



## Targeting lysyl-oxidase (LOX) may facilitate intramural periarterial drainage for the treatment of Alzheimer's disease

Louise Kelly<sup>a,\*</sup>, Matthew Macgregor Sharp<sup>a</sup>, Isabelle Thomas<sup>b</sup>, Christopher Brown<sup>a</sup>, Matthew Schrag<sup>c,d,e</sup>, Lissa Ventura Antunes<sup>c,d,e</sup>, Elena Solopova<sup>c,d,e</sup>, José Martinez-Gonzalez<sup>f,g,h</sup>, Cristina Rodríguez<sup>g,h</sup>, Roxana Octavia Carare<sup>a,\*</sup>

<sup>a</sup> Faculty of Medicine, University of Southampton, Southampton, United Kingdom, UK

<sup>b</sup> Cardiff University, UK

<sup>c</sup> Department of Neurology, Vanderbilt University Medical Center, Nashville, TN, USA

<sup>d</sup> Vanderbilt Memory and Alzheimer's Center, Vanderbilt University, Nashville, Tennessee, United States of America

<sup>e</sup> Vanderbilt Brain Institute, Vanderbilt University, Nashville, Tennessee, USA

<sup>f</sup> Instituto de Investigaciones Biomédicas de Barcelona (IIBB-CSIC), Barcelona, Spain

<sup>g</sup> CIBER de Enfermedades Cardiovasculares, Instituto de Salud Carlos III, Madrid, Spain

<sup>h</sup> Institut d'Investigació Biomèdica Sant Pau (IIB SANT PAU), Barcelona, Spain

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### ABSTRACT

Alzheimer's disease is the commonest form of dementia. It is likely that a lack of clearance of amyloid beta (A $\beta$ ) results in its accumulation in the parenchyma as A $\beta$  oligomers and insoluble plaques, and within the walls of blood vessels as cerebral amyloid angiopathy (CAA). The drainage of A $\beta$  along the basement membranes of blood vessels as intramural periarterial drainage (IPAD), could be improved if the driving force behind IPAD could be augmented, therefore reducing A $\beta$  accumulation. There are alterations in the composition of the vascular basement membrane in Alzheimer's disease. Lysyl oxidase (LOX) is an enzyme involved in the remodelling of the extracellular matrix and its expression and function is altered in various disease states. The expression of LOX is increased in Alzheimer's disease, but it is unclear whether this is a contributory factor in the impairment of IPAD in Alzheimer's disease. The pharmacological inhibition of LOX may be a strategy to improve IPAD and reduce the accumulation of A $\beta$  in the parenchyma and within the walls of blood vessels.

### Cerebral amyloid angiopathy and Alzheimer's disease

Alzheimer's disease is the commonest form of dementia and its ultimate cause remains unknown. The hallmarks of AD consist of the extracellular deposition of amyloid-beta (A $\beta$ ) as plaques and in the walls of blood vessels as cerebral amyloid angiopathy (CAA), in addition to the intracellular deposition of hyperphosphorylated tau [1]. Approaches consisting of lowering the production of A $\beta$  or removing aggregated and oligomeric soluble A $\beta$  by immunisation against A $\beta$  have been extensively trialled, yet only a single recent clinical trial has shown an improvement in clinical outcomes so far [2–5], and the magnitude of the improvement in that trial was small and offset by concerns about toxicity. The series of failures suggest that either A $\beta$  is not an appropriate target [6], or that the treatments were administered too late and neurodegeneration was too advanced by the time these trials took place [2]. Active immunisation

against A $\beta$  results in the removal of plaques and worsening of CAA [7].

Most Alzheimer's disease cases are sporadic. Ageing is the greatest risk factor [8], followed by apolipoprotein E4 genotype [9] and cardiovascular disease [10]. In familial forms of Alzheimer's disease, mutations cause the overproduction of pathological A $\beta$ , leading to its accumulation within the parenchyma and blood vessels [11]. This suggests that in sporadic cases with no clear genetic cause, the clearance and degradation of A $\beta$  is impaired, resulting in the pathological accumulation of A $\beta$  in the extracellular spaces as plaques or CAA [12,13]. Plaques are composed mainly of A $\beta$ 42 but vascular amyloid contains predominantly A $\beta$ 40 [14]. Postmortem studies show that up to 80% of Alzheimer's brains [15,16] and 20% of aged non-Alzheimer's brains [16] have CAA. Moderate to severe CAA can be identified clinically as small foci of bleeding in cortical areas with iron-sensitive magnetic resonance imaging sequences and is detectable in 20–30% of patients

\* Corresponding author.

