhesus monkey ( <i>Macaca mulatta</i> )	<b>DFQ</b>	<b>QVVS</b>	<b>SITW</b>	12-HpETE	12-HpETE
Baboon ( <i>Papio anubis</i> )	<b>DFQ</b>	<b>QVVS</b>	<b>SMTW</b>	12-HpETE	n.d.
Cattle ( <i>Bos taurus</i> )	<b>DFQ</b>	<b>QVVS</b>	<b>SITW</b>	12-HpETE	12-HpETE
Pig ( <i>Sus scrofa</i> )	<b>DFQ</b>	<b>QVVS</b>	<b>SITW</b>	12-HpETE	12-HpETE
Rat ( <i>Rattus norvegicus</i> )	<b>DLQ</b>	<b>KAMS</b>	<b>NVWV</b>	12-HpETE	12-HpETE
Mouse ( <i>Mus musculus</i> )	<b>DLQ</b>	<b>KVMS</b>	<b>NVWV</b>	12-HpETE	12-HpETE

**Table 5**

Sequence determinants of ALOX15B and ALOX12B orthologs and reaction specificity of arachidonic acid oxygenation. The one letter code of amino acid nomenclature was used and the sequence determinants are indicated in bold face. In human ALOX15B amino acid numbering is D603 and V604. In human ALOX12B amino acid numbering is G441.

Species	Jisaka determinants	Coffa/Brash determinants
Human ( <i>Homo sapiens</i> )	TCDVIL	SIGRA
Chimpanzee ( <i>Pan troglodytes</i> )	TCDIIL	SIGRA
Gorilla ( <i>Gorilla gorilla</i> )	TCDIIL	SIGRA
Orangutan ( <i>Pongo abelii</i> )	TCDVIL	SIGRA
Gibbon ( <i>Nomascus leucogenys</i> )	ASLQMS	SIGRA
Rhesus monkey ( <i>Macaca mulatta</i> )	TCDVIL	SIGRA
Baboon ( <i>Papio anubis</i> )	TCDVIL	SIGRA
Cattle ( <i>Bos taurus</i> )	TCDVVI	SIGRA
Pig ( <i>Sus scrofa</i> )	TCDVVI	SIGRA
Rat ( <i>Rattus norvegicus</i> )	TCDVII	SIGRA
Mouse ( <i>Mus musculus</i> )	SSYHII	SIGRA

**Evolution of specificity determinants in ALOX15B and ALOX12B orthologs:** The triad concept of reaction specificity is not applicable for all LOX isoforms [163] and for ALOX15B orthologs other sequence determinants have been identified. Human ALOX15B exhibits a singular positional specificity converting arachidonic acid exclusively to 15S-HpETE [218]. In contrast, the mouse enzyme oxygenates arachidonic acid to 8S-HpETE [219] and site-directed mutagenesis indicated that substitution of Tyr603 and His604 to the corresponding residues present at these positions in human ALOX15B (Y603D+H604V) leads to complete shift in the positional specificity of arachidonic acid oxygenation from 8S- to 15S-HpETE formation. The inverse mutagenesis strategy leads to partial alterations [45]. When we compared the ALOX15B sequences of different mammals (Table 5) we found that chimpanzee, gorilla, orangutan, rhesus monkey, baboon, pig and even rats have an Asn-Val or Asn-Ile motif at this position and thus, they are similar to the human enzyme. In contrast, the mouse sequence has a Tyr-His motif at this position. In gibbons these positions are occupied by Leu-Gln but the functional consequence of this amino acid exchange needs to be explored experimentally.

Multiple sequence alignments of a large number of different LOX sequences [164] suggested that S-LOXs carry an Ala at a defined position (Ala402 of human ALOX15) in their primary structure. In contrast, R-LOXs carry a Gly at this position (Gly441 for human ALOX12B) and mutagenesis studies suggested that an Ala-to-Gly exchange induces major alterations in the enantioselectivity of several LOX-isoforms [164]. Although this concept was applicable for a large number of LOX isoforms [220] zebrafish LOX-1 (zbfLOX1), which contains a Gly at this position, converts arachidonic acid mainly to 12S-HpETE [161]. A sequence alignment of various ALOX12B of different mammals indicated strong conservation of the corresponding Gly residue during mammalian evolution (Table 5).

**Lipoxygenases in extinct human subspecies:** *Homo neanderthalensis* is the closest evolutionary relative of modern humans and for a long time they were widely distributed over large parts of Europe and western Asia [221]. During the later parts of their history Neanderthals settled as far east as southern Siberia and as far south as the Middle East [222]. Neanderthal individuals got in contact with modern humans and the question of whether Neanderthals have interbred with modern humans has been discussed controversially for many years [223]. Comparison of the nuclear genomes of *H. neanderthalensis* [224,225] and *H. sapiens* suggested that Neanderthals played a role in the genetic ancestry of today's humans outside Africa since 1–3% of the DNA of today's Europeans originate from Neanderthals. However, it remains a matter of discussion how intense the original mixing was and where and

when it took place [223]. When the first draft sequence of the *H. neanderthalensis* genome (1.3-fold genome coverage) was published a number of genomic regions, which are different between ancient and modern humans have been identified [224]. Among them was the cysteinyl leukotriene receptor 2 (CLTR2), the expression of which appeared to be corrupted in *H. neanderthalensis* [224]. In addition, when we analyzed the genomic sequence data and searched for human LOX orthologs we found [226] that the genes encoding for ALOX12 and ALOXE3 appeared to be dysfunctional because of premature stop codons interrupting the open reading frame. However, more precise genomic sequence data (42-genome coverage), which were recently obtained from a *H. neanderthalensis* fossil found in the Denisovan cave [224,225], did not confirm these defects [227]. Thus, it was concluded that *H. neanderthalensis* had fully functional leukotriene signaling.

