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Cathepsin K: both a likely biomarker and a new therapeutic target in lymphangioleiomyomatosis?

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Abstract

Lymphangioleiomyomatosis (LAM) is a rare disease characterized by cystic lung destruction and lymphangiomas and is associated with a high risk of osteoporosis-related bone fractures. Its diagnosis is based on pulmonary anatomo-pathological criteria combined with chest computed tomography. Vascular endothelial growth factor-D is the only serum diagnostic biomarker used in clinical practice, while inhibition of the mammalian target of rapamycin (mTOR) pathway by rapamycin is currently the only reference therapy for LAM. Human cathepsin K (CatK), a potent collagenase predominantly found in osteoclasts, is considered a valuable target for anti-osteoporosis and bone cancer therapy. Recently, CatK, which is overexpressed in lung cysts, was proposed as a putative LAM biomarker. Moreover, CatK may play a role in the LAM pathophysiology by contributing to pulmonary cystic destruction and bone degradation. Accordingly, targeting the collagenolytic activity of CatK by exosite-binding inhibitors, in combination with mTOR inhibition, could represent an innovative therapeutic option for reducing lung destruction in LAM.

Keywords: Bourneville’s disease, biomarker, cathepsin, protease inhibitors, lung, lymphangioleiomyomatosis, protease, rapamycin (Sirolimus)



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CATHEPSIN K: AN OVERVIEW

Introduction

Cysteine cathepsins (cathepsins B, C, F, H, K, L, O, S, V, X, and W) are a group of eleven structurally related papain-like proteases (clan CA, family C1) in humans (MEROPS database; <http://merops.sanger.ac.uk>). These enzymes are primarily found in acidic endosomal/lysosomal compartments where they partake in various cellular processes (i.e., non-specific intracellular protein degradation and turnover, autophagy, and immune responses)^[1,2]. Cysteine cathepsins, which are expressed either ubiquitously or with tissue- and cell-type specificity, are also found outside lysosomes under specific conditions. When dysregulated, they often contribute to a wide range of pathophysiological events^[3,4]. Cathepsin K (CatK), which was discovered in the 1990s, is a highly potent collagenase with a restricted cell distribution. Its predominant expression in osteoclasts has promptly suggested specialized functions, including bone and articular cartilage resorption^[5]. Moreover, CatK, which is overexpressed in osteoporosis and bone cancers, has been validated as an attractive target for anti-osteoporosis therapy and anti-tumor treatments^[6-10]. Meanwhile, it is gaining increasing interest due to its potential roles in other physiological and pathological processes, including rare diseases.

First, we summarize contemporary knowledge of the molecular aspects of CatK (genomic organization, tissue expression, functional and structural characteristics, substrate specificity, and regulation of its activity) and review interventional strategies currently developed to selectively prevent the uncontrolled collagenolytic activity of CatK in osteoporosis and bone cancer. Then, following a concise description of lymphangioleiomyomatosis (LAM), a rare human disease, we discuss both the interest in using CatK as a new LAM biomarker and in targeting CatK to reduce lung destruction in LAM. Indeed, given the severity of the disease and the ensuing loss of lung function that might be associated with CatK expression level in LAM, inhibition of CatK could represent an additional therapeutic option, in addition to inhibition of the mammalian target of rapamycin (mTOR) pathway.

Cathepsin K: molecular and biological characteristics

Human CatK is encoded by a single gene [Cathepsin-K (CTSK), ~12.1 kb], localized on chromosome 1q21, such as cathepsin S (CatS)^[11]. CTSK gene expression is organized and tightly controlled at multiple steps, and likely involves the interaction of several transcription factors that are activated by cytokines. For instance, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-13 positively regulate the synthesis of CatK, while transforming growth factor (TGF)- β 1 and IL-10 may repress its expression level (for review:^[5,11]).

CatK (EC 3.4.22.38) is a monomeric endopeptidase (~24 kDa) that shares a common papain-like structure, consisting of two globular domains folded together to form a “V”-shaped active site cleft in the center. Although the catalytic triad Cys²⁵, His¹⁵⁹, Asn¹⁷⁵ (papain numbering) is well conserved, mapping studies of the crystal structure of CatK have revealed differences in the substrate binding subsites (labeled Sn-Sn’), which are located on both sides of the substrate’s scissile bond (corresponding to the complementary positions Pn-Pn’)^[12]. Notably, the residues forming the S2 binding pocket of CatK, which is the major determinant of its substrate specificity, are crucial for its preference for hydrophobic and aliphatic amino acids (for review:^[5]). Both the binding pattern and substrate specificity of CatK were previously detailed by Lecaillon *et al.*^[5]. Importantly, CatK displays an unusual ability among cysteine cathepsins to accept Pro in the S2 pocket, a recognition residue of collagens. Based on these data, selective substrates and activity-based probes for CatK have been developed^[13-16].