E-mail address: [l.kelly@soton.ac.uk](mailto:l.kelly@soton.ac.uk) (L. Kelly).

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with Alzheimer's disease [17,18]. Since increasing age does not result in an increase in the production of A $\beta$ , the pathogenesis of CAA reflects a failure of clearance of A $\beta$  and interstitial fluid from the ageing brain [13].

Considering the probable impairment of degradation and clearance of A $\beta$  in patients with sporadic Alzheimer's disease, it is perhaps not surprising that anti-A $\beta$  drugs and vaccines have not proven efficacious thus far. A $\beta$  is removed from the brain via several mechanisms, including proteolytic degradation, degradation by glial cells and clearance to the blood [19–23] which become less effective with age [24–26]. CAA is usually composed, primarily, of A $\beta$ 40. However, analysis of the brains of patients who took part in the AN1792 clinical trial [27] discovered that patients immunised against A $\beta$ 42 displayed a far higher incidence of CAA, which also contained proportionally more A $\beta$ 42 [28]. The A $\beta$ 42 in the vessels may have originated from the parenchymal pool of A $\beta$  and its drainage via the periarterial pathway was impaired. This indicates that an important limiting factor for A $\beta$  removal from the brain is along the walls of blood vessels. Because the majority of patients with Alzheimer's disease and a significant proportion of those with non-Alzheimer's dementia develop CAA, the removal and drainage of A $\beta$  via the blood vessels is an essential area for future research.

### Intramural periarterial drainage and CAA

The central nervous system (CNS) lacks a traditional lymphatic system. The two major fluids of the CNS, cerebrospinal fluid and interstitial fluid, follow different drainage pathways. Cerebrospinal fluid drains primarily to the blood via arachnoid villi and granulations [29]. There is no doubt that enlarged perivascular spaces are key radiological signs in cerebral small vessel disease and multiple sclerosis, or even present in normal ageing. The problem is that the concept of an actual space adjacent to the vessel wall present in the normal brain is wrong. Histological studies in humans [30] as well as electron microscopy studies in humans [31] demonstrate there are no spaces as such, but rather a compartment filled with extracellular matrix adjacent to the vessel wall and composed of the basement membranes of the astrocyte end feet fused with the basement membranes of the leptomeningeal adventitia. It is this compartment that is labelled as "perivascular space". Experimental *in vivo* studies using rodents demonstrate that this compartment is used for glymphatic entry/convective influx of CSF into the brain [32]. Interstitial fluid drains from the parenchyma along the basement membranes of capillaries, arterioles and arteries of the CNS [33,34] as intramural periarterial drainage (IPAD) [32]. Failure of IPAD leads to the deposition of A $\beta$  in the basement membranes of capillaries and arteries as cerebral amyloid angiopathy (CAA) [35]. IPAD occurs within the basement membranes against the direction of blood flow rather than alongside vessels. Ultrastructural analysis has shown that tagged A $\beta$  injected into the hippocampal parenchyma enters the wall of the capillary and travels along basement membranes surrounding the smooth muscles of arterioles and arteries, within the tunica media of the vessel [36]. IPAD is driven by the contractions and relaxation of the smooth muscle cells of vessels, which drive the fluid and solutes within the basement membranes in the opposite direction to the flow of blood [37]. As CAA progresses, it causes a range of structural changes in the vessels, including impairment of vascular smooth muscle function and survival, thickening of the vessel wall, loss of autoregulation and occasional rupture. The drainage of A $\beta$  from the brain may be improved if the driving force behind IPAD could be augmented, thus reducing the accumulation of A $\beta$ . The worsening of CAA and the presence of A $\beta$ 42 in the CAA after immunisation against A $\beta$  suggests that A $\beta$ 42 is solubilised from plaques and entrapped in the IPAD pathways [38].