The Denisovan cave in the Altai Mountain in southern Siberia is a rich source of human fossils and paleontological excavations have been going on in this cave for many years. Recently, a distal manual phalanx [228], a proximal pedal phalanx [229] and a molar tooth [230] were excavated from different sublayers of layer 11 (tooth from sublayer 11/1, manual phalanx from sublayer 11/2, pedal phalanx from sublayer 11/4). This data indicate that the corresponding individuals lived in this cave about 50,000 years ago and that the finger individual was somewhat older than the two other fossils. The pedal phalanx came from the 4th or 5th toe of a human adult individual and its morphological traits linked it with both *H. neanderthalensis* and *H. sapiens* [225]. In contrast, the morphology of the two other fossils suggested an independent human subspecies, which was named *Homo denisovan*. Comparison of mitochondrial DNA preparations from this manual phalanx [228] and mitochondrial DNA prepared from previous *H. neanderthalensis* fossils [231] and from *H. sapiens* clearly indicated evolutionary differences between these three human subspecies but also suggested a common ancestor. Since evolutionary conclusions drawn from mitochondrial genomes are always risky, nuclear DNA was extracted from the manual phalanx and the genome was sequenced at high (>40-fold) genome coverage [232]. Taken together, high quality genomic sequences of two different archaic human subspecies (*H. neanderthalensis*, *H. denisovan*) and of modern humans (*H. sapiens*) are currently available for detailed structural and functional comparison. When we aligned on the amino acid level the LOX sequences of all *H. sapiens* LOX isoforms with those of *H. denisovan* we only observed subtle structural differences [227]. Neither of the detected amino acid exchanges was at positions impacting iron coordination or reaction specificity. Some of the observed mutations were at positions, for which a high degree of genetic variability (SNPs or rare mutations) has been observed among different human individuals [233]. Since only one *H. denisovan* individual has been sequenced so far, it remains unclear whether the observed amino acid exchanges are unique for this individual.

## 5. Genetic variability of human leukotriene signaling

The leukotriene signaling pathway has been introduced during evolution on the bony fish level (perhaps somewhat earlier) and has been conserved in all higher vertebrates. The modern human (*H. sapiens*) has six functional LOX isoforms and a number of other genes that are involved in leukotriene signaling. During the last decade various Genome-wide association studies (e.g., HapMap, 1000 Genome, COSMIC etc.) have been performed, and genetic variations in the leukotriene signaling cascade have been associated with certain diseases, such as bronchial asthma, Alzheimer's disease, atherosclerosis and different forms of cancer. Although a large number of variations have been described only a few of them

**Table 6**

Non-synonymous coding variations in enzymes and receptors of the LOX pathway.

Enzyme/receptor (UniProt-Code)	Total	SNPs (allele frequency >1%)	Missense mutations (allele frequency <1%)	Nonsense mutations
<i>Lipoxygenases</i>				
ALOX5 (P09917)	69	1	68	0
ALOX15 (P16050)	94	2	84	8
ALOX12 (P18054)	67	2	64	1
ALOX15B (O15296)	86	6	76	4
ALOX12B (O75342)	69	1	68	0
ALOXE3 (Q9BYJ1)	83	2	75	6
<i>Other leukotriene synthesizing enzymes</i>				
LTA4H (P09960)	42	1	39	2
LTC4S (Q16873)	10	0	10	0
<i>Leukotriene receptors</i>				
CYSLTR1 (Q9Y271)	49	0	49	0
CYSLTR2 (Q9NS75)	71	1	69	1
LTB4R (Q15722)	16	0	16	0
LTB4R2 (Q9NPC1)	43	2	41	0
GPR17 (Q13304)	67	0	66	1
P2RY12 (Q9H244)	35	1	34	0
GPR99 (Q96P68)	63	0	58	5
Total	864	19	817	28

have functionally been characterized. Most alterations are either located in intronic, in the promoter, in intergenic DNA regions or they are synonymous in nature. In this part of the review we will discuss the functional consequences of gene variations involved in human leukotriene signaling. We therefore mainly focus on distribution, structural localization and functional consequences of coding non-synonymous variations in genes of the LOX pathway, and we try to summarize the current knowledge about functional relevant promoter, 5' and 3' UTR variations as well. In total more than 800 non-synonymous variations have been found in different genes of the LOX pathway but only 19 are defined as SNPs with an allele frequency >1% (Table 6).

### 5.1. Genetic variability of human lipoxygenases

#### 5.1.1. ALOX5 (5-lipoxygenase)

The human ALOX5 gene is located on chromosome 10 and spans about 71.9 kbp consisting of 14 exonic and 13 intronic sequences [234]. It lacks TATA and CCAT boxes in the promoter but contains eight GC boxes, and five of them are arranged in tandems to be recognized by the transcription factors Sp1 and Egr1 [235,236]. Previous clinical studies have shown that naturally occurring mutations in the ALOX5 gene can lead to the deletion or insertion of Sp1-binding sites, and the hALOX5 promoter genotype might impact anti-asthmatic therapy [237,238]. As a possible molecular reason for this observation a reduced level of hALOX5 mRNA and LTC<sub>4</sub> in patients with a certain genotype (non5/non5) was discussed [239]. In addition, mutations in the tandem GC boxes were associated with an increased arterial intima-media thickness and with higher plasma levels of C-reactive protein suggesting a role of this genetic variation in atherogenesis [240]. This conclusion was supported by another study, in which the Sp1 promoter variations were associated with an increased risk of coronary artery disease [241]. The molecular basis for these statistical associations has not been explored in detail. It was suggested, that the promoter variations might impact the biosynthesis of various arachidonic acid and eicosapentaenoic acid derived eicosanoids [242]. In addition, ALOX5 promoter variations have been associated with a decreased risk for colon and rectal cancer [243,244] but there was indication for an involvement in Alzheimer's disease as well [245].

