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## **The interest of oral calcium loads test in the diagnosis and management of pediatric nephrolithiasis with hypercalciuria: experience from a tertiary pediatric centre**

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## Double lipoxygenation of polyunsaturated fatty acids of nutritional interest

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### Abstract.

Double lipoxygenation of polyunsaturated fatty acids having at least three methylene-interrupted double bonds can be made by two lipoxygenases, e.g. 5- and 12-LOX, or 15-LOX only, followed by reduction of the hydroperoxide products through the glutathione peroxidase action. Several biological activities have been reported for such a double 15-LOX product of docosahexaenoic acid, called protectin DX to differentiate it from protectin D1, a stereo and geometric isomer described for its potent anti-inflammatory potential. The geometric characteristic of the double lipoxygenase products is the conjugated triene *E,Z,E* (*trans,cis,trans*), which appears crucial in their biological activities. A focus is also done on single lipoxygenation of mono-hydroxylated products first made by aspirin-treated cyclooxygenase-2. The resulting (*R,S*)-diOH, *E,Z,E* conjugated trienes, instead of the (*S,S*)-diOH isomer in case of double lipoxygenation, seem to be even more active for some biological effects, making biologically relevant the single lipoxygenation in aspirin-treated situations.

### Introduction.

Lipoxygenases oxygenate polyunsaturated fatty acids (PUFA) with at least two *cis/Z* double bonds separated by one methylene group. They produce a hydroperoxide product with a *cis,trans/Z,E* conjugated diene [1].

Although lipoxygenases have been first described in plants, initially in soya bean [2], many articles relate to animal cell lipoxygenation, especially with arachidonic acid (ArA) as a substrate [3]. The main lipoxygenases have been reported to oxygenate carbons 5, 12, or 15 of ArA to make the corresponding hydroperoxy-eicosatetraenoates (HpETEs), further reduced into hydroxy derivatives (HETEs) by glutathione peroxidases [4]. A peculiar pathway relates to 5-lipoxygenation of ArA into 5-HpETE, further transformed into the 5,6-epoxy derivative, as catalyzed by the same enzyme [5]. This 5,6-epoxy derivative has been called leukotriene A<sub>4</sub> (LTA<sub>4</sub>) as it was first described in leukocytes, and owns a conjugated triene. In those cells, LTA<sub>4</sub> is quickly hydrolyzed into 5(*S*),12(*R*)-diOH-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid, named leukotriene B<sub>4</sub> (LTB<sub>4</sub>), exhibiting potent pro-inflammatory activity by promoting polynuclear neutrophil chemo-attraction [6]. It is noteworthy that such a double oxygenated product from ArA is a single, not a double, lipoxygenase product.

### Double lipoxygenation of 5,8,11,14-eicosatetraenoic acid (20:4 $\omega$ 6/ArA).

An early described double lipoxygenase product of ArA is a LTB<sub>4</sub> stereo and geometric isomer, made by both 5- and 12-lipoxygenases, present in blood leukocytes and platelets, respectively. The reduced product of 5-HpETE, 5-HETE from leukocytes, or the reduced product of 12-HpETE, 12-HETE from platelets, may be further converted by platelet 12-

LOX, or leukocyte 5-LOX, by a transcellular process, and reduced into the same final product 5(*S*),12(*S*)-diOH-6*E*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid [7].

Interestingly enough, when those two stereo and geometric isomers were evaluated for their putative capacity of inhibiting platelet aggregation, comparatively to various HETEs, it was clear that the LTB<sub>4</sub> isomer (that we call LTB<sub>x</sub>) shared the inhibition of platelet aggregation induced by HETEs, whereas LTB<sub>4</sub> was totally inactive [8]. At that time, we hypothesized that the absence of inhibitory activity by LTB<sub>4</sub> might be due to its 12(*R*) stereochemistry instead of 12(*S*) in LTB<sub>x</sub>, because of the (*S*) stereochemistry of all active HETEs tested.

Another example of double lipoxygenase product from ArA is 8(*S*),15(*S*)-diOH-5*Z*,9*E*,11*Z*,13*E*-eicosatetraenoic acid made by 15-LOX, and further reduction of the primary hydroperoxides [9].

