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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 that is 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 anatomopathological criteria combined with chest computed tomography. VEGF-D is the only serum diagnostic biomarker used in the clinic, while inhibition of the 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 as 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 take part in the LAM pathophysiology by participating in pulmonary cystic destruction and bone degradation. Accordingly, targeting 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 proteins degradation and turnover, autophagy, and immune responses)<sup>[1,2]</sup>. Cysteine cathepsins, which are expressed either ubiquitously or with tissue and cell-type specificity, are also found outside lysosomes under specific conditions, contributing most often when they are dysregulated to a wide range of pathophysiological events<sup>[3,4]</sup>. 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<sup>[5]</sup>. Moreover, CatK, which is overexpressed during osteoporosis as well in bone cancers, was validated as an attractive target for anti-osteoporosis therapy and anti-tumor treatments<sup>[6-10]</sup>. Meanwhile, CatK is gaining a growing interest according to potential other roles in physiological and pathological processes, including rare diseases.

First, we will summarize contemporary knowledge on molecular aspects of CatK (genomic organization, tissue expression, functional and structural characteristics, substrate specificity, 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 will discuss both the interest in using CatK as a new LAM biomarker and in targeting CatK for reducing lung destruction in LAM. Indeed, according to 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 be an additional therapeutic option, besides inhibition of the mTOR (mammalian target of rapamycin) pathway.

### Cathepsin K: molecular and biological characteristics

Human CatK is encoded by a single gene (CTSK, ~12.1 kb), localized on chromosome 1q21, like cathepsin S (CatS)<sup>[1]</sup>. 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)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukins (IL)-6 and -13 positively regulate the synthesis of CatK, while transforming growth factor (TGF)- $\beta$ 1 and IL-10 may repress its expression level (for review:<sup>[5,11]</sup>).

CatK (EC 3.4.22.38) is a monomeric endopeptidase (~24 kDa), which shares the common papain-like structure, which consists of two globular domains folded together to give a “V”-shaped active site cleft configuration in the middle. Albeit the catalytic triad Cys<sup>25</sup>, His<sup>159</sup>, Asn<sup>175</sup> (papain numbering) is well conserved, mapping studies of the crystal structure of CatK revealed differences in the substrate binding subsites (labeled Sn-Sn') that are located on both sides of the substrate's scissile bond (corresponding to the complementary positions Pn-Pn')<sup>[12]</sup>. Notably, residues forming the S2 binding pocket of CatK, its major determinant of substrate specificity, are crucial for its prevalence for hydrophobic and aliphatic amino acids (for review:<sup>[5]</sup>). Both binding pattern and substrate specificity of CatK were previously detailed by Lecaillon *et al.*<sup>[5]</sup>. 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 of CatK were developed<sup>[13-16]</sup>.

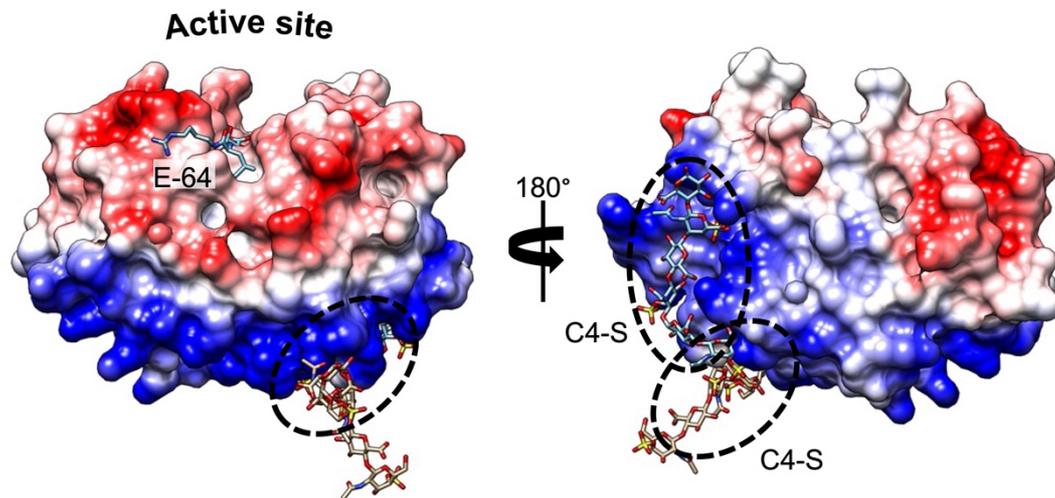
Compared to other human proteases, CatK displays the unique potency to cleave within the triple helix of type I and II collagens<sup>[17]</sup>. Furthermore, the presence of chondroitin 4-sulfate (C4-S), a glycosaminoglycan