### Cerebrovascular basement membranes

The conduits for IPAD are cerebrovascular basement membranes. The basement membranes are specialised forms of extracellular matrix

composed of glycoproteins and proteoglycans that are aligned along the abluminal side of endothelia separating the endothelia from pericytes/smooth muscle cells and pericytes/smooth muscle cells from astrocytes, forming membranes between different cell types [39] (Fig. 1). Basement membranes consist of highly cross-linked complexes of collagen IV, laminin, fibronectin, nidogen/entactin, and heparan sulphate proteoglycans. The formation of these cross-links is catalysed by the activity of lysyl oxidase (LOX) (Fig. 1). Both collagen IV and laminin are critical for basement membrane stability and can self-assemble into sheet-like structures interacting with each other by nidogen/entactin. LOX, an extracellular matrix amine oxidase, converts amines into highly reactive aldehydes that spontaneously form covalent crosslinks between fibrillar collagens and elastins, ensuring extracellular matrix structural integrity [40]. Each cell type of the perivascular compartment contributes to the composition of the extracellular matrix by producing structural and functional diversity between the basement membranes of different vessel types [41]. Cells interact with the extracellular matrix by the association of the transmembrane proteoglycan dystroglycan and integrin adhesion receptors with laminin networks [42]. Dystroglycan is expressed in astrocytes, neurons and endothelial cells. Integrins are present on all cell types involved in the formation of the blood-brain barrier. Integrin adhesion receptors regulate signalling pathways but also anchor cells in place regulating their motility [43].

Ageing and cerebrovascular diseases are associated with changes in the basement membrane. Degradation, splitting, duplication, thickening and presence of abnormal inclusions have been observed in animal models of ageing and subarachnoid haemorrhage [44] [45,46], in aged human brains [45,46] and in humans following ischaemic strokes with haemorrhagic transformations [47] which is possibly due to an increase in the expression of proteolytic enzymes [48].

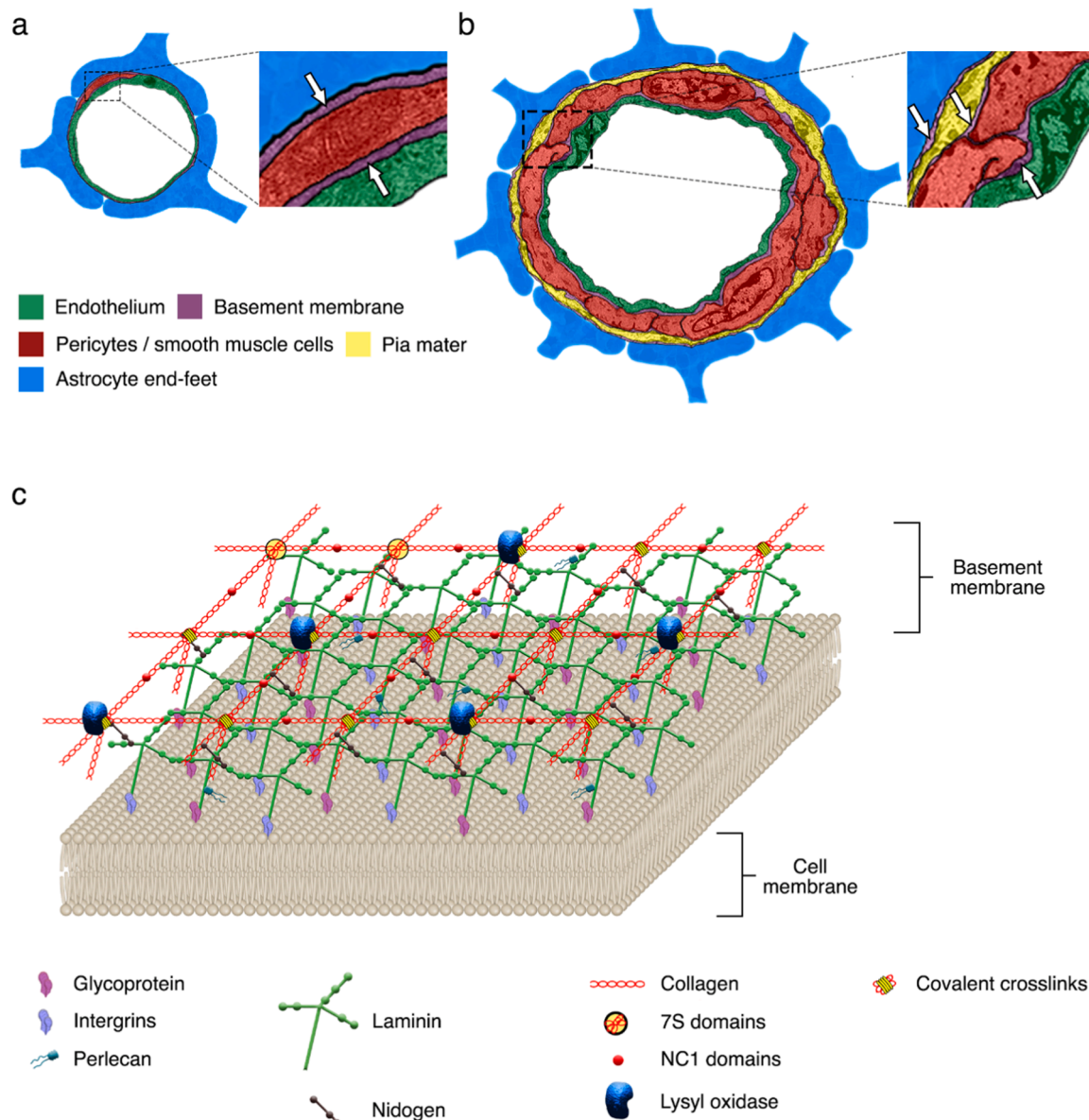
Remodelling of the brain microvasculature and biochemical alterations to basement membranes are common in ageing and neuropathological conditions. Prominent fibrosis, associated with degeneration of vascular smooth muscle cells [49,50] and alterations to basement membrane proteins such as collagen IV have been reported in ageing [51], Parkinson's disease [52] and Alzheimer's disease [53]. In Alzheimer's disease, the development of CAA has even been attributed to biochemical changes to basement membrane proteins that directly interact with A $\beta$ . These proteins either promote A $\beta$  aggregation (perlecan, fibronectin and agrins) [54,55] or inhibit A $\beta$  accumulation by destabilising its fibrilization (laminin, nidogens and collagen IV) [56]. In the early stages of Alzheimer's disease, thickening of the basement membrane [57] is accompanied by increased levels of collagen IV, perlecan and fibronectin [58] but in later stages, collagen IV is reduced [59] and heparan sulphate proteoglycans such as agrin are increased [60,61] creating an environment that favours A $\beta$  aggregation as CAA. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved in the turnover of basement membranes. Enzymes such as LOX are involved in morphological changes of basement membranes [62].