Compared with other human proteases, CatK displays a unique potency to cleave within the triple helix of type I and II collagens^[17]. Furthermore, the presence of chondroitin 4-sulfate (C4-S), a glycosaminoglycan (GAG) predominantly expressed in bone and cartilage, is required to promote its collagenolytic activity by forming high molecular complexes^[18]. The formation of an active complex between the negatively charged C4-S and specific positively charged residues of CatK (thereby shaping a so-called exosite located opposite the active site) is unique among human cysteine cathepsins [Figure 1]. In the absence of GAG, the ability of CatK to cleave triple helical collagen fibrils is impaired. Accordingly, it has been hypothesized that selective inhibitors able to disrupt the CatK/C4-S complex by targeting the C4-S binding exosite could inhibit its collagenase activity without impairing other regulatory peptidase and protease activities (see further section). In addition to collagens, CatK hydrolyzes other extracellular matrix (ECM) components such as elastin fibers and aggrecans^[19]. CatK is a lysosomal protease that is mainly active at an acidic pH of 5.5 and rapidly inactivated at neutral pH. Nevertheless, lung macrophages and osteoclasts secrete CatK, which remains active in the pericellular space due to the presence of H⁺-ATPase pump or Na⁺/H⁺ exchanger^[20,21]. In addition to its primary location in bone, high levels of CatK have also been detected in synovial fibroblasts, aortic smooth muscle cells, macrophages, and epithelial cells, while CatK messenger RNA (mRNA) levels increase in other tissues such as the ovary, synovia, heart, skin, and lungs following scarring or inflammation^[10,22,23]. Beyond its collagenolytic activity and its involvement in ECM remodeling, CatK also contributes to the maturation of thyroid hormones by participating in the processing of thyroglobulin^[24,25]. Moreover, it plays a pivotal protective role in lung homeostasis through its ability to proteolytically inactivate TGF- β 1^[26,27]. Over the last decade, a paradigm shift from viewing cysteine cathepsins as “simple” protein-degrading enzymes to recognizing them as key signaling molecules has emerged^[28], as demonstrated by the crucial roles of cysteine cathepsins (including CatK) in diverse cell-signaling cascades [e.g., peroxisome proliferator-activated receptor- γ (PPAR- γ) /caspase-8-mediated cell apoptosis, TGF- β signaling pathway]^[29-33].

Cathepsin K in osteoporosis, bone cancer and oral diseases

Besides its biological roles in bone turnover, skeletal, heart, lung and intestinal development, and reproduction, CatK expression may be dysregulated in bone resorption disorders such as osteoporosis and bone metastasis^[5]. CatK has been identified as the main osteoclastic bone-resorbing protease, and its overexpression is markedly related to extensive bone loss, which is highlighted by the presence of N-telopeptide of type I collagen (NTx) fragments (typical products of CatK cleavage) in the urine or serum of osteoporotic patients^[6]. Since inhibition of the CatK active site could prevent bone resorption without disturbing bone formation, the protease has become an attractive and validated target for anti-resorptive drug development^[34]. Moreover, abnormally high expression of CatK in various organs is intertwined with the extensive hydrolysis of elastin fibers or collagens in several cancers, such as prostate, breast, lung, and bone cancers^[8,10]. CatK, which contributes to tumor progression through extracellular degradation of ECM proteins, cytokine inactivation/activation, or pro-matrix metalloproteinases processing, has been proposed for differential prognostic purposes.

Oral and maxillofacial abnormalities have been observed in *Ctsk*(-/-) mice. Similarly, close relationships between defective CatK and oral diseases have been identified in patients with pycnodysostosis^[35], as well as in those with periodontitis, peri-implantitis, tooth movement, oral and maxillofacial tumors, root resorption, and periapical disease (see for review^[36]). Extensive histological and ultrastructural changes in cementum, a part of the periodontium, have been observed, which might be linked to compromised proteolytic activity of CatK^[37]. Mutations in the human *CLCN7*, which encodes voltage-gated chloride channel 7 (so-called ClC-7), lead to osteopetrosis associated with deformities in craniofacial morphology and marked tooth dysplasia. Loss of *CLCN7* function also results in lysosomal storage in the brain and jaw, and its surrounding tissues, which has been associated with CatK downregulation^[38].