### **Double lipoxygenation of 4,7,10,13,16,19-docosahexaenoic acid (22:6 $\omega$ 3/DHA).**

Although DHA has been found to be doubly oxygenated by soybean lipoxygenase (15-LOX) and further reduced into 10(*S*),17(*S*)-diOH-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-22:6 [10], substantial work has been done about a single lipoxygenase product of DHA which has been defined as 10(*R*),17(*S*)-diOH-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-22:6, called protectin D1, and reported for its anti-inflammatory activity [11], also called neuroprotectin D1 for its action in neuroprotection [12]. Also, its position isomer 7(*R*),14(*S*)-diOH-4*Z*,8*E*,10*E*,12*Z*,16*Z*,19*Z*-22:6, called maresin 1, has been described with similar anti-inflammatory potential [13].

Accounting for the blood platelet inhibitory activity of LTB<sub>x</sub> [8], we looked at the potential activity of 10(*S*),17(*S*)-diOH-4*Z*,7*Z*,11*E*,13*Z*,15*E*,19*Z*-22:6, **which is a double lipoxygenation product of DHA through 15-LOX, as 8(*S*),15(*S*)-diOH-5*Z*,9*E*,11*Z*,13*E*-20:4 from arachidonic acid [9].** It was made by soybean lipoxygenase, then reduced by NaBH<sub>4</sub>, followed by proper structural characterization. We found this compound is able to inhibit platelet aggregation by the physiological agent collagen [14], and called it PDX to differentiate it from PD1. We further characterized this activity by comparison with other double (*S*) di-OH conjugated *E,Z,E* conjugated trienes, globally called poxytrins (PUFA oxygenated trienes). We also found that poxytrins may decrease platelet aggregation by both inhibiting the cyclooxygenase activity, meaning the generation of PGH<sub>2</sub>/TxA<sub>2</sub>, and the aggregating power of the latter [15]. We have more recently extended such observations for two *E/Z/E* conjugated trienes from alpha-linolenic acid (18:3 $\omega$ 3), called linotrin [16]. Surprisingly, we found that the (*R*),(*S*)-diOH isomer linotrin was slightly more potent than the double (*S*) **isomer**, in agreement with what we found earlier with the 10(*R*),17(*S*) isomer of PDX [15]. **This suggests that, in addition to the *E/Z/E* conjugated triene inhibitory effect on platelet aggregation, the stereochemistry of the hydroxyl groups may affect such inhibitory activity.** This agrees with earlier findings that show that all chemically synthesized (*R*) stereoisomers of mono-hydroxy derivatives of PUFA were slightly more potent than the corresponding (*S*) stereoisomers to inhibit thromboxane-induced platelet aggregation [17]. Considering that (*R*) stereoisomers may be first generated by aspirin-treated cyclooxygenase-2 (COX-2) [18,19], a further lipoxygenation of the aspirin-treated COX-2 products might produce (*R*),(*S*) di-OH derivatives, with anti-aggregating activity when an *E/Z/E* conjugated triene occurs between the two hydroxyl groups. **The 10(*R*),17(*S*) as well as 10(*S*),17(*R*) isomers of PDX might be then called AT-PDX.**

Since these observations, PDX has been described active in several other biological systems. Chronologically, PDX has been reported to inhibit the replication of influenza virus [20], although the Authors called it PD1 instead of PDX, except in material & methods. These Authors recognized the biological activity of PDX against the influenza virus in a further review [21]. **For this anti-viral activity, the (*E,Z,E*) conjugated triene might be crucial as resolvins D1 and D2, with *E,E,Z,E* and *E,Z,E,E*, conjugated tetraenes, were totally inactive**

[20]. In addition, it has been described that PDX is able to restore insulin sensitivity in obese mice [22], and to suppress hepatic gluconeogenesis [23]. In terms of inflammation status, PDX has been shown to inhibit ROS production by human neutrophils [24], to reduce neutrophil extravasation [25], and to increase the M2 macrophages polarization [26]. More recently, PDX has been shown to prevent H<sub>2</sub>O<sub>2</sub>-mediated oxidative stress in vascular endothelium [27], and improve palmitate-induced hepatic insulin resistance [28].

### **Relevance of PDX in the brain.**

PD1 has been reported to exert neuroprotective effects, and for that reason was also named neuroprotectin D1 (NPD1) [12]. Looking at the presence of PDX in normal rat brain, we found it within brain homogenates, as well as its homolog from ArA, namely 8(*S*),15(*S*)-diHETE. However, their amounts were around ten times lower than that of mono-hydroxy derivatives from ArA and DHA. Whereas LTB<sub>4</sub> could be detected at two-fold lower amounts than its corresponding double lipoxygenation product analog, 8(*S*),15(*S*)-diHETE, PD1, the equivalent of LTB<sub>4</sub> homolog, was undetectable [29]. However, we were able to detect PD1 in brain from rats submitted to the inflammatory bacterial component lipopolysaccharide (unpublished results). This suggests that PD1 might be produced in inflammation states only.