The morphological and physiological changes in cerebrovascular basement membranes are essential for IPAD and therefore the efficient removal of A $\beta$ . It is therefore imperative to understand the biology of the regulatory processes of basement membranes during ageing and with risk factors identified for Alzheimer's disease.

### Facilitating the clearance of amyloid from the brain

Experimental studies demonstrate that the efficiency of IPAD in clearing solutes found in interstitial fluid from the CNS decreases with age, most likely due to arterial stiffness and arteriosclerosis [63]. Arterial stiffness has been associated with the progression of Alzheimer's disease [64]. Improving the compliance and contractions of the smooth muscle cells driving IPAD may improve the clearance of A $\beta$  and either prevent its accumulation or allow its full removal from the brain.

Intervening in the remodelling of basement membranes via MMPs or



**Fig. 1.** Schematic diagram of a typical cerebral capillary (a) and arteriole (b). Basement membrane (purple) align along the abluminal side of endothelia (green) separating endothelia from pericytes/smooth muscle cells (red) and pericytes/smooth muscle cells from astrocytes (blue) (enlarged insets in a & b). Basement membranes consist of highly crosslinked complexes of collagen IV, laminin, fibronectin, nidogen/entactin, and heparan sulphate proteoglycans (c). Lysyl oxidase reinforces collagen IV networks by forming covalent crosslinks at 7S domains.

TIMPs may not be feasible due to their inability to act locally and the risk of systemic effects. However, it may be possible to act upon LOX, also involved in the remodelling of the extracellular matrix. Described in 1968 as an enzyme responsible for cross-linking collagen and elastin by converting lysine to allycine [65], the role of LOX in maintaining and stabilising the basement membrane of peripheral blood vessels is well established [66]. It remains to be seen if the effects of LOX on cerebral arteries are similar to those in systemic arteries. Ultimately the goal is to facilitate vasomotion, not just contractility and this may require a fine balance between LOX, MMPs and TIMPs.

