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1	The abnormal accumulation of heparan sulfate in patients with
2	mucopolysaccharidosis prevents the elastolytic activity of cathepsin V
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39	Running title: Regulation of cathepsin V activity by heparan sulfate
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51 Abstract:

52 Mucopolysaccharidosis (MPS) are rare inherited diseases characterized by accumulation of 53 lysosomal glycosaminoglycans, including heparan sulfate (HS). Patients exhibit progressive 54 multi-visceral dysfunction and shortened lifespan mainly due to a severe cardiac/respiratory 55 decline. Cathepsin V (CatV) is a potent elastolytic protease implicated in extracellular matrix 56 (ECM) remodeling. Whether CatV is inactivated by HS in lungs from MPS patients remained 57 unknown. Herein, CatV colocalized with HS in MPS bronchial epithelial cells. HS level 58 correlated positively with the severity of respiratory symptoms and negatively to the overall 59 endopeptidase activity of cysteine cathepsins. HS bound tightly to CatV and impaired its 60 activity. Withdrawal of HS by glycosidases preserved exogenous CatV activity, while 61 addition of Surfen, a HS antagonist, restored elastolytic CatV-like activity in MPS samples. 62 Our data suggest that the pathophysiological accumulation of HS may be deleterious for 63 CatV-mediated ECM remodeling and for lung tissue homeostasis, thus contributing to 64 respiratory disorders associated to MPS diseases.

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66 Keywords: protease, glycosaminoglycan, MPS, lung, elastin

67 68

69 Abbreviations:

70 AEBSF: 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; CA-074: N-(L-3-trans-71 propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; Cat : cathepsin, CS : chondroitin 72 sulfate; DMMB : 1,9-dimethylmethylene blue; DS : Dermatan sulfate; DTT: dithiothreitol, E-73 64 : trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; ECM : extracellular matrix, 74 EDTA : ethylenediaminetetraacetic acid; GAG : glycosaminoglycan; GlcA : glucuronic acid; 75 GlcNAc : N-acetyl-D-glucosamine; GRSS : global respiratory symptoms severity; HA : hyaluronic acid; HS : heparan sulfate; IF : Immunofluorescence; KS : keratan sulfate; MD : 76 77 molecular dynamics; MMP : matrix metallo-proteinase; MMTS : *S*-methyl 78 thiomethanesulfonate; MPS: mucopolysaccharidosis; PAS: periodic acid schiff; PMSF: 79 phenylmethylsulfonyl fluoride; ProCat : procathepsin; SPRI : surface plasmon resonance 80 imaging; Surfen : bis-2-methyl-4-amino-quinolyl-6-carbamide hydrate; Z-Phe-Arg-AMC : Z-81 Phe-Arg-7-amido-4-methylcoumarin.

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84 **1. Introduction**

Mucopolysaccharidoses (MPS) are a group of seven rare inherited metabolic diseases 85 86 associated with deficiencies in enzymes involved in the degradation of glycosaminoglycans 87 (GAGs). MPS type I (MPS-I) is a lysosomal disease caused by a deficiency of the α-L-88 iduronidase, which is essential to correct the metabolism of both dermatan sulfate (DS) and 89 heparan sulfate (HS). MPS-I is commonly classified into three clinical syndromes from severe 90 to attenuated form: Hurler, Hurler-Scheie, and Scheie. MPS-II (or Hunter syndrome) is caused 91 by a deficiency of lysosomal iduronate-2-sulfatase, which is critical for the degradation of 92 both HS and DS, by cleaving their O-linked sulfate. MPS-III (also named Sanfilippo 93 syndrome) is caused by a deficiency in one of the four enzymes involved in the degradation of 94 HS and is divided into four subtypes : heparan N-sulfatase (type IIIA), α -N-95 acetylglucosaminidase (type IIIB), acetyl CoA: α-glucosaminide acetyltransferase (type IIIC), 96 and N-acetylglucosamine-6-sulfatase (type IIID) (Stapleton et al., 2018). The subsequent 97 accumulation of these GAGs participates in cellular dysfunction, resulting in progressive 98 multi-visceral and tissue damages (Muenzer, 2011). The hallmarks of most MPS-I, II, and III phenotypes are represented by a wide spectrum of clinical severity, including 99 100 cardiorespiratory diseases, which for the most severe forms of MPS diseases remain the 101 leading cause of early mortality (Arn et al., 2015; Berger et al., 2013; Muhlebach et al., 2011). 102 Thickened depositions/secretions in airways and interstitium due to an abnormal 103 accumulation of extracellular matrix (ECM) components can further exacerbate obstruction 104 (Batzios et al., 2013; Leighton et al., 2001; Pal et al., 2018; Rutten et al., 2016). Under these 105 circumstances, the exact molecular mechanisms by which GAG accumulation ultimately leads 106 to lung disease manifestations have not been clearly elucidated (Iozzo & Gubbiotti, 2018). 107 Therefore, a better understanding of factors affecting airways dysfunction may lead to novel 108 strategies for improving respiratory function in patients with MPS.