(GAG) predominantly expressed in bone and cartilage, is required to promote its collagenolytic activity by forming high molecular complexes<sup>[18]</sup>. The formation of an active complex between the negatively charged C4-S and specific positively charged residues of CatK (and shaping a so-called exosite, which is 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 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<sup>[19]</sup>. CatK is a lysosomal protease that is mainly active at acidic pH 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<sup>+</sup>-ATPase pump or Na<sup>+</sup>/H<sup>+</sup> exchanger<sup>[20,21]</sup>. In addition to its primary location in bones, high levels of CatK were also detected in synovial fibroblasts, aortic smooth muscle cells, macrophages, and epithelial cells, while CatK mRNA levels were increased in other tissues such as ovary, synovia, heart, skin, and lungs, following scarring or inflammation<sup>[10,22,23]</sup>. In addition to its collagenolytic activity and its involvement in ECM remodeling, CatK takes part in the maturation of thyroid hormones, participating in the processing of thyroglobulin<sup>[24,25]</sup>. Moreover, CatK plays a pivotal protective role in lung homeostasis via its ability to proteolytically inactivate TGF-β1<sup>[26,27]</sup>. In the last decade, a paradigm shift from the concept of “simple” protein-degrading enzymes to key signaling scissors was proposed<sup>[28]</sup>, as demonstrated by the paramount importance of cysteine cathepsins (including CatK) in diverse cell signaling cascades (e.g., PPAR-γ/caspase-8-mediated cell apoptosis, TGF-β signaling pathway)<sup>[29-33]</sup>.

### **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 like osteoporosis and bone metastasis<sup>[5]</sup>. 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 (NTx) collagen fragments (a typical product of CatK cleavage) in the urine/serum of osteoporotic patients<sup>[6]</sup>. Since inhibition of CatK active site could prevent bone resorption without perturbing bone formation, the protease has become an attractive and validated target for anti-resorptive drug development<sup>[34]</sup>. Moreover, abnormally high expression of CatK in various organs intertwines with the massive hydrolysis of elastin fibers or collagens in numerous cancers such as prostate, breast, lung, and bone cancers<sup>[8,10]</sup>. CatK, which contributes to tumor progression via extracellular degradation of ECM proteins, cytokines inactivation/activation or pro-matrix metalloproteinases processing, was proposed for differential prognostic purposes.

Oral and maxillofacial abnormalities were found in *Ctsk*(-/-) mice. Similarly, close relationships between defective CatK and oral diseases were identified in patients with pycnodysostosis<sup>[35]</sup>, but also in patients with periodontitis, peri-implantitis, tooth movement, oral and maxillofacial tumor, root resorption, and periapical disease (see for review:<sup>[36]</sup>). Extensive histological and ultrastructural changes of cementum, a part of the periodontium, were observed, which might be linked to compromised proteolytic activity of CatK<sup>[37]</sup>. Otherwise, mutations of 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. Also, loss of *CLCN7* function resulted in lysosomal storage in the brain as well as in the jaw and its surrounding, which has been associated with CatK downregulation<sup>[38]</sup>.