When we quantified the genetic variability in the coding region of the human ALOX5 gene on the basis of the sequence data of the 1000 human genome project [233], we observed 69 non-synonymous coding variations but only the Glu254Lys exchange

(rs2228065) had an allele frequency >1% and thus, qualifies as SNP (Table 6). This SNP has been associated with an increased risk for bronchial asthma in two independent case-control studies [246,247]. Interestingly, this SNP is more frequent in African-Americans (allele frequency >10%) when compared with other ethnic groups and in Europeans it is much less abundant. Its low frequency in the European population appears to be the most probable reason why this SNP has never been considered for European case-control studies [248–250]. Although the Glu254Lys exchange occurs with increased frequency in patients suffering from bronchial asthma the functional basis for this relation remains unclear. It was hypothesized that the non-conservative Glu254Lys exchange destabilizes the 3D-structure [246]. When we expressed human wildtype ALOX5 and its corresponding Glu254Lys mutant as recombinant protein we found that the mutant enzyme exhibited a reduced (60% residual activity) catalytic activity [251]. On the other hand, the reaction specificity of the enzyme was not impacted by this amino acid exchange. Whether the rather subtle alterations in catalytic activity induced by the Glu254Lys exchange significantly contribute to the statistical correlation is difficult to judge, and partial functional inactivation of the enzyme should rather decrease the risk for asthma. In additional case-control studies this SNP was not significantly correlated with breast and colon cancer [243,252].

When we localized the non-synonymous coding variations identified in the frame of the 1000 human genome project [233] in the 3D-structure of human ALOX5 [12], we found that neither the iron ligands [253] nor amino acids that are important for the reaction specificity [164,254] were affected by genetic variations. Moreover, there are no nonsense mutations leading to interruption of the open reading frame reported in the database.

The substrate-binding pocket of hALOX5 is an elongated cavity, which appears to be sealed at both ends in the ligand free protein. Phe178 and Tyr182 (FY-cork) close the cavity at one end and Trp148 may block the opposite entrance to the cavity [12,255]. In addition to that, the enzyme activity can be regulated by certain factors (Ca<sup>2+</sup>, phosphatidyl choline, ATP, coactosin-like protein) and for some of these ligands the binding mechanisms have been described [256–259]. Neither of these functionally important amino acids were affected by any of the genetic variations [251].

Human ALOX5 can be regulated by reversible phosphorylation of Ser271, Ser523, and Ser663 [260–263]. There is a rare genetic variation at Ser271 in the 1000 human genome database (TMP\_ESP\_10\_45920561, allele frequency <0.1%), which induced



on the protein level a Ser271Ile exchange. This mutation should prevent enzyme phosphorylation at this position. Since phosphorylation of Ser271 was suggested to prevent nuclear export of the protein [262,263] this rare mutation might be of functional relevance.

#### 5.1.2. ALOX15 (12/15-lipoxygenase)

The hALOX15 gene is located on chromosome 17 and spans about 10.7 kb consisting of 14 exonic and 13 intronic sequences. It also lacks TATA and CCAT boxes but contains several GC rich sequences [264]. A genetic promoter polymorphism (–292 C/T) leads to introduction of an additional Sp1 transcription factor-binding site. This promoter polymorphism apparently enhances transcription of the ALOX15 gene, which might be relevant for atherogenesis [265,266]. However, in a European case-control study there was no significant association between this promoter variation and myocardial infarction [267]. Other ALOX15 promoter polymorphisms were associated with the extent of carotid plaque formation [268]. For one of these variations (rs2255888) a significant difference in the *in vitro* promoter activity was reported [269] but it remains unclear whether this might also be the case *in vivo*. Moreover, ALOX15 promoter polymorphisms appeared to be important for the pathogenesis of aspirin-exacerbated respiratory disease [270].

Human ALOX15 consists of 662 amino acids [271]. In the 1000 human genome database 94 non-synonymous coding variations were found (Table 6). Two of them, Pro617Ser (rs41432647) and Thr560Met (rs34210653), have an allele frequency of >1% (SNPs) whereas the vast majority are rare mutations. We analyzed the functional consequences of selected genetic variations. For Thr560Met (rs34210653) and Gly422Glu (rs61099320) a complete loss in catalytic activity was reported [272,273]. More detailed analysis of the structural basis for the loss in catalytic activity suggested that the amino acid exchanges induced partial misfolding of the enzyme. For other naturally occurring rare mutations no functional consequences could be identified [251]. Heterozygous carriers of the Thr560Met mutations had an increased risk for developing coronary artery disease [267,274] and similar effects can be expected for other hALOX15 loss-of-function variations.

The 1000 human genome database involves eight nonsense mutations in the ALOX15 gene and when these nonsense alleles occur homozygously the corresponding individuals should be devoid of ALOX15. His360, His365, His540, His544, and the C-terminal Ile662 constitute the direct iron ligands of hALOX15 [275,276] but neither of these residues were affected by any genetic variation. Rabbit and human ALOX15 exhibit dual positional specificity of arachidonic acid oxygenation, and the amino acid residues Phe352, Ile417/Met418 and Ile592 play a major role for this enzyme property [215,277]. Searching the 1000 human genome database we found a rare variation at Phe352, in which the bulky Phe is mutated to a less space-filling Leu (rs143365387). According to the triad concept [44] this enzyme variant should be a 12-lipoxygenating enzyme species. These data indicate that homozygous allele carriers express a 12-lipoxygenating ALOX15 (as it is the case in mice) and it would be of major interest to find out whether such individuals have any functional defects. Unfortunately, this variation has only a low allele frequency and thus, the search for homozygous allele carriers is rather laborious and the existence of such individuals is even questionable. Human ALOX15 seems to follow the Ala-to-Gly [164] concept but for the corresponding residue Ala403 there is no genetic variation described in the SNP databases.