The difference between LTB<sub>4</sub> that we found in normal rat brain, and PD1 that could not be detected, might putatively be considered as resulting from the difference between 5-LOX and 15-LOX. 5-LOX, involved in the generation of LTB<sub>4</sub>, is known to exhibit a dual activity, making 5-HpETE and converting it to LTA<sub>4</sub>, its 5,6-epoxy derivative [5]. **Apart from the reticulocyte 15-LOX, which has been reported to favor the formation of 14,15-epoxy-5Z,8Z,10E,12E-20:4 (called 14,15-leukotriene A4) from 15-HpETE [30], the latter 15-LOX product is preferentially reduced into 15-HETE by cell glutathione peroxidase (GPx).** We may then speculate that in normal situations, cell GPx activity is strong enough to reduce hydroperoxides, **like 15-HpETE and 17-HpDoHE from 20:4 and 22:6, respectively,** as soon as they are produced. In contrast, in oxidative stress situations, as we may expect in inflammation, the overall altered GPX activity would allow some of those hydroperoxides to escape the reduction, allowing the formation of some 16,17-epoxy derivative from the unstable 17-HpDoHE, as a kind of « stabilization ». We might then speculate that such an epoxide formation followed by PD1 generation is an adaptation to oxidative stress to generate this anti-inflammatory protectin.

Since PDX seems to be present in normal brain, we may anticipate a biological function that we could point out by increasing its local concentration. For transferring PDX to the brain, we have prepared a lipid vector, already used for DHA. The structured phospholipid to transfer DHA to the brain is 1-acetyl,2-docosahexaenoyl-glycerophosphocholine (AceDoPC), a mimetic and stabilized form of 1-lyso,2-docosahexaenoyl-glycerophosphocholine (LysoPC-DHA) [31]. Indeed, LysoPC-DHA is now considered as the physiological vector of DHA to the brain [32] and preferred to DHA because of the specific transporter Mfsd2a for the former [33]. The AceDoPC mimetic with PDX in place of DHA, called AceDoxyPC, has been prepared [34], but its use to increase the PDX brain concentration remains to be investigated.

### **Conclusion.**

The double lipoxygenase products of PUFA, requiring at least three methylene-interrupted double bonds may be produced in physiological situations. Among them, PDX, a 15-LOX double **oxygenation** product from DHA, has been described with diverse biological activities, suggesting that such products from other PUFA might be of biological interest. Shared biological activities of stereoisomers produced by single lipoxygenation of (*R*)-OH derivatives **formed** by aspirin-treated COX-2 **from** PUFA, also opens perspectives in the

worldwide population using aspirin in different situations (Figure 1 summarizes the conclusion).

### Figure legend.

Figure 1 shows two pathways leading to *E,Z,E* conjugated trienes from polyunsaturated fatty acids (PUFA). Figure 1A relates to PUFA having at least three methylene-interrupted double bonds, and Figure 1B is specific for docosahexaenoic acid (DHA). One pathway (on the left of each Fig 1A and 1B) involves a double lipoxygenation, either by the same enzyme (15-lipoxygenase) or two enzymes (5- and 12-lipoxygenases), and reduction of the hydroperoxide intermediates by glutathione peroxidase (GPx). The resulting compound is a double (*S*) dihydroxy (diOH) product. The other pathway (on the right of each Fig 1A and 1B) involves first a mono-hydroxylation by aspirin-treated cyclooxygenase-2 (COX-2), and then a lipoxygenation followed by the hydroperoxide reduction by GPx of the LOX product. The hydroxylation by aspirin-treated COX-2 providing a (*R*) mono-hydroxy derivative, the final product is a (*R,S*)- or (*S,R*)-diOH derivative with a *E,Z,E* conjugated triene as well. Both types of products exert diverse biological activities, as shown for protectin DX (PDX) obtained by double lipoxygenation of docosahexaenoic acid (DHA), and **AT-PDX formed by aspirin-treated COX-2 followed by 15-lipoxygenase action on DHA**. Successive conjugated dienes and trienes are shown in blue in Fig 1B). Considering human blood platelet functions, various double lipoxygenase products, globally called poxytrins (PUFA oxygenated trienes) share both the inhibition of prostaglandin H<sub>2</sub>/thromboxane A<sub>2</sub> production and action.

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