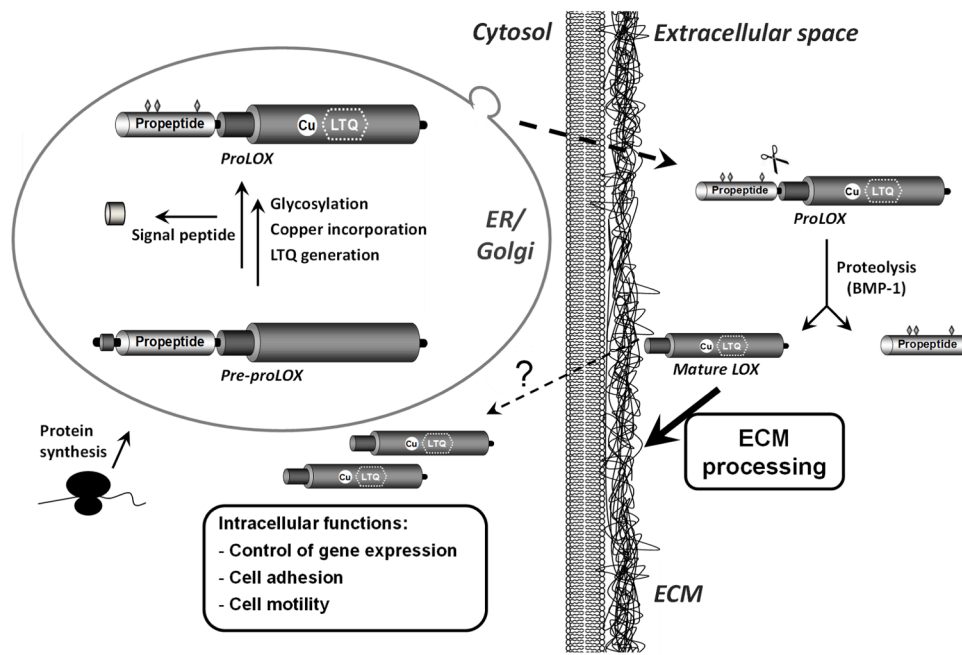
**Lessons learned from research into peripheral roles of LOX**

LOXs consist of a family of extracellular copper-containing enzymes comprised by five closely related isoenzymes, LOX and LOX-like (LOXLs; LOXL1-4). LOXs are responsible for the oxidative deamination of specific ε-amino groups of lysine and hydroxylysine residues in

collagen and elastin chains, the first step in the covalent cross-linking of the extracellular matrix, which is critical for the maintenance of the tensile and elastic properties of connective tissues [67,68]. Although its primary function lies in extracellular matrix maturation, several recent reports have highlighted that this family of isoenzymes are involved in multiple biological activities (Fig. 2) [67,69]. The ubiquitous functions of LOXs along with the existence of active intracellular forms of LOXs suggest that their dysregulation might affect numerous pathophysiological processes, particularly those involved in vascular disorders.

In the vascular wall, LOX family members are expressed in endothelial cells, vascular smooth muscle cells and fibroblasts [67]. These enzymes have been involved in different processes underlying the onset and progression of atherosclerosis and restenosis; their inhibition has been linked to the destructive remodelling characteristic of arterial dissection and aneurysms in the peripheral vascular system [67]. However, there is a paucity of information about the contribution of LOXs to cerebrovascular function.