Human and rabbit ALOX15 are capable of oxygenating membrane lipids [278]. Surface exposed hydrophobic amino acids such as Tyr15, Leu70, Leu71, Lys180 and Leu194 have been implicated in membrane binding [279] and there are genetic variations of cor-

responding residues in the human 1000 genome database. The Tyr15His exchange (rs13306168) has a global frequency of 0.6% whereas no frequency data are available for the Leu194Pro exchange (rs11867874). Unfortunately, there are no functional data for the corresponding mutated proteins. Mutagenesis studies and molecular dynamic simulations of enzyme-substrate complexes suggested that Arg402 and Phe414 may be involved in substrate binding [280,281]. For Arg402 two rare mutations with a global frequency <1% have been identified in the 1000 human genome database [Arg402Gln (TMP\_ESP\_17\_4536752) and Arg402Trp (rs144038526)]. However, the corresponding mutant protein did not exhibit major functional defects [251].

#### 5.1.3. ALOX12 (platelet-type 12-lipoxygenase)

The human ALOX12 gene is located on chromosome 17 and spans a region of 14.6 kbp [282]. Its promoter involves five GC-boxes and carries two Sp1-binding sites [283]. Two genetic promoter variations (rs3840880, rs9897850) have been associated with an increased risk of osteoporosis [284] whereas another variation (rs11571327) was related to melanoma [285]. In addition, another ALOX12 promoter variation (rs9904779) was associated with earlier onset of menopause but there is no evidence for statistical association of this SNP with cardiovascular diseases [286,287].

Human ALOX12 contains 663 amino acids and was the first mammalian lipoxygenase described back in 1974 [60]. In the 1000 human genome database there are 67 non-synonymous coding variations for this enzyme (Table 6). Two of them, Gln261Arg (rs28395860) and Asn322Ser (rs434473) have an allele frequency <1% [251]. The Gln261Arg SNP was associated with osteoporosis, schizophrenia and type 2 diabetes [284,288,289] while the Asn322Ser SNP has been related to early onset of menopause and schizophrenia [287,289]. Unfortunately, the molecular basis for these statistical correlations remains unclear and corresponding mutagenesis studies on the recombinant enzyme did not provide convincing data for functional defects [251,290].

When we searched the 1000 human genome database for genetic variations of functionally important amino acid residues (iron ligands, position- and stereospecificity determinants) we did not find any genetic variations at these positions. However, there is one nonsense variation (Arg348stop, rs141346813) in the 1000 human genome database and homozygous carriers of this allele should be devoid of ALOX12. As for hALOX15 [280] Arg402 of hALOX12 may play a role for substrate binding [281] and Arg402Gln exchange (rs143539715) is a naturally occurring rare enzyme variant. However, the functional consequences of this amino acid exchange have not been explored yet.

#### 5.1.4. ALOX15B (15-lipoxygenase-2)

The gene for hALOX15B is located on chromosome 17 and spans about 10.1 kb [218]. The hALOX15B promoter has several Sp1 sites. The enzyme was suggested as negative cell-cycle regulator (tumor-suppressor activity) and Sp1-binding appeared to upregulate tumor suppressor function [291,292].

The enzyme consists of 676 amino acids (Isoform D) and was first discovered in 1997 [218]. 86 non-synonymous coding variations have been identified in the 1000 human genome database and six of them [251] have an allele frequency >1% (Table 6): Pro77Ala (rs78230493), Ala311Val (rs148602477), Arg486His (rs9895916), Arg635Gln (rs61730298), Gln656Arg (rs4792147), and Ile676Val (rs7225107). The Arg486His and Ile676Val exchanges could not be associated to coronary artery disease [293] but heterozygous allele carriers of the Gln656Arg SNP seem to have a decreased risk for developing the disorder. However, mutagenesis studies on the recombinant protein did not provide any evidence for major functional defects of these mutations [293]. The C-terminal Ile676 is a direct iron ligand and Ile676Val

is a SNP with a frequency of 9.6% but mutagenesis studies did not reveal major functional alterations [251]. There are four nonsense variations described in the 1000 genome database but neither of them had an allele frequency >1%.

The hALOX15B exhibits a singular positional specificity, as it converts arachidonic acid almost exclusively to 15-HpETE, but the mutations Asp603Tyr+Val604His convert the enzyme partially to an 8-lipoxygenating isoform [45,218]. In the 1000 genome database there is a rare natural occurring variant of Val604 (rs192653434) with a global frequency of 0.1% but the functional consequences of this amino acid exchange have not been studied in detail. Human ALOX15B carries an Ala at the Coffa-Brash position and therefore it has been classified as S-LOX [164]. In the 1000 human genome database there is a rare genetic variation at this position (rs140152561) leading to an Ala-to-Asp exchange. We found that this amino acid exchange constitutes a loss-of-function mutation and that the catalytic inactivity of the mutant enzyme may be caused by a partial misfolding of the protein [251]. ALOX15B is highly expressed in human macrophages [294]. Lentivirus-mediated expression silencing of the enzyme in LDL-receptor deficient mice and siRNA induced down-regulation of ALOX15B expression in human primary macrophages reduced lipid deposition in the vessel wall and impaired secretion of inflammatory cytokines. Thus, the enzyme was considered a pro-atherogenic protein [295]. Since human and mouse ALOX15B orthologs exhibit different reaction specificities with arachidonic acid it remains unclear whether these data can be directly translated into the human situation.

#### 5.1.5. ALOX12B (12R-lipoxygenase)

The gene encoding for the hALOX12B is also located on chromosome 17 and spans about 15 kb [296]. The promoters of human and mouse orthologs contain several GC boxes but lack TATA and CCAT boxes [202,296,297]. There is currently no information available as to the functionality of genetic promoter variations.