109 Lysosomal cysteine cathepsins are papain-like proteases (eleven members in human: 110 cathepsins B, C, F, H, L, K, O, S, V, X and W) that play key roles in numerous physiological 111 processes in particular the degradation and recycling of endocytosed bulk protein (Lecaille et 112 al., 2002). Beside their expression in endolysosomal system, cathepsins are also found in 113 cytosol or nucleus and can be secreted (Reiser et al., 2010; Vidak et al., 2019). Cathepsins 114 participate in ECM remodeling by degrading major structural proteins (e.g., collagens I, II and 115 IV and elastin), specialized adhesion proteins (e.g., fibronectin, fibrillin, and laminins) and 116 proteoglycan (aggrecan) (Vizovišek et al., 2019). Their uncontrolled proteolytic activity (e.g., 117 protease/inhibitor and redox imbalance) is associated with various human diseases including

118 cardiovascular diseases, immune defects, bone and cartilage diseases (osteoporosis, 119 rheumatoid arthritis), cancer, and lung inflammatory pathologies (emphysema, fibrosis, 120 asthma, chronic obstructive pulmonary disease) (for review: Brömme & Wilson, 2011; 121 Lalmanach et al., 2020). Moreover cathepsins are relevant biomarkers and promising 122 therapeutic targets for numerous diseases (Kramer et al., 2017; Vizovišek et al., 2020). 123 Cathepsins B, C, H, and L are ubiquitously found in mammalian tissues while cathepsins S, 124 K, W, and notably cathepsin V (CatV) exhibit a more restricted expression pattern. CatV (also 125 called CatL2) that shares 80% protein sequence identity with human CatL is mostly expressed 126 in cornea, testis, epidermis, colon, and thymus (Adachi et al., 1998; Brömme et al., 1999; 127 Santamaría et al., 1998; Tolosa et al., 2003). Deficiency in murine CatL, the orthologue of 128 human CatV, induces myocardial fibrosis probably due to a reduced collagenolytic activity 129 (Spira et al., 2007). A 2-fold reduction of CatV protein level was detected in bronchoalveolar 130 lavage fluids of patients with pulmonary sarcoidosis compared to healthy subjects, suggesting 131 an association to lung matrix homeostasis under physiological and pathological conditions 132 (Naumnik et al., 2014). Human CatV displays a 3-fold higher elastolytic activity than CatK 133 and CatS (Yasuda et al., 2004). In addition, GAGs including chondroitin 4-/6-sulfate (C4-S 134 and C6-S), heparin (Hep) and DS decreased in vitro the elastolytic activities of both CatV and 135 CatK and to a lesser extent CatS. Similarly, the digestion of type II collagen by CatK is 136 impaired by the presence of high HS and DS concentrations in osteoclasts from MPS-I mouse 137 model, which likely contributes to MPS-I bone pathology (Wilson et al., 2009). Previous 138 reports demonstrate that abnormal expression and/or activity of cathepsins B, S, and K 139 correlate with major clinical manifestations in MPS (for review: De Pasquale et al., 2020). 140 Nevertheless, it remains unknown whether cysteine cathepsins, and especially elastolytic 141 CatV, contribute to respiratory disorders in MPS.

142

143 Hypotheses

The proteolytic activity of some cysteine cathepsins is tightly controlled by the formation of a complex between their highly positively charged exosites and specific GAGs (for review: Novinec et al., 2014). Our hypothesis is that i*n situ* HS levels in patients with MPS interfere with the proper elastolytic activity of CatV and that this inhibition could be prevented. To address this issue, we evaluated the protein level of CatV and the HS content in respiratory secretions (sputum and tracheal aspirates) of patients with MPS-I, II, and III and unaffected individuals. Also, we characterized CatV elastolytic activity in the presence of HS. Finally, we demonstrated that Surfen, a small HS antagonist, markedly restored the endogenouselastolytic CatV-like activity in MPS samples.