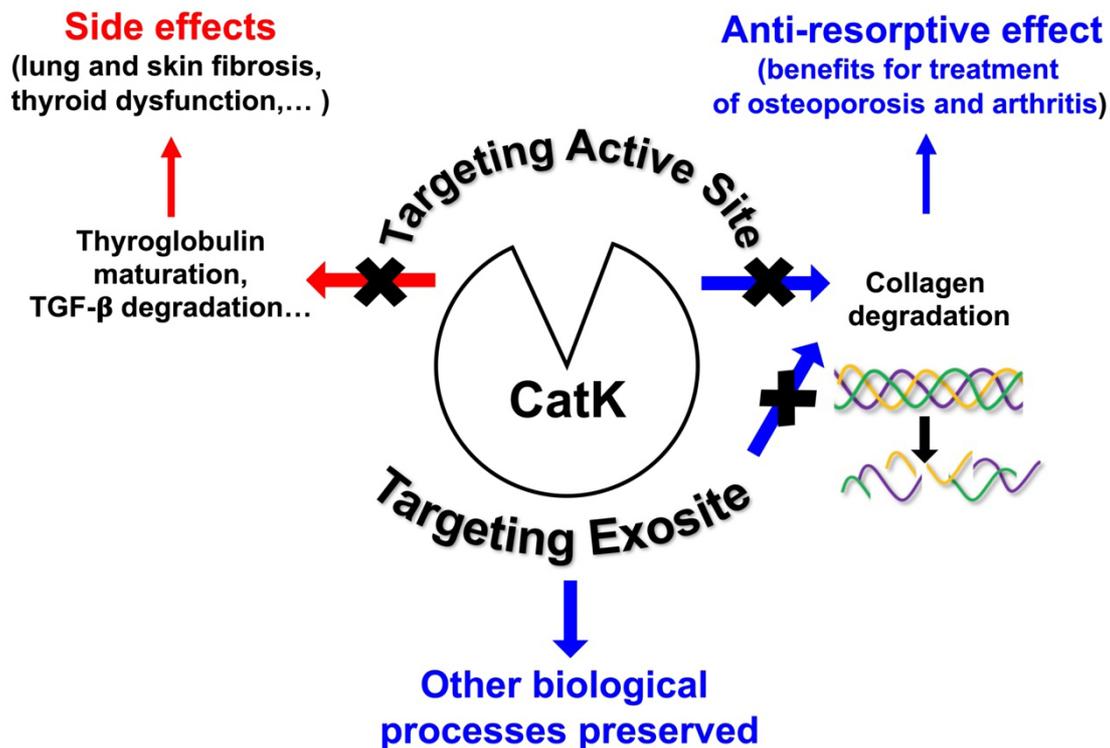
**Figure 1.** Complex formation between CatK and C4-S. Electrostatic surface distribution of human CatK. Blue and red areas represent positively and negatively charged surface domains of CatK, respectively (Chimera software). The spots of two C4-S-binding sites (exosites) (PDB accession codes 3C9E and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

Thus, from 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 which have been tested in preclinical or clinical trials so far exclusively target the active site of CatK, thus blocking both its collagenolytic activity and other peptidase activities such as maturation of thyroid hormones and TGF- $\beta$ 1 hydrolysis. Therefore, blocking its entire active site may cause unwanted side effects [Figure 2]. Accordingly, Merck & Co had to discontinue their 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 (preservation of 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 increases the risk of cardiovascular side effects, specifically stroke<sup>[39]</sup>. For an exhaustive update of preclinical and clinical trials conducted with CatK inhibitors, see a recent review:<sup>[34]</sup>. Thus, new pharmacological approaches are urgently needed to design novel inhibitors of human CatK to impair its collagenase activity but not 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 focusing on finding 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)<sup>[40,41]</sup>. It should be noted that inhibition by 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 a murine model of postmenopausal and senile osteoporosis<sup>[42]</sup>.