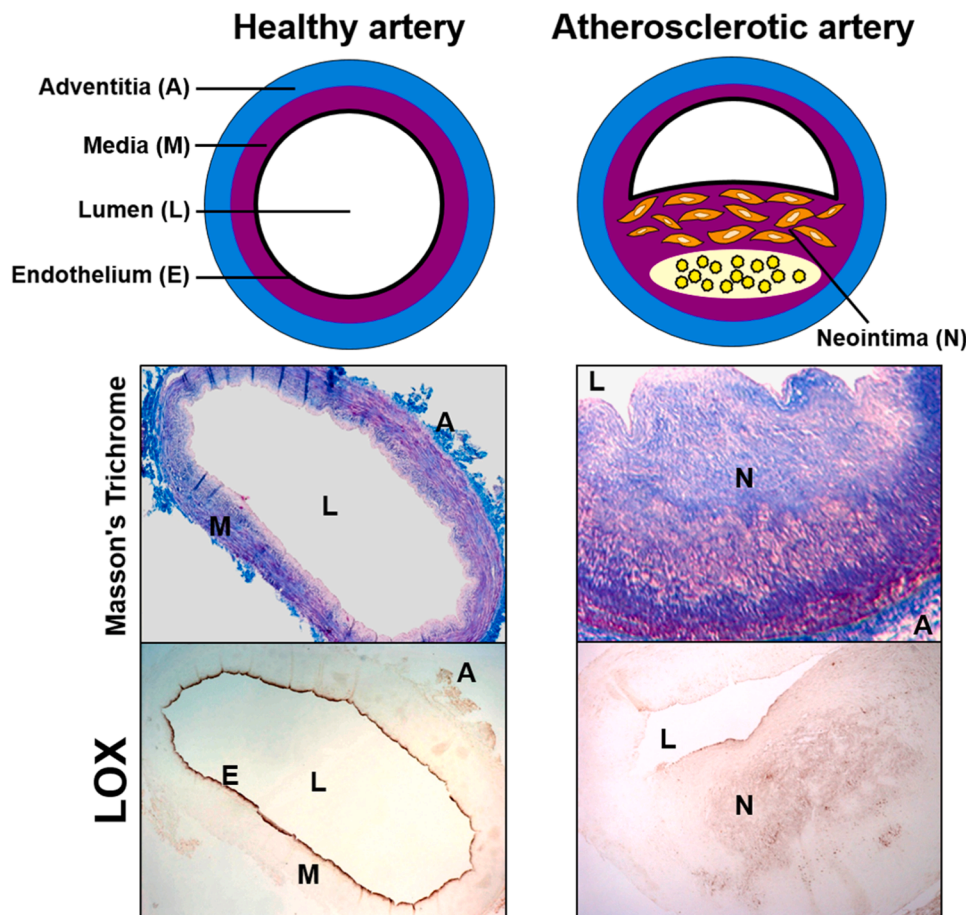
**Fig. 2. LOX synthesis and processing.** LOX is synthesized as a pre-proenzyme. In the endoplasmic reticulum (ER) and Golgi it is subjected to post-translational modifications, including signal peptide cleavage, copper incorporation, lysine tyrosylquinone (LTQ) generation and enzyme glycosylation. Then, the proLOX form translocates into the extracellular space, where it is proteolytically processed by bone morphogenetic protein (BMP-1), yielding the mature and enzymatically active LOX form and its pro-peptide. Mature LOX contributes to extracellular matrix (ECM) maturation through elastin and collagen crosslinking. In turn, intracellular mature LOX forms have been detected in the cytoplasm and nucleus, participating in the regulation of different cellular processes such as the control of gene expression and cell adhesion and motility.

**Contribution of LOXs to vascular aneurysm development**

Genetic sequencing in humans [70] as well as experimental work in rodents [71] support a role for dysregulation of LOX in the pathogenesis of aneurysms. It is now recognised in the literature that overexpression

or genetic mutations of LOX are involved in different aspects of the pathologies of arterial wall. It is not known which stage of the pathological process and this needs to be determined in future studies.

LOX may represent a valuable approach to preserve vascular integrity. Low expression of LOX has been detected in patients with cerebral



**Fig. 3. LOX in the vascular wall.** The scheme in the upper panel depicts the structure of a healthy vessel (left) and an atherosclerotic artery (right). Vascular layers (adventitia, media and endothelium) and lumen are indicated. In atherosclerotic lesions, vascular smooth muscle cell proliferation and migration leads to the formation of a neointimal tissue with a fibrous cap enriched in vascular smooth muscle cells and collagen (purple) covering a lipid core (yellow) containing inflammatory cells, cholesterol and macrophage-derived foam cells. Masson's trichrome staining of coronary arteries show vascular remodelling associated with atherosclerotic lesions, characterized by collagen deposition (purple; middle panel). In the bottom panel, note the strong staining of LOX found in collagen rich areas of atherosclerotic lesions, while, in healthy arteries, LOX is detected in the endothelium.

aneurysms and in animal models of this disease associated to an exacerbated inflammatory response [72]. Furthermore, polymorphisms in LOXL2 are associated with increased susceptibility to intracranial aneurysms [73]. Similarly, the development of abdominal aortic aneurysms (AAA) in preclinical models is frequently associated with a decrease in LOX expression [74,75]. In fact, genetic or pharmacologic inhibition of LOX induces AAA in different animal models [71,76], while local LOX overexpression promotes the regression of experimentally established AAA [75]. These effects seem to be isoform selective since ablation of LOXL1 in mice has no impact on aortic diameter [77]. More interestingly, recent research uncovers the major contribution of LOX to the sexual dimorphism of AAA, reporting higher aortic expression of LOX in women and how LOX inhibition in mice abolishes the protection against angiotensin II-induced AAA exhibited by females [78]. Likewise, downregulation of LOX has also been described in patients affected by aortic dissections of ascending aorta [79,80], while genetic variants of LOX seem to predispose to thoracic aneurysms [70].