153 **2. Material and methods:**

154 2.1. Reagents- Benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl coumarin (Z-Phe-Arg-AMC) 155 was purchased from R&D Systems (R&D Systems Europe, Abingdon, UK). E-64 (1-3-156 carboxy-trans-2-3-epoxypropionyl-leucylamido-(4-guanidino)-butane), Pepstatin A, EDTA, 157 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Pefabloc), S-methyl 158 thiomethanesulfonate (MMTS) and Elastin-Congo Red conjugate were obtained from Sigma-159 Aldrich (Saint Quentin Fallavier, France). CatB inhibitor N-(L-3-trans-160 propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074) and CatL inhibitor (N-161 (4-biphenylacetyl)-S-methylcysteine-(D)-Arg-Phe- β -phenethylamide, a.k.a CatL inh. 7) 162 (Chowdhury et al., 2002) were from Calbiochem (Merck Millipore, France). CatS inhibitor 163 (morpholinourea-leucinyl- homophenylalanine-vinyl-sulfone phenyl inhibitor, LHVS) was a kind gift from Dr. J. H. McKerrow (Skaggs School of Pharmacy and Pharmaceutical 164 165 Sciences, University of California, San Diego, USA). Odanacatib (CatK inhibitor) was from 166 Selleck Chemicals (Houston, TX, U.S.A.). Heparan sulfate (HS) from bovine kidney (~14 167 kDa), chondroitin 4-sulfate (C4-S) from bovine trachea (20-30 kDa), chondroitin 6-sulfate 168 (C6-S) from shark cartilage (~63 kDa), dermatan sulfate (DS) from porcine intestinal mucosa 169 (~14 kDa), dermatan sulfate (DS) from porcine intestinal mucosa (~14 kDa), heparin (Hep) 170 from porcine intestinal mucosa (15-17 kDa), hyaluronic acid (HA) from Streptococcus equi 171 (~1400 kDa) were from commercial source (Sigma-Aldrich). Perlecan isolated from basement 172 membrane extract (derived from Engelbreth-Holm-Swarm mouse sarcoma) and aggrecan 173 from bovine articular cartilage were supplied by Sigma-Aldrich. Insoluble elastin from bovine 174 neck was a kind gift from Dr. Laurent Duca (UMR CNRS 7369 MEDyC, Reims, France). 175 Surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide hydrate) came from Sigma-Aldrich.

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177 2.2. Enzymes- Heparinases I, II, III, Δ -4,5-glycuronidase, and chondroitinase B, all from 178 Flavobacterium heparinum were purchased from Grampenz (Upper Ardoe, Aberdeen, 179 Scotland). Dnase I from bovine pancreas was purchased from Sigma-Aldrich. Recombinant 180 human procathepsin V (proCatV) was cloned in the pPic9K yeast expression system and 181 expressed in *Pichia pastoris* as previously described (Brömme et al., 1999). The K20G CatV 182 mutant was generated with the following primers, forward: 5'-183 AAGAATCAGGGACAGTGTGGTTCTTGTT-3', and 5'reverse:

184 CACTGGCGTCACGTAGCCTT-3', using the pPic9K vector carrying the wild-type proCatV 185 sequence as a template. The expression of the recombinant wild-type (wt) CatV and K20G 186 CatV mutant, extraction, as well as renaturation, purification and processing into active form 187 were carried out as previously described (Du et al., 2013). Unless stated otherwise, enzymatic 188 assays for cathepsin activity were carried out in 100 mM sodium acetate buffer, pH 5.5 189 containing 2 mM dithiothreitol (DTT). Cathepsin activity was recorded using as substrate Z-190 Phe-Arg-AMC (λ_{ex} =350 nm, λ_{em} =460 nm, Spectramax Gemini spectrofluorometer, Molecular 191 Devices, Saint Grégoire, France). The active site concentrations of wild-type CatV and K20G 192 CatV mutant were determined using E-64 (Barrett et al., 1982).

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194 2.3. MPS-I lung biopsy- Postmortem lung tissue samples from a 2-year old female with MPS
195 type I were kindly provided by Dr Michael McDermott (Department of Histopathology,
196 Dublin, Ireland). Autopsy findings suggested a cardiopulmonary pathology in combination
197 with transplant related pulmonary venopathy (Gupta et al., 2013). Samples were further used
198 for staining, immunohistological (IHC) and immunofluorescence (IF) examinations.