This LOX isoform consists of 701 amino acids and was first described in mouse and human skin [202,296,297]. 69 non-synonymous coding variations have been published in the 1000 human genome database (Table 6) but neither of them leads to a premature stop codon. There is a rare mutation in the ALOX12B gene (rs140063508) leading to a His398Tyr exchange. Since His398 is an immediate iron ligand this mutation might impact the catalytic activity of the enzyme. However, since Tyr carries a phenolic OH-group it might well function as iron ligand but functional studies on the corresponding mutant enzyme are still pending. There are no nonsense mutations in ALOX12B described in the 1000 human genome database and except for the iron coordinating His398 neither of the other functional important amino acid residues (other iron ligands, Coffa-Brash determinant) are affected by genetic variations.

Newborn Alox12b knockout mice die shortly after delivery because of dehydration [29] and a naturally occurring mutation in this gene induces similar skin defects [298]. In humans, genetic variations in the hALOX12B gene have been related to the development of autosomal recessive congenital ichthyosis [31,299–302]. The molecular mechanisms for the involvement of LOX isoforms in skin development are not completely understood but there is a testable mechanistic concept, which involves enzymatic oxygenation of skin specific ceramide, subsequent decomposition of the hydroperoxy derivatives and preferential hydrolysis of the oxidized lipids [303,304].

Some of the genetic variations observed in the above referenced association studies have been picked up in human 1000 genome project but except for Pro127Ser (rs72842957), which has a global frequency of 1.1%, all others are classified as rare enzyme mutations. These data are consistent with the fact that ARCI is a rare

skin disease (incident rate 1:200,000–300,000 Europeans and Northern Americans). Unfortunately, the Pro127Ser variant is not expressed in HEK 293 cells and thus, no functional data are currently available [31]. If this variation with its relatively high global allele frequency of >1% would be a strong inducer of ARCI a much higher prevalence of this skin disease should be expected. Only minor functional consequences are predicted for the Pro127Ser exchange since it is located at the enzyme surface far from the active site as suggested by structural modeling [251].

#### 5.1.6. ALOXE3 (epidermal lipoxygenase-3)

The human ALOXE3 gene is also located on chromosome 17 and spans about 23.1 kb [305]. Similar to other LOXs the hALOXE3 promoter lacks TATA and CCAT motifs but contains several GC boxes. So far, no promoter variations have been associated with major diseases.

Like ALOX12B this LOX isoform was discovered in mouse and human skin [201,305]. It consists of 711 amino acids and has a molecular weight of 80.6 kDa. It is an unusual LOX isoform and exhibits dominant hydroperoxide isomerase activity converting hydroperoxides (HpETEs) to specific epoxyalcohols [306,307]. However, under certain conditions it also exhibits dioxygenase activity [308,309].

83 non-synonymous coding variations have been published in the human 1000 genome database (Table 6) but only two of them (Ile515Val, rs3027205; Arg678Cys, rs143246503) have a global allele frequency >1% (3.0% and 1.9%). Ile515Val exchange is a conserved amino acid substitution and thus, no major functional consequences are expected. In contrast, Arg678Cys is non-conservative but its functional relevance can hardly be predicted. As for the ALOX12B gene genetic variations of ALOXE3 have been associated with autosomal recessive congenital ichthyosis [31,299–302] and some of these mutations were also found in the 1000 human genome database. The Ile515Val exchange was found with higher frequency in ichthyosis patients but it remains unclear whether this mutation impacts the enzyme functionality. Six nonsense variations have been identified in the ALOXE3 gene, which lead to C-terminally truncated enzyme variants. One of them (Arg211stop; rs141340759) has also been identified in a genome-wide melanoma study [310].

### 5.2. Genetic variability of other leukotriene synthesizing enzymes

#### 5.2.1. Leukotriene A4 hydrolase (LTA4H)

The gene encoding for hLTA4H is located on chromosome 12 and spans about 42.7 kb [311–313]. Although the 5'-upstream region of the gene has not been explored for promoter activity, genetic variations in this region (rs2660845, rs2540477) have been related to an altered response to Montelukast treatment of asthma patients and to coronary artery disease [314–316]. The SNP rs2660845 was previously described in the 6-fold haplotype “HapE” and the ten-fold haplotype “HapK”. HapE is protective in female depression and in subclinical atherosclerosis [317,318]. HapK seems to increase the risk for myocardial infarction [319].

This enzyme consists of 610 amino acids and has a MW of 69 kDa [313]. It catalyzes the final step in the biosynthesis of LTB<sub>4</sub>. In the 1000 human genome database there are 42 non-synonymous coding variations (Table 6) but only one of them (Glu481Asp, rs45630739) has a global frequency of >1% (1.6%). This is a conserved amino acid exchange and the crystal structure of hLTA4H [13] indicated that this residue is located on the enzyme surface. LTA4H is a bifunctional enzyme that has an epoxide hydrolase activity for LTA<sub>3</sub>, LTA<sub>4</sub> and LTA<sub>5</sub> [320,321] but also harbors a zinc-dependent aminopeptidase activity with a zinc binding motif [322]. Three amino acids (His295, His299 and Glu318) form the Zn-binding cluster and mutagenesis studies indicated that the loss of

one of these residues blocks the catalytic activity [323–326]. LTA4H undergoes suicidal inactivation during LTA4 hydrolysis and as molecular reason for the loss in catalytic activity chemical modification of Tyr378 was suggested [327,328]. Additional mechanistic studies indicated the importance of the following amino acids for the functionality of the enzyme: Gln136, Glu271, Glu296, Asp375, Arg563, and Lys565 [6,329–331]. When we searched the 1000 human genome database we did not detect any genetic variations for these residues. However, we found two premature stop variants as rare mutations with an allele frequency of <0.1%.

### 5.2.2. Leukotriene C4 synthase (LTC4S)

The gene encoding for leukotriene C4 synthase is located on chromosome 5 and spans about 2.5 kb [332,333]. The promoter involves potential cis-regulatory sequences but their *in vivo* functionality remains to be explored [334]. One promoter polymorphism (rs730012) was associated with a higher susceptibility of the allele carriers for ischemic stroke and with an altered response to the anti-asthma treatment with Montelukast [314,335].