## LYMPHANGIOLEIOMYOMATOSIS: CLINICAL FEATURES, DIAGNOSIS AND CURRENT THERAPY

### Clinical features

Lymphangioliomyomatosis (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 during a genetic disease, tuberous sclerosis (also known as



**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 by exosite-binding inhibitors.

Bourneville's disease)<sup>[43,44]</sup>. The prevalence of the sporadic form is estimated to be 3.4-7.8/million adult women with an incidence of 0.23 to 0.31/million women/year<sup>[45]</sup>. 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, up to 30%. This pathology affects almost exclusively women in the period of genital activity, with a median age of 35 years at the time of diagnosis. LAM is considered as a slowly progressive neoplastic disease, responsible for the proliferation of cells derived from smooth muscle cells in the lymphatic pathways, particularly in the lungs, resulting in progressive cystic lung destruction responsible for the deterioration of respiratory function<sup>[46]</sup>. The clinic is mainly marked by recurrent pneumothorax, progressively worsening dyspnea on exertion, then chronic obstructive respiratory failure. The respiratory function (i.e., Forced Expiratory Volume in the first second (FEV<sub>1</sub>) and Diffusing Capacity of the lung for carbon monoxide (DLCO) is correlated with chest Computed Tomography (CT) and histopathological abnormalities. In addition, renal angiomyolipoma injuries are observed, which can sometimes be responsible for fatal hemorrhagic events. Since the risk is correlated with the size of the tumors, regular monitoring is therefore required<sup>[47]</sup>.

### 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 is based on the association of cystic cavities and disseminated proliferation of abnormal/ immature smooth muscle cells (LAM cells). LAM cells express smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA), desmin, vimentin<sup>[46]</sup> and hormone receptors for estrogen and progesterone<sup>[48]</sup>. Moreover, LAM cells are characterized by their reactivity with mouse monoclonal antibody HMB45 (i.e., Human Melanoma Black), which was originally developed against human melanoma<sup>[49]</sup> and is currently used as a diagnostic marker of LAM<sup>[50]</sup>. The origin of these cells is still unknown, and the main

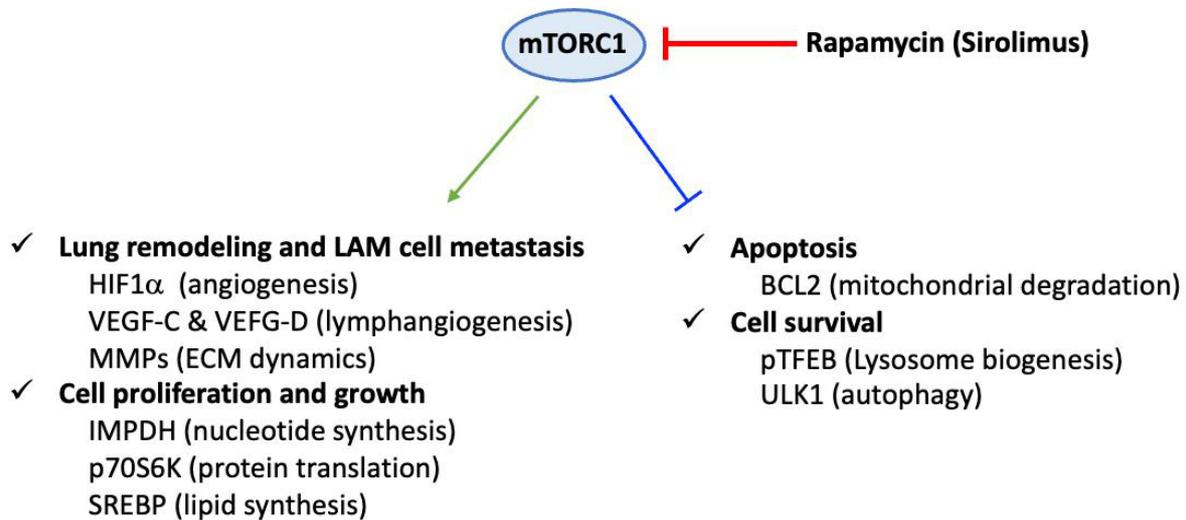
hypothesis is uterine origin with migration via the lymphatic pathways. The mechanisms involved in the pathophysiology of LAM are misunderstood. In particular, they encompass lymphangiogenesis, which is notably associated with the overexpression of lymphatic growth factors, vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGF R3, a marker of lymphatic endothelial cells<sup>[51]</sup>. The level of blood VEGF-D, which is significantly increased in LAM, is currently the only serum biomarker for LAM 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 during tuberous sclerosis concern two tumor suppressor genes: tuberous sclerosis complex 1 (TSC1) and 2 (TSC2), coding respectively for hamartin and tuberin<sup>[52]</sup>. The TSC1/TSC2 complex allows the inhibition of the intracellular signaling pathway involved in cell growth and proliferation, the mTOR-S6k1 [mammalian target of rapamycin (mTOR)-S6 kinase 1] pathway<sup>[53]</sup>. In the case of sporadic LAM, these mutations are sometimes found at the somatic level and *de novo*. They are found in lung and kidney lesions, the most common mutation being that affecting the TSC2 gene. Mutations in these genes lead to a loss of inhibitory function of the hamartin-tuberin complex and are responsible for the constitutive activation of the mTOR pathway and subsequent abnormal cell proliferation and growth as well as lung remodeling [Figure 3]<sup>[54]</sup>. Contrariwise, the constitutive activation of Raptor-containing mTORC1 leads to downregulation of apoptosis and cell survival<sup>[55]</sup>.