199 2.4. Lung biological samples from MPS and non-MPS patients- Sputum and tracheal aspirates 200 (respiratory specimens) from MPS patients (N=11, including 2 MPS-I, 5 MPS-II, and 4 MPS-201 III) and non-MPS patients (controls, N=9) were collected in the Pediatric and Intensive Care 202 Units of the University Hospital of Tours, France. Respiratory-related disorders were 203 evaluated by three independent clinicians for MPS patients as part of routine follow-up. Based 204 on the analysis criteria from early comprehensive literature surveys (Berger et al., 2013; 205 Muhlebach et al., 2011), a global respiratory symptoms severity (GRSS) evaluation was 206 assessed for each patients regarding four sub-types: ear-nose-throat symptoms (chronic 207 rhinitis or sinusitis, otitis, adeno-tonsillar hypertrophy, hearing loss, macroglossia, stridor), 208 pulmonary symptoms (dyspnea, wheezing, cough, sputum, asthma, bronchitis, pneumonia), 209 clinical symptoms of obstructive sleep apnea, and skeletal abnormalities causing restrictive 210 lung disease (scoliosis, kyphosis, ribcage narrowing, chest wall deformity). The GRSS was 211 graduated from 0 to 4, corresponding to the number of respiratory disorder sub-types reported 212 for each patient. This study was approved by the French National bioethical authorities (n°ID-213 RCB: 2019-A01361-56) and informed written consent was obtained from parents of each 214 participant. Non-MPS patients were intubated for cardiac surgery or severe head trauma. 215 None of the patients in the control group had chronic respiratory disease before admission or 216 breathing symptoms during hospital stay. Tracheal aspirations of MPS and control groups

217 were taken during an orotracheal intubation of the patient in the operating room or in the 218 Pediatric Intensive Care Unit (University Hospital of Tours, France). Samples were 219 aseptically weighed, instantly diluted at 1 g/10 mL in a preservative buffer (100 mM sodium 220 acetate, pH 5.0 plus the peptidase inhibitors 0.5 mM PMSF, 0.5 mM EDTA, 40 µM pepstatin 221 A, and 1 mM MMTS) (Naudin et al., 2011), then centrifuged for 10 min at 5,000 g at 4°C. 222 Resulting cell-free supernatants were collected, aliquoted and stored at -80°C. The total 223 protein quantification of supernatants was performed by BCA assay (ThermoFisher 224 Scientific).

225 2.5. Immunohistological and immunofluorescence studies in MPS-I lung tissue sections-226 Samples were fixed in neutral-buffered, 10% formalin solution and processed by standard methods for further histological examinations. Conventional immunohistochemistry was 227 228 performed on automatic BenchMark XT (Roche). The lung parenchyma was stained with 229 Hematoxilin-Eosin-Safran (HES) (Tissue-Tek Prisma, Sakura Finetek Europe). Periodic Acid 230 Schiff (PAS) was used to detect polysaccharides. In parallel, anti-CD68 (a monocyte and 231 macrophage marker, 1:200, Dako) was used as control. Finally, digital microscopic images 232 were acquired from scanned slides (Hamamatsu Nanozoomer 2.0RS, magnification: x200 to 233 x400) and processed with the Hamamatsu NDP.view 2.0 software. The immunohistochemical 234 detections of CatV and HS were obtained without antigen retrieval after removing paraffin 235 with xylene. Tissue sections were rehydrated by sequential washings with ethanol and water. 236 Briefly, the endogenous peroxidase activity was blocked by incubating sections in 3% H₂O₂ 237 solution for 20 min. Non-specific binding sites were blocked with 5% BSA in PBS for 1 h at 238 room temperature. Sections were then incubated overnight at 4°C with goat anti cathepsin V 239 (1:400 dilution in PBS with 5% BSA, R&D Systems), and mouse anti heparan sulfate (10E4: 240 1:400 dilution in PBS with 5% BSA, Amsbio, Cambridge, MA, USA). The slides were 241 washed and further incubated with peroxidase-conjugated goat anti-mouse or mouse anti-goat 242 IgG (1:1,000, R&D Systems) for 1 h. Peroxidase activity in tissue sections was visualized as 243 brown color using 3, 3'-diaminobenzidine as a substrate solution. Lung sections were 244 counterstained with Gill's hematoxylin for 1 min, dehydrated, cleared in xylene, and mounted 245 in Vectamount permanent medium (Vector Labs, Peterborough, U.K.)