Human LTC4S is an 18 kDa enzyme with 150 amino acids which belongs to the family of membrane-associated Proteins in Eicosanoid and Glutathione (MAPEG) metabolism. It catalyzes the reaction between LTA4 and glutathione (GSH) to LTC4. 10 different non-coding variations are published in the 1000 human genome database but none of them has an allele frequency >1% (Table 6).

Based on the crystal structures of the enzyme the catalytic importance of Arg104 was suggested [16,336]. Interestingly, when we searched the publically available SNP databases we found a rare mutation (rs13162195), which induces an Arg104Lys exchange. Mutagenesis studies at this position have shown that exchanges of Arg104 to Ala, Ser, Thr, and Lys caused almost complete loss in catalytic activity [337]. The observation that the Arg104Lys exchange induces such dramatic functional consequences is rather surprising because of the conservative character of this amino acid substitution. Nevertheless, on the basis of this functional data one has to conclude that homozygous allele carriers of this non-synonymous SNP should completely lack functional cys-LT signaling. Thus, such individuals should be protected from bronchial asthma and other allergic diseases. However, since this SNP is a rare mutation, which only occurred once in the Human 1000 Genome Database the design of a meaningful large scale epidemiological study is somewhat problematic. In addition to Arg104, Ser28, Ser111 and Trp116 have been implicated in the catalytic cycle [16,336,338]. For Ser28 a rare mutation (TMP\_ESP\_179222609) was identified in the SNP databases and this nucleotide exchange leads to a Ser28Leu exchange. This mutation removes a hydroxyl group and thus corrupts a phosphorylation site.

## 5.3. Genetic variability of leukotriene receptors

### 5.3.1. Cysteinylleukotriene receptor 1 (CysLTR1)

The gene encoding for hCYSLTR1 is located on X chromosome and spans about 56 kbp [339,340]. Little is known about transcriptional regulation of the gene, but a 4-fold promoter polymorphism haplotype (rs321029, rs2806489, rs7066737, and rs2637204) causes a significant decrease in transcription of the CYSLTR1 gene when compared with wildtype individuals [341]. *In vitro* studies indicated that the polymorphism rs2806489 reduces promoter activity by factor 2 [342] but *in vivo* confirmation of the effect is lacking. Other CYSLTR1 promoter polymorphisms appear to be involved in atopy and bronchial asthma [343–345].

The CYSLTR1 belongs to the family of G-protein coupled receptors (GPCRs) consisting of 337 amino acids. It contains 7-transmembrane spanning helices and binds various ligands: LTD4 ≫ LTE4 = LTC4 [346]. In the 1000 human genome database

49 non-synonymous genetic variations are described but none of them has an allele frequency >1%. Among them there is no nonsense variation (Table 6). Little is known about the molecular details of ligand binding and intracellular signaling and thus, predictions on functional consequences of structural alterations are problematic. The naturally occurring Gly300Ser exchange (CM072979) was associated with increased risks for atopy and asthma. Apparently, the Gly300Ser receptor has an increased affinity for LTD4 [347] suggesting more intense leukotriene signaling for allele carriers. Owing to its low allele frequency in the normal population this genetic variation has not been picked up in the Human 1000 Genome Project and is neither listed in the NCBI SNP database. However in the population of the Tristan da Cunha islands its allele frequency exceeds 10%. The Tristan da Cunha islands are localized in the southern Atlantic 2000 km apart from Saint Helena, 2400 km apart from the western shore of South Africa and more than 3000 km apart from South America. They are the most isolated inhabited islands on earth. The main island has a north–south length of about 11 km, and an area of 98 km<sup>2</sup>. Today 80 families are living on its main island and the total population comprises about 300 permanent residents. The genetic pool of the permanent residents has been derived from only 15 common ancestors, which makes the population an interesting group for analyzing the inheritance of certain genetic defects [348].

### 5.3.2. Cysteinylleukotriene receptor 2 (cysLTR2)

The gene encoding for CYSLTR2 is located on chromosome 13 and spans about 2.5 kb [200]. Although there is little knowledge about transcriptional regulation of CYSLTR2 there are genetic polymorphisms in the promoter (rs7324991), in the 3'UTR (rs912278, rs34494076) and in the downstream region of the gene (rs2407249), resulting in an increased mRNA and protein expression [349]. *In vitro* reporter gene experiments indicated higher activity of the mutated promoter when compared with the wild-type constructs [349]. Promoter and 3'UTR polymorphisms were associated with aspirin intolerance in asthma but the molecular basis for this interesting statistical correlation is not clear [350].

The CYSLTR2 is also a typical GPCR with 7-transmembrane spanning helices consisting of 346 amino acids. It binds Cys-LTs with different affinities in the following order: LTC4 = LTD4 ≫ LTE4 [351]. Searching the 1000 human genome database we found 71 non-synonymous genetic variations but only the Ser236Leu mutant (rs61735175) had an allele frequency >1% (Table 6). This amino acid exchange is rather conservative in nature and PolyPhen2 predictions suggested only minor effects on protein function (Horn et al., unpublished data). In addition, there is one nonsense variation (Trp104stop, COSM139999) but its low allele frequency suggests that homozygous allele carriers may hardly exist. Little is known about functionally important amino acids in CYSLTR2 but a genetic variation Met201Val (rs41347648) appears to be associated with atopy [352] and with bronchial asthma [353]. Met-to-Val exchanges are rather conservative and thus, only minor functional consequences may be predicted. However, experimental data suggested impaired receptor affinity for LTD4 [353].

### 5.3.3. Leukotriene B4 receptor 1 (BLT1)

The BLT1 gene is located on chromosome 14 and spans about 6.5 kb [354]. The promoter lacks typical TATA and CAAT boxes but contains one GC rich sequence, which can be activated by Sp1 [355]. No genetic promoter variations have been associated with diseases. BLT1 is a GPCR with 7-transmembrane spanning helices consisting of 352 amino acids [356]. There are 16 non-synonymous coding variations in the 1000 human genome database but none of them has an allele frequency >1%. There is no nonsense mutation leading to the formation of a truncated receptor (Table 6). Little is known about the functional relevance of important amino

acids but Thr308 may be involved in BLT1 desensitization [357]. However, there is no genetic variation in the 1000 human genome database affecting this residue.