### Current therapy

Recently, purine and pyrimidine nucleotide analogues and immune checkpoint inhibitors were advocated as potential therapeutic avenues to induce LAM cell death (see for review:<sup>[55]</sup>). Likewise, preliminary surveys of statins and a cyclo-oxygenase-2 inhibitor of the coxib family (celecoxib) were reported, but supplemental studies with larger LAM cohorts are needed to validate clinical effectiveness. Also, progesterone as well as pharmacological inhibitors (oestrogen receptor modulators, gonadotropin-releasing hormone agonists, aromatase inhibitors) were attempted as potential treatments for LAM but gave unreliable and unconvincing results<sup>[55]</sup>. Conversely, several clinical studies have pointed to a beneficial effect of mTOR inhibitors, especially rapamycin, on LAM [Figure 3]. Rapamycin (a.k.a., Sirolimus), a macrolide compound naturally produced by the bacterium *Streptomyces hygroscopicus*, is currently the reference treatment for this pathology<sup>[56]</sup>. Nevertheless, its effect, which remains only suspensive, is associated with many side effects, notably an increased risk of infection following the immunosuppression induced by treatment with Sirolimus<sup>[57]</sup>. Also, Sirolimus can be responsible for hematological disorders with a very frequent probability of pancytopenia associated with the risks of an infectious event<sup>[56]</sup>. Although rapamycin favors stabilization of lung function and improves quality of life, the cessation of rapamycin treatment results in recurrence of the disease progression, highlighting the imperative need to identify novel targets and contemporary LAM treatments (see for review:<sup>[58]</sup>).

### CATHEPSIN K: A NEW LAM BIOMARKER?

An early immunohistochemical study reported a strong CatK immunoreactivity, which was specifically restricted to lymphangioleiomyomatosis specimen, compared to other lung samples from angiomyolipomas and diverse lung diseases (sarcoidosis, organizing pneumonia, usual interstitial pneumonia, emphysema) used as controls<sup>[59]</sup>. The specificity of CatK as a putative LAM marker seemed appropriate, according to other pulmonary  $\alpha$ -SMA-expressing cells that are immuno-negative for CatK or exhibit only a discrete CatK immunoreactivity, such as myofibroblasts in fibroblast foci of usual interstitial pneumonia<sup>[59]</sup>. Moreover, these  $\alpha$ -SMA-expressing cells do not represent diagnostic problems because of their distinctive morphologies and immunophenotypes. Following this study, authors proposed that CatK could be a useful additional biomarker for diagnosis in some ambiguous cases. Moreover, they suggested for the first time that CatK can contribute to the progressive remodelling of lung parenchyma observed in LAM. In an outstanding study, it was hypothesized that LAM nodule-derived proteases cause cyst formation and tissue