### LOXs in the onset and progression of atherosclerosis: arterial stiffness

*In vitro* and *in vivo* studies of atherosclerosis support the hypothesis that inhibition of LOX activity may contribute to the endothelial dysfunction elicited by cardiovascular risk factors and inflammatory cytokines, impairing endothelial barrier integrity [68,81,82]. While the downregulation of LOX seems to be involved in the early stages of atherosclerosis development [83], expression of LOX is enhanced in advanced plaques from different animal models [84,85] (Fig. 3). Inhibition of LOX using  $\beta$ -aminopropionitrile (BAPN) [65,86] reduced the extent of atherosclerosis, limiting macrophage infiltration [85]. The enhanced expression of LOX in calcified areas from human advanced atherosclerotic lesions [87] (Fig. 3) and in fibrotic regions from human carotid endarterectomies in which higher LOX expression is coincident with more stable plaques, supports its role in the healing process that limits plaque rupture [88]. The potential contribution of LOX and LOXL2 to neovascularization and thrombosis [89–91] and the ability of LOX to control vascular smooth muscle cell proliferation and calcification [87,92] add further complexity to the intricate mechanisms through which LOX could influence plaque progression and stability.

Arterial stiffness, which is a predictor of cardiovascular events, is closely associated with atherosclerosis, hypertension and ageing and has also been linked to brain microvascular damage and the deposition of A $\beta$  [93–95]. The altered composition and structure of the extracellular matrix impacts on the mechanical properties of the vascular wall and is a major determinant of vascular stiffness. In this context, recent research has uncovered the relationship between the disturbance of the expression and/or activity of LOXs with changes in vascular compliance [96]. Analysis in transgenic mice that overexpress human LOX in vascular smooth muscle cell (TgLOX<sup>VSMC</sup>) demonstrated that LOX triggers an increase in vascular stiffness and alters the structure of elastin in mesenteric arteries [96]. These effects rely on the enhanced vascular LOX activity and production of reactive oxygen species exhibited by these animals. Hypertension appears to enhance the expression of vascular LOX; the enhanced LOX activity is partially responsible for the higher vascular stiffness and the disturbance of elastin structure characteristic of hypertensive animal models [96]. LOXL2 is associated with vascular stiffening and hypertension; blockade of LOXL2 expression prevents the age-dependant increase in vascular stiffness and delays the onset of hypertension [97]. We are unaware to date of specific studies addressing the contribution of LOXs to cerebral arterial stiffness and its impact on the brain microvascular function and perivascular amyloid clearance.

Late interventions in AD are not likely to yield results, as at that stage the smooth muscle cell function and structure are compromised. Early interventions focussing on improving vasomotion and the function of cerebrovascular smooth muscle cells promise to improve both perfusion

as well as IPAD. Inhibitors of LOX may help with delaying or preventing arterial wall stiffness and preserving arterial wall compliance.

### LOX and the brain

The role of LOX and LOXL in the cerebral vessels is less established than in the periphery. However, animal studies investigating the expression of LOX in various disease states have shown that LOX likely has a role in maintaining cerebral health. The inhibition of LOX following spinal cord injury in mice accelerated their functional recovery and improved some outcomes, suggesting that LOX may be involved in scar formation at injury sites [98]. Another study demonstrated that LOX has increased activity in rat experimental traumatic brain injury sites [99]. Conversely, a low copper diet or inhibition of LOX was found to increase the risk of cerebral haemorrhage in mice [100]. Taken together, these studies suggest that increased LOX expression may be a natural response to injury or lesions of the CNS.

Few studies have investigated how LOX expression and function is affected in neurodegenerative diseases. While investigating a role for LOX in amyotrophic lateral sclerosis, Li et al. found that LOX expression and functional activity was increased in some brain regions, such as the brainstem, spinal cord and cortex of SOD1 mutant mice [101]. The expression of LOX is increased in brains from patients with Alzheimer's and non-Alzheimer's dementia and LOX was identified in amyloid plaques and capillaries [102]. LOX is associated with senile plaques and CAA of both Alzheimer's patients and patients carrying the hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D) mutation, an autosomal dominant disease characterised by severe CAA [103]. A coding variant in LOXL4 was recently identified in a kindred with autosomal dominant AD and was reported to delay the onset of symptoms by 9 years [104]. These studies suggest that LOX has a disease modifying role in the progression of disease.