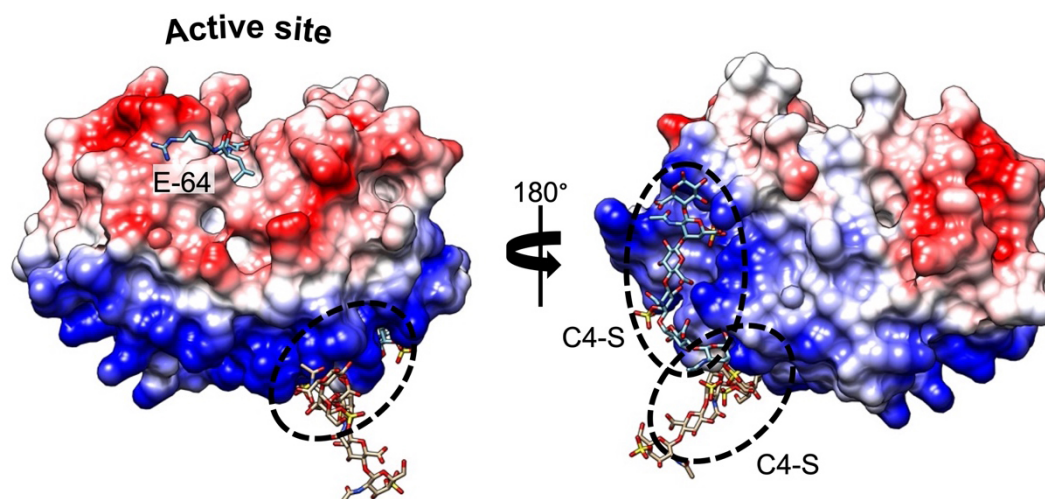


Figure 1. Complex formation between CatK and C4-S. Electrostatic surface distribution of human CatK. Blue and red areas represent positively and negatively charged surface domains of CatK, respectively (generated using Chimera software). The locations of two C4-S-binding sites (exosites) (PDB accession codes 3C9E and 4N8W) are marked by dashed circles, away from the active site. The pan-inhibitor of cysteine proteases, E-64, is located in the active site of the enzyme. The peptidase is shown in surface representation, while C4-S and E-64 are shown as sticks. CatK: Cathepsin K; C4-S: chondroitin 4-sulfate; PDB: protein data bank.

Based on the X-ray crystal structure of CatK, numerous efforts have been made to develop potent, selective, and orally deliverable CatK inhibitors. However, all synthetic inhibitors tested in preclinical or clinical trials so far exclusively target the active site of CatK, thereby blocking both its collagenolytic activity and other peptidase activities such as maturation of thyroid hormones and TGF- β 1 hydrolysis. Consequently, blocking the entire active site may lead to unwanted side effects [Figure 2]. Accordingly, Merck & Co. had to discontinue its phase III clinical trials for osteoporosis treatment using Odanacatib, the most promising drug targeting CatK. Compared with other anti-resorptive agents, Odanacatib effectively limited osteoclast-mediated bone resorption without suppressing remodeling (preserving bone formation). However, despite its ability to increase bone mineral density (BMD) and improve bone strength in the spine and hip in postmenopausal women with osteoporosis, Odanacatib also increased the risk of cardiovascular side effects, specifically stroke^[39]. For a comprehensive update on preclinical and clinical trials conducted with CatK inhibitors, see the recent review:^[34]. Thus, new pharmacological approaches are urgently needed to design novel human CatK inhibitors that impair its collagenase activity without affecting other physiological regulatory proteolytic activities. One possible strategy that has emerged in recent years and could help overcome the problems associated with on-target toxicity is to focus on developing either exosite or allosteric inhibitors, such as tanshinones, which are ectosteric inhibitors isolated from the roots and rhizomes of the Chinese medicinal herb *Salvia miltiorrhiza* Bunge (Danshen)^[40,41]. It should be noted that inhibition via gene therapy could also limit adverse off-target effects. Indeed, systemic delivery of a bone-targeting recombinant adeno-associated virus, serotype 9 (rAAV9), which can deliver to osteoclasts an artificial microRNA (rAAV9.amir-ctsk), counteracts bone loss and improves bone mechanical properties in murine models of postmenopausal and senile osteoporosis^[42].