After ligand binding GPCRs activate intracellular G-proteins [358] and there are several genetic variations in the activating loop but neither of them has an allele frequency >0.1%. Helix 8 of the receptor is required for G-protein activation [359,360] and several leucines appear to be important for receptor expression [361]. However, none of these residues is affected by any genetic variation.

#### 5.3.4. Leukotriene B4 receptor 2 (BLT2)

The BLT2 gene is located on chromosome 14 and spans about 6.3 kb [362–364]. Little is known on transcriptional regulation of BLT2 and no promoter polymorphisms have been associated with any disease.

BLT2 is also a GPCR with 7-transmembrane spanning helices and consists of 389 amino acids [363,364]. It binds different LTB4 derivatives [365] with high affinity (LTB4 > 12-epi-LTB4 > LTB5 > LTB3). 43 non-synonymous coding variations were found in the 1000 human genome database and two of them (Table 6), Arg80Gln (rs150416614) and Asp196Gly (rs1950504), have an allele frequency >1%. PolyPhen2 predictions suggest that Asp196Gly has only minor functional consequences while Arg80Gln might be deleterious (Horn et al., unpublished data). This residue is located in a putative cytoplasmic domain and thus, additional experiments are required to characterize the functional impact. There is no nonsense variation leading to truncated receptor.

#### 5.3.5. Other leukotriene receptors (GPR17, P2RY12 and GPR99)

The gene encoding for GPR17 is located on chromosome 2. It spans about 6.8 kb and consists of two coding exons and an additional exon containing the 5' UTR region [366,367]. Little is known about the functional elements in the promoter region of this gene and currently no promoter polymorphisms have been associated with any disease. The gene encodes for a classical 7-transmembrane G-protein coupled receptor (GPCR) consisting of either 339 or 367 amino acids [367]. It selectively binds uracil nucleotides and cysLTs [368], and is a negative regulator of the cysLTR1 response to LTD4 [369]. There are 67 non-synonymous genetic variations for this receptor published in the 1000 genome database but none of them has an allele frequency >1% (Table 6). There is a single nonsense variation (Trp6stop, rs61742898) leading to a C-terminally truncated protein but it remains unclear whether this truncation is of functional importance.

The gene encoding for P2RY12 is located on chromosome 3 and spans about 47.4 kb. It consists of two exons separated by a single intron [370]. The receptor also belongs to the family of the GPCRs and consists of 342 amino acids [370]. Analyzing the 1000 genome database we found that there are 32 non-synonymous genetic variations but only one of them (Glu330Gly, rs16846673) qualifies as SNP (Table 6). However, PolyPhen2 prediction suggests that this mutation has only minor functional consequences (Horn et al., unpublished data). Mutations in the P2RY12 gene have been discovered in patients with platelet-type bleeding disorder-8 and congenital bleeding disorder [371,372] and these mutants appear to impact receptor functionality [372]. Unfortunately, it remains unclear whether these patients have aberrant leukotriene signaling.

The GPR99 (GPR80, OXGR1) gene is located on chromosome 13 and spans about 9 kb. It consists of two exons separated by a single intron [373]. Like the P2Y receptor it belongs to the GPCRs and consists of 337 amino acids [374]. It has been implicated in citric cycle regulation [375] but it also binds LTE4 [376]. Analyzing the 1000 genome database we found that there are 58 non-synonymous genomic variations but none of them qualifies as SNP (Table 6). In addition, there are five nonsense variations [Arg148stop

(COSM1677754), Tyr198stop (rs370630287), Arg240stop (ss1349524372), Gln310stop (COSM432637), and Ser315stop (COSM126856)] but neither of them has an allele frequency >1%. Unfortunately, little is known on the functionality of different amino acids for ligand binding at the GPR99 receptor and thus, it is difficult to predict the functional consequences of amino acid substitutions.

## 6. Conclusions

LOXs are lipid-peroxidizing enzymes and require molecular oxygen for their activity which was virtually absent at the onset of life about 3 Bya. About 650 Mya the oxygen was enriched in the atmosphere owing to the photosynthetic activity of cyanobacteria in the oceans and of primitive land plants reaching a concentration of 35% about 300 Mya. Next, the oxygen level decreased again reaching today's steady state concentration of 21% around 250 Mya. The increase in atmospheric oxygen concentration was one of the driving forces for Cambrian species explosion, which dramatically increased the biodiversity on the planet. It might well be that LOXs as oxygen metabolizing enzymes have first been introduced during this time window of life development and the occurrence of LOXs in today's cyanobacteria [65] might be supportive for this hypothesis. However, the currently available sequence data suggest that only a minor share of cyanobacteria contain LOX genes and thus, it must be concluded that these enzymes may not be essential for the evolution of life. Today, LOX occur in two (bacteria, eucarya) of the three domains of life but a continuous development of this class of enzymes during early evolution of life is rather unlikely. In fact, for some prokaryotic LOXs horizontal gene transfer has been suggested [9,63] and there is the possibility of lineage-specific loss of LOX genes in other bacterial species. The combination of these complex gain-of-function and loss-of-function events makes the search for the most ancient LOX isoforms problematic. In plants LOXs are widely distributed. They are already found in algae, which constitute the most primitive photosynthesizing plant-like eucarya but they also occur in fungi. Algae occurred during evolution of life already 1.6–1.5 Bya and are considered the most ancient producers of polyunsaturated fatty acids, the major substrate of LOXs. However, in this time window the oxygen concentration in our atmosphere was rather low and thus, despite the presence of PUFAs a true LOX reaction should not have taken place. Thus, oxylipin biosynthesis in algae and fungi [101,102] should have been introduced later on. In protozoa, LOX-like sequences have been reported but functionality of the corresponding enzymes remains unclear. The low degree of LOX distribution in protozoa suggests that the enzymes may not be essential for survival of the corresponding species. In lower metazoa a number of functional LOXs have been identified as fusion and/or non-fusion proteins [135,136,141] and sequence searches [145] suggested the occurrence of functional LOXs in additional invertebrate species (Table 2). However, it is somewhat difficult to estimate the percentage of LOX-containing invertebrates among all invertebrates because only a limited number of completely sequenced invertebrate genomes are currently available. In *Chordata* LOXs are more frequently distributed. Potential LOX sequences appear to be present in primitive *Chordata* such as *Cephalochordata* and *Tunicata* as indicated by the genomic sequences of selected model organisms (*B. floridae*, *C. intestinalis*). However, it remains unclear whether the corresponding proteins are functional LOXs and if other representatives of these subphyla also carry LOX genes. All higher vertebrates (cartilaginous fish, bony fish, amphibia, reptilia, and birds) contain LOX-like sequences and although no functional data are currently available for most of them the existence of true LOXs in these animals are likely. In mammals LOX sequences are widely distributed and functional LOX isoforms