Mutations in LOXL1 have been associated with pseudoexfoliation syndrome and pseudoexfoliation glaucoma [105] in multiple populations, such as Spanish [106], Chinese [107], Finnish [108] and Icelandic [109]. These are age-related disorders characterised by the accumulation of fibrillar deposits in the eye, affecting up to 30% of people aged over 60 [110]. Several studies conclude that the risk of Alzheimer's disease is higher in pseudoexfoliation patients [111,112], though there has been some disagreement [113]. However, A $\beta$  has been identified in the aqueous humour from people with pseudoexfoliation syndrome [114,115]. Given that up to 91% of cases of pseudoexfoliation syndrome, an age-related disease itself, are strongly associated with three polymorphisms in LOXL1 [105], it is possible that LOXL1 polymorphisms may be associated with an increased risk of Alzheimer's disease or CAA although one study denies such association [116]. Clusterin (Apolipoprotein J) is involved in the pathology of both Alzheimer's disease and pseudoexfoliation syndrome, and is a possible explanation for the link between these two diseases [117].

### The role of LOX in CAA

Evidence presented so far suggests that in terms of brain vascular health following injury or insult, increased LOX is associated with disease and inhibition of LOX improves outcomes. Taking into consideration the association of LOX with Alzheimer's pathology and CAA, and the expression of LOX associated with the thickness of the basement membranes of blood vessels, it is probable that increased expression of LOX contributes to vascular dysfunction in ageing, the failure of IPAD, and development of CAA. A valuable tool for the study of IPAD and pathogenesis of CAA could be an animal model that overexpresses LOX [92].

Most studies investigating the role of the LOXs in the brain have focused on LOX rather than its homologs, LOXL1–4 [118]. A developmental study using immunohistochemistry to assess LOX and LOXL protein expression in mice at different ages found that they are

co-localised in some peripheral tissues, such as the skin and aorta, but not in other areas, such as the kidney and stomach [119]. Analysis of the brain revealed LOX and LOXL expression varied throughout different regions and cell types, with LOX expression stronger on endothelial capillary cells and pyramidal cells of the cortex and LOXL expression dominating in the commissural nerve fibres of the cortex and Purkinje cells of the cerebellum. Within the hippocampus, the expression of LOXL was much stronger than that of LOX in the pyramidal cell layer [119]. Although homologs, the processing and regulation of LOX [120] and LOXL [121] is different. Given the expression of both LOX and LOXL in endothelial cells of capillaries within the cortex [119], it is possible that an altered expression of either one of these enzymes could have a role in vascular disease in the brain. Since LOXL and LOX have widespread but differential distribution within the hippocampus and capillary endothelium, it is important to investigate the role of both LOX and LOXL when determining their contribution to the pathogenesis of CAA and Alzheimer's disease.

Inhibition of LOX may be a possible therapeutic strategy to improve brain health in diseases of the cerebrovasculature. BAPN is an irreversible inhibitor of LOX and LOXL which is widely used in pre-clinical studies [65,86,122]. BAPN has been used in clinical studies only in limited ways due to its substantial toxicity, including the risk of large vessel rupture [123,124]. Newer drugs, synthesised to be less toxic than BAPN, could prove beneficial to those with CAA or other vascular diseases of the brain. One such new drug is PXS-5505 already in Phase 1 clinical trials for myelofibrosis and cancer (ClinicalTrials.gov Identifier: NCT04676529) [125].

## Conclusion

The majority of research into the function of LOX and LOXLs has established the role of LOX in the peripheral cardiovascular system. Taken together, the evidence suggests that increased activity of LOX in particular, within the cerebrovascular system, could be part of a key mechanism of arteriosclerosis. Since the arteries of the brain are responsible for both IPAD and perfusion of fluid and solutes, arteriosclerosis forms the basis of both vascular dementia and the CAA of Alzheimer's disease. LOX therefore represents an attractive new target for reducing the stiffness of cerebral arterioles, thus improving both the perfusion of the brain as well as IPAD.

## CRedit authorship contribution statement

**Louise Kelly:** Conceptualization, Writing – original draft, Writing – review & editing. **Matthew Macgregor Sharp:** Writing – review & editing. **Isabelle Thomas:** Writing – review & editing. **Christopher Brown:** Writing – review & editing. **Matthew Schrag:** Writing – review & editing. **Lissa Ventura Antunes:** Writing – review & editing. **Elena Solopova:** Writing – review & editing. **José Martínez-González:** Writing – review & editing. **Cristina Rodríguez:** Writing – review & editing, Funding acquisition. **Roxana Octavia Carare:** Conceptualization, Writing – review & editing, Supervision.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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