LYMPHANGIOLEIOMYOMATOSIS: CLINICAL FEATURES, DIAGNOSIS AND CURRENT THERAPY

Clinical features

LAM is a rare multisystemic disorder that belongs to the group of cystic pulmonary diseases and mainly affects young women. This disease frequently progresses to chronic respiratory failure. It can occur sporadically or in association with a genetic disorder, tuberous sclerosis (also known as Bourneville's

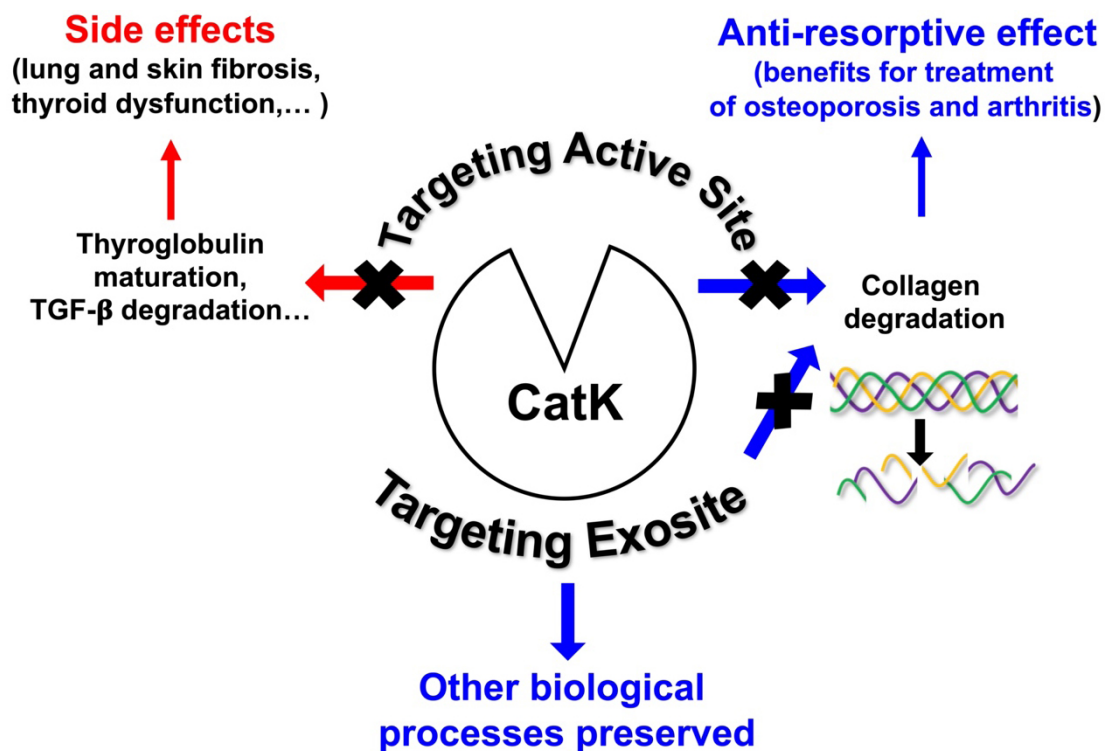


Figure 2. Selective inhibition of the collagenolytic activity of cathepsin K by targeting its exosite. Schematic drawing summarizing the overall strategy to target cathepsin K using exosite-binding inhibitors. CatK: Cathepsin K; TGF: transforming growth factor.

disease)^[43,44]. The prevalence of the sporadic form is estimated to be 3.4-7.8 per million adult women, with an incidence of 0.23 to 0.31 per million women per year^[45]. In France, 320 cases of patients with LAM were recorded in April 2021 (RE-LAM-CE: National Register of Lymphangioleiomyomatosis). It is more common in people with tuberous sclerosis, occurring in up to 30% of cases. This pathology affects almost exclusively women of reproductive age, with a median age of 35 years at diagnosis. LAM is considered a slowly progressive neoplastic disease characterized by the proliferation of cells derived from smooth muscle cells within the lymphatic pathways, particularly in the lungs, resulting in progressive cystic lung destruction that leads to deterioration of respiratory function^[46]. Clinically, this disease is mainly marked by recurrent pneumothorax, progressively worsening dyspnea on exertion, and eventually chronic obstructive respiratory failure. Respiratory function [i.e., forced expiratory volume in one second (FEV₁) and diffusing capacity of the lung for carbon monoxide (DLCO)] correlates with chest computed tomography (CT) and histopathological abnormalities. In addition, renal angiomyolipoma lesions are often observed, which can sometimes cause fatal hemorrhagic events. Since the risk is correlated with tumor size, regular monitoring is therefore required^[47].