**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, lung remodeling as well apoptosis and cell survival are altered. This scheme corresponds to a non-exhaustive summary, emphasizing some key proteins which are specifically linked to mTOR-dependent under-expression or over-expression in LAM cells. Representative upregulated molecules: HIF-1 $\alpha$  (hypoxia-inducible factor 1 $\alpha$ ), 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).

damage<sup>[60]</sup>. A gene expression profiling analysis was performed in whole lung tissue. The authors showed that CatK gene expression was 40-fold overexpressed in LAM compared with control lung tissue, while immunohistochemistry confirmed overexpression of CatK protein in LAM tissue. Consistently, CatK immunoreactivity is predominantly co-localized with LAM-associated fibroblasts in lung nodules. Also, CatK is overexpressed in renal angiomyolipomas found in LAM, which relate to “perivascular epithelioid cell lesions” (PEComa)<sup>[61]</sup>. Recently, the sensitivity of both CatK and HMB-45 were compared as potent diagnostic markers for pulmonary LAM. The percentage of LAM cells expressing CatK was significantly higher than for HMB45 and overall expression was significantly higher, confirming that CatK is a more sensitive immunohistochemical marker than HMB45 in diagnosing pulmonary LAM<sup>[62]</sup>.

### 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 is also found throughout the body and contributes to ECM remodeling processes in numerous chronic inflammatory diseases<sup>[63]</sup>. Although the expression level of C4-S is currently not known in LAM, the total amount of GAG is increased in lung diseases, including idiopathic pulmonary fibrosis (IPF) or mucopolysaccharidosis (MPS), another rare disease<sup>[63,64]</sup>. Alongside its immunoreactivity, active CatK was detected in LAM-associated fibroblasts. Also, active secreted CatK was found in extracellular medium under in vitro conditions. It is well established that monocyte-derived macrophages acidify their pericellular environment via vacuolar-type H<sup>+</sup>-ATPases, thus enabling them to maintain cysteine cathepsins, including CatK, in their active form<sup>[21]</sup>. Similar extracellular acidification may exist within LAM nodules, because of 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 indirect impairment of CatK activity<sup>[60]</sup>.

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<sup>[65,66]</sup> and may have synergistic benefits with mTOR inhibition in LAM to reduce detrimental proteases activity, including CatK. Interestingly, markers for LAM cells, fibroblasts, lymphatics, and mast cells were examined by dual immunohistochemistry, quantitated in LAM nodules, and compared with clinical features and prospective lung function loss. Levels of CatK were negatively correlated with FEV<sub>1</sub> and DLCO, while a higher reactivity to the mTOR complex 1 activation marker was associated with a better lung function response to rapamycin<sup>[67]</sup>. Taken together, results suggested that an increase in CatK expression is associated with severity of the disease and loss of lung function. At last, the higher prevalence of osteoporosis in LAM than in the general population strengthens the interest in specifically targeting CatK<sup>[68]</sup>. Interestingly, some recent reports suggested that overexpression of CatK is associated with mTOR upregulation<sup>[69]</sup>, while CatK could promote tumor cell proliferation, invasion and migration, and its mechanism may be related to mTOR signaling pathway<sup>[70]</sup>. On the other hand, expression of cysteine cathepsins (including CatK and CatS) is associated, via the inhibition of mTOR, with a decreased phosphorylation of TFEB (transcription factor EB), a central regulator of autophagy/lysosomal biogenesis<sup>[34,71]</sup>. Therefore, although there is a scarcity of experimental data, the possibility that rapamycin may have an impact on CatK expression cannot be ruled out.

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

## DECLARATIONS

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### Authors' contributions

Draft the paper: Lalmanach G, Lecaille F, Marchand-Adam S

Prepared the figures: Lecaille F, Lalmanach G

Wrote the paper: Lalmanach G

Revised the paper: Saidi A, Pronost M

All authors 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

Not applicable.

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