have been identified in a large number of highly developed mammalian species including many primates.

ALOX5 constitutes the key enzyme of leukotriene biosynthesis. Since putative ALOX5-like sequences have been identified in *Cephalochordata* and *Tunicata* leukotriene signaling might already have been present in these primitive *Chordata*. When we searched the genome databases of *B. floridae* and *C. intestinalis* we found a CYS-LTR1-like (XP002126547.2) and a LTA4H-like (XP002123481.1) sequence in the genome of *C. intestinalis* supporting this hypothesis. However, this is not the case for *B. floridae*, which is evolutionary more closely related to vertebrates. Moreover, the LOX sequences found in the genomes of these two species are more similar to coral LOXs than to ALOX5 isoforms of higher vertebrates (data not shown) and thus, leukotriene biosynthesis in *C. intestinalis* may be unlikely. To answer the question of whether primitive *Chordata* already have functional leukotriene signaling more direct experimental data and additional genomic sequences for other species representing this evolutionary stage are required. However, from the observation that both cartilaginous and bony fish (ray- and lobe-finned) appear to have all major enzymes and receptors of the leukotriene cascade it may be concluded that this signaling pathway has been introduced during evolution of life earlier than previously expected [158]. In this context it would be interesting to find out whether extinct fish species like armoured jawless fishes (*Ostracodermata*) or the primitive jawed fishes (*Placodermata*) carried functional genes for enzymes and receptors of leukotriene signaling. Genomic data on these extinct species would be helpful to close this gap of knowledge.

## 7. Perspectives

During the past decade the genomes of an increasing number of recent and extinct living organisms have been sequenced at high genome coverage and these high quality sequence data provide a suitable basis for detailed sequence comparisons to conclude the evolutionary relatedness of different species and their phylogenetic origin. However, a major problem for such conclusions is the lack of functional data for many of the published sequences. For instance, it remains unclear of whether or not the LOX-like sequences identified for the *Acanthamoeba Polyphaga Mimivirus* (YP\_003986553.1) encodes a functional LOX enzyme. The sequence data are strongly supportive but sometimes it simply takes a single amino acid exchange to convert a catalytically active protein into an inactive enzyme (see Section 5.1.2). However, since the sequence is now known for this and other potential LOX isoforms the corresponding enzymes can be expressed as recombinant proteins and characterized with respect to their protein chemical and enzymatic properties. Similarly, it is of particular evolutionary interest to find out whether the LOX-like sequences identified in lower *Chordata* (*B. floridae*, *B. belcheri*) and in lower *Vertebrata* (*P. marinus*) encode for functional LOX isoforms. For *Amphibia*, *Reptilia* and *Aves* this review focused on selected model organism (*Xenopus Silurana tropicalis*, *A. mississippiensis*, *G. gallus*) because of space constraints. However, these restrictions might lead to misleading conclusions since other representatives of these evolutionary classes may be different. Thus, the genomes of additional class members should be screened for the presence of LOX-like sequences. The chemical stability of DNA and the improvement of the DNA sequencing techniques nowadays allows sequencing of complete genomes from excavated fossils representing extinct species. A single bone (manual phalanx) found in the Denisovan cave in south Siberia was sufficient to obtain the complete genomic sequence of a new relative of modern humans, *H. denisovan* [232]. This bone was conserved in the cave for more than 50,000 years but its DNA was well preserved so that a more than 40-fold genome coverage

was reached during sequencing. Similar results might be obtained with even older plant and animal fossils particularly if the fossils are conserved in permafrost environments or in deserts. Sequencing the DNA remnants of *Mammuthus* or even of different dinosaurs will provide valuable information on the occurrence of LOX and leukotriene signaling in these animals, which dominated nature in ancient time.

In modern humans leukotrienes have been implicated in the pathogenesis of inflammatory and hyperproliferative diseases [6,10] and thus, defects in leukotriene biosynthesis and in leukotriene signaling are expected to impact severity and duration of the disease. Consequently, naturally occurring mutations in the genes involved in leukotriene signaling are likely to impact the individual susceptibility for a specific disease and/or the responsiveness to therapeutic strategies. There are a number of correlation studies, which related specific alterations in leukotriene relevant genes to the development of a number of disorders or to the responsiveness of certain therapies. Although some studies showed significant differences, in most cases the causal chains have not been identified. The major reason for lacking causality is the fact that no reliable functional data are currently available for most genetic variations. Thus, in many cases it remains unclear of whether the naturally occurring mutants lead to defective or improved leukotriene signaling and this information is of major importance for interpretation of the clinical correlations.

## Conflict of interest

The authors state that there is no conflict of interest with this work.

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