Diagnosis and LAM markers

Definitive diagnosis is based on tissue (most often lung) biopsy and/or the association of a clinical picture and a characteristic chest CT appearance. Histopathological diagnosis relies on the presence of cystic cavities and the disseminated proliferation of abnormal or immature smooth muscle cells (LAM cells). LAM cells express smooth muscle α -actin (α -SMA), desmin, vimentin^[48] and hormone receptors for estrogen and progesterone^[48]. Moreover, LAM cells are characterized by their reactivity with mouse monoclonal antibody HMB45 (i.e., Human Melanoma Black), originally developed against human melanoma^[49] and currently used as a diagnostic marker of LAM^[50]. The origin of these cells is still unknown, and the main hypothesis

suggests a uterine origin with migration via the lymphatic pathways. The mechanisms involved in the pathophysiology of LAM are not fully understood. In particular, they involve lymphangiogenesis, which is notably associated with the overexpression of lymphatic growth factors, vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGF R3, a marker of lymphatic endothelial cells^[51]. The level of blood VEGF-D, which is significantly increased in LAM, is currently the only serum biomarker for its diagnosis. Indeed, high levels of serum VEGF-D (> 800 pg/mL) are specifically observed in LAM, among pulmonary cystic pathologies. The pathophysiology of LAM also includes a genetic component. Mutations associated with tuberous sclerosis involve two tumor suppressor genes: tuberous sclerosis complex 1 (TSC1) and TSC2, which encode hamartin and tuberin, respectively^[52]. The TSC1/TSC2 complex inhibits the intracellular signaling pathway involved in cell growth and proliferation, the mTOR-S6 kinase 1 (S6K1) pathway^[53]. In sporadic LAM, these mutations are sometimes somatic and *de novo*, found in lung and kidney lesions, with the most common mutation affecting the TSC2 gene. Mutations in these genes result in the loss of inhibitory function of the hamartin-tuberin complex, leading to constitutive activation of the mTOR pathway, abnormal cell proliferation and growth, and lung remodeling [Figure 3]^[54]. Conversely, constitutive activation of Raptor-containing mTORC1 leads to downregulation of apoptosis and enhanced cell survival^[55].

Current therapy

Recently, purine and pyrimidine nucleotide analogs and immune checkpoint inhibitors have been proposed as potential therapeutic avenues to induce LAM cell death (see for review:^[55]). Likewise, preliminary surveys of statins and a cyclo-oxygenase-2 inhibitor of the coxib family (celecoxib) have been reported, but additional studies with larger LAM cohorts are needed to validate clinical effectiveness. Progesterone, as well as pharmacological inhibitors (oestrogen receptor modulators, gonadotropin-releasing hormone agonists, aromatase inhibitors), has also been attempted as a potential treatment for LAM; however, these interventions produced unreliable and unconvincing results^[55]. Conversely, several clinical studies have demonstrated a beneficial effect of mTOR inhibitors, especially rapamycin, on LAM [Figure 3]. Rapamycin (also known as Sirolimus), a macrolide compound naturally produced by the bacterium *Streptomyces hygroscopicus*, is currently the reference treatment for this condition^[56]. Nevertheless, its effect, which remains only suspensive, is associated with many side effects, notably an increased risk of infection due to immunosuppression induced by treatment with Sirolimus^[57]. Sirolimus can also cause hematological disorders, with a very frequent risk of pancytopenia associated with susceptibility to infections^[56]. Although rapamycin promotes stabilization of lung function and improves quality of life, cessation of rapamycin treatment results in recurrence of the disease progression, highlighting the urgent need to identify novel targets and contemporary LAM treatments (see for review:^[58]).

CATHEPSIN K: A NEW LAM BIOMARKER?

An early immunohistochemical study reported a strong CatK immunoreactivity, which was specifically restricted to LAM specimens, compared with other lung samples from angiomyolipomas and various lung diseases (sarcoidosis, organizing pneumonia, usual interstitial pneumonia, emphysema) used as controls^[59]. The specificity of CatK as a putative LAM marker seemed appropriate, since other pulmonary α -SMA-expressing cells are immunonegative for CatK or exhibit only discrete CatK immunoreactivity, such as myofibroblasts in fibroblast foci of usual interstitial pneumonia^[59]. Moreover, these α -SMA-expressing cells do not pose diagnostic challenges because of their distinctive morphologies and immunophenotypes. Following this study, Chilosi *et al.* proposed that CatK could serve as a useful additional biomarker for diagnosis in some ambiguous cases^[59]. They also suggested, for the first time, that CatK can contribute to the progressive remodelling of lung parenchyma observed in LAM. In a landmark study, it was hypothesized that LAM nodule-derived proteases cause cyst formation and tissue damage^[60]. A gene expression profiling analysis was performed on whole lung tissue. Dongre *et al.* demonstrated that CatK gene expression was

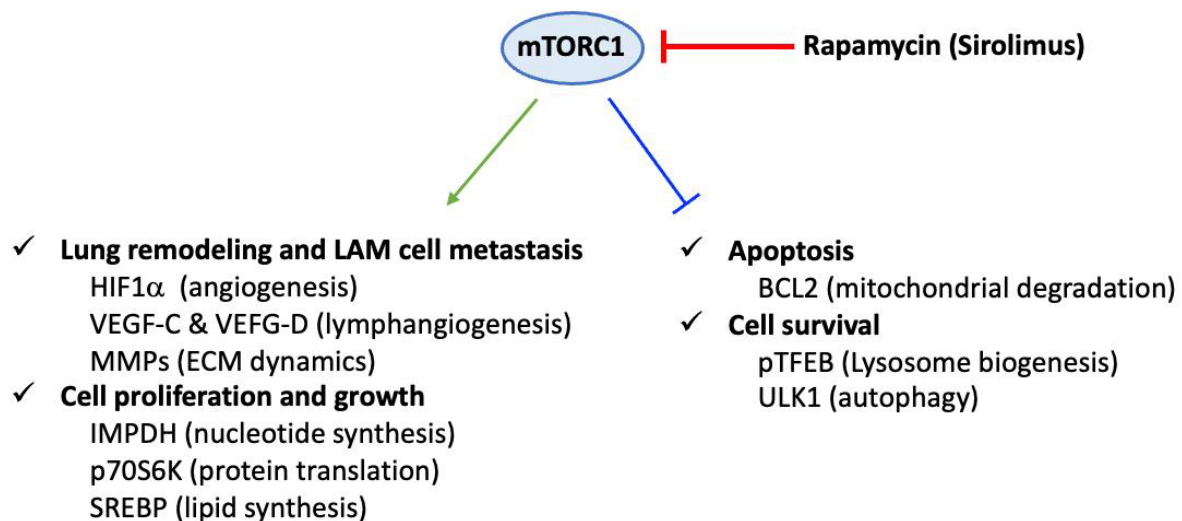


Figure 3. Dysregulation of mammalian target of rapamycin (mTOR) C1 signaling in LAM pathogenesis. Constitutive activation of the Raptor-containing mTOR leads to dysregulated mTOR signaling pathways in LAM cells. Consequently, cell proliferation, cell growth, and lung remodeling, as well as apoptosis and cell survival, are altered. This scheme corresponds to a non-exhaustive summary, emphasizing some key proteins that are specifically linked to mTOR-dependent underexpression or overexpression in LAM cells. Representative upregulated molecules: HIF-1 α (hypoxia-inducible factor 1 α), VEGF (vascular endothelial growth factor)-C and VEGF-D, MMP (matrix metalloproteinase), IMPDH (inosine 5'-monophosphate dehydrogenase), p70S6K (70 kDa ribosomal protein S6 kinase), SREBP (sterol regulatory element-binding protein) and downregulated molecules: BCL2 (B cell lymphoma 2), pTFEB (phosphorylated form of transcription factor EB), ULK1 (unc-51-like autophagy activating kinase 1). LAM: Lymphangioleiomyomatosis; ECM: extracellular matrix.

40-fold higher in LAM compared with control lung tissue, while immunohistochemistry confirmed overexpression of CatK protein in LAM tissue^[60]. Consistently, CatK immunoreactivity is predominantly co-localized with LAM-associated fibroblasts in lung nodules. CatK is also overexpressed in renal angiomylipomas found in LAM, which correspond to “perivascular epithelioid cell lesions” (PEComa)^[61]. Recently, the sensitivity of both CatK and HMB-45 was compared as diagnostic markers for pulmonary LAM. The percentage of LAM cells expressing CatK was significantly higher than that for HMB45, and overall expression was significantly greater, confirming that CatK is a more sensitive immunohistochemical marker than HMB45 in diagnosing pulmonary LAM^[62].

CATHEPSIN K: AN ADDITIONAL THERAPEUTIC TARGET IN LYMPHANGIOLEIOMYOMATOSIS?

C4-S binding is mandatory to promote the collagenolytic activity of CatK, as stated before (paragraph 1.2). C4-S is predominantly expressed in bone and cartilage, but it is also found throughout the body and contributes to ECM remodeling processes in numerous chronic inflammatory diseases^[63]. Although the expression level of C4-S in LAM is currently unknown, the total amount of GAG is increased in lung diseases, including idiopathic pulmonary fibrosis (IPF) and mucopolysaccharidosis (MPS), another rare disease^[63,64]. In addition to its immunoreactivity, active CatK was detected in LAM-associated fibroblasts. Moreover, active secreted CatK was found in the extracellular medium under in vitro conditions. It is well established that monocyte-derived macrophages acidify their pericellular environment via vacuolar-type H⁺-ATPases, enabling them to maintain cysteine cathepsins, including CatK, in their active form^[21]. Similar extracellular acidification may occur within LAM nodules, due to both expression of membrane transporters (carbonic anhydrases, monocarboxylate transporters and sodium-bicarbonate co-transporters) and mTOR dysregulation, which induces a metabolic dependence on aerobic glycolysis (Warburg effect). Acidification paralleled CatK activity, and both were forcefully compromised by sodium bicarbonate co-transporter inhibitors or carbonic anhydrase inhibitors. Impressively, inhibition of mTOR by rapamycin

also abrogated acidification in a cell culture model by acting on cell metabolism rather than solely on the transporters themselves. Thus, inhibition of extracellular acidification may be an alternative therapy for LAM by indirectly impairing CatK activity^[60].

As previously mentioned, inhibition of the mTOR pathway by rapamycin is currently the only reference therapy for LAM. This beneficial effect may be related to the suppression of Warburg metabolism and extracellular acidification. Accordingly, pharmacological inhibitors of carbonic anhydrases and sodium-bicarbonate co-transporters have been successfully used in preclinical cancer models^[65,66] and may have synergistic benefits with mTOR inhibition in LAM, reducing detrimental protease activity, including CatK. Interestingly, markers for LAM cells, fibroblasts, lymphatics, and mast cells were examined by dual immunohistochemistry, quantified in LAM nodules, and compared with clinical features and prospective lung function loss. Levels of CatK were negatively correlated with FEV1 and DLCO, whereas higher reactivity to the mTOR complex 1 activation marker was associated with better lung function response to rapamycin^[67]. Taken together, these results suggest that increased CatK expression is associated with disease severity and loss of lung function. Furthermore, the higher prevalence of osteoporosis in LAM compared with the general population underscores the interest in specifically targeting CatK^[68]. Some recent reports suggest that overexpression of CatK is associated with mTOR upregulation^[69], while CatK may promote tumor cell proliferation, invasion, and migration, potentially via the mTOR signaling pathway^[70]. On the other hand, expression of cysteine cathepsins (including CatK and CatS) is associated, through inhibition of mTOR, with a decreased phosphorylation of TFEB (transcription factor EB), a central regulator of autophagy/lysosomal biogenesis^[34,71]. Therefore, although experimental data remain scarce, the possibility that rapamycin may affect CatK expression cannot be ruled out.

To summarize, combining the current treatment with rapamycin with inhibition of the collagenolytic activity of CatK by ectosteric inhibitors could be more effective than mTOR inhibition alone. Indeed, a cooperative therapeutic outcome in reducing lung destruction in LAM could be expected compared with single-target therapy. In our opinion, such a dual approach is conceivable despite the lack of experimental evidence at this hypothetical clinical stage.

DECLARATIONS

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Authors' contributions

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Wrote the paper: Lalmanach G

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All authors have read and approved the final manuscript.

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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