Immunofluorescence (IF) assays on CatV and HS: Non-specific binding sites were blocked
with PBS containing 5% BSA and 5% goat serum for 2 h, at room temperature. Sections were
then incubated overnight at 4°C with goat anti-CatV (1:100 dilution in PBS containing 5%
BSA and 5% goat serum) and mouse anti-HS 10E4 (1:100 dilution in PBS containing 5%

250 BSA and 5% goat serum). Slides were washed and further incubated with fluorescein-labeled 251 anti-mouse IgM antibody (1:2,000, Molecular Probes) or anti-goat IgG NorthernLights[™] 252 NL637-conjugated antibody (1:2,000, R&D Systems) for 1 h. Lung sections were 253 counterstained with DAPI (4',6-diamidino-2-phenylindole) for 1 min, and mounted in 254 Vectamount permanent medium (Vector Labs., Peterborough, U.K). Microscopic images 255 were acquired (Carl Zeiss Axio vert A1 inverted microscope, magnification: x200) and 256 processed with the ZEISS microscope software, ZEN 2 Lite blue and repeated three times on 257 three separate tissue sections. The lack of cross reactivity of anti-CatV antibody was checked 258 by western blot analysis on human cathepsins B, K, L, and S (100 ng) as described elsewhere 259 (Sage et al., 2013).

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261 2.6. Cathepsin V and sulfated GAGs in MPS biological samples- Following running of MPS 262 cell-free supernatants (50 µg of total protein/well; 12% SDS-PAGE under reducing 263 conditions) CatV was immunodetected by a mouse anti CatV primary antibody (1:1,000) 264 incubated overnight at 4°C. Horseradish peroxidase (HRP)-conjugated anti-IgG antibody 265 (1:5,000) was incubated for 1 h at room temperature, and immune-positive bands were 266 visualized using an enhanced chemiluminescence assay kit (ECL Plus Western blotting 267 detection system; Amersham Biosciences, UK). Assays (triplicate) were repeated at least 268 three independent times. In addition, a quantitative analysis of CatV was performed in MPS 269 and non-MPS cell-free supernatants using a sandwich ELISA DuoSet kit (R&D Systems).

Sulfated GAGs were quantified with 1,9-dimethylmethylene blue (DMMB, Sigma-Aldrich), (Barbosa et al., 2003). Absorbance was measured at 654 nm (Cary 100 spectrophotometer, Agilent, France) and all values were reported to a final protein content (100 μ g). Finally, concentrations of sulfated GAGs were determined by using a calibration curve of similarly treated commercial C4-S solutions (1-10 μ g/mL). In parallel, CS, DS and HS content in MPS and non-MPS biological samples were determined by ELISA (AMS Biotechnology, Abingdon, UK) (n=3, triplicate).

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278 2.7. Titration of cathepsins in MPS and non-MPS lung biological samples- Overall
279 endopeptidase cysteine cathepsin concentration in cell-free supernatants (sputum and tracheal
280 aspirates: 30 µg of total protein) from MPS and non-MPS patients, as reported elsewhere
281 (Naudin et al., 2011). Assays were performed in triplicates and repeated at least three times.

282

283 2.8. Activity of exogenous cathepsin V in the presence of biological samples- The activity of 284 recombinant CatV (0.5 nM) was measured in vitro at 37 °C without or with increasing 285 amounts of cell-free supernatants (corresponding to 3-300 ng of total protein) from sputum 286 and tracheal aspirates of MPS patients in 100 mM sodium acetate buffer, pH 5.5, 0.01% 287 Brij35, 10 mM DTT, using Z-Phe-Arg-AMC (20 µM) as a substrate. The activity was 288 measured by spectrofluorometry (λ_{ex} =350 nm, λ_{em} =460 nm). The same experiment was 289 carried out with biological samples pretreated with glycosidases (heparinase I, II, and III, 290 chondroitinase B, and Δ -4,5-glycuronidase) (180 mU/mL), sodium chromate (10 mM), and 291 Dnase I (100 mU/mL) during 48 h at 37°C under vigorous agitation. Controls were performed 292 with non-MPS patients. All kinetic measurements were made in triplicates and repeated three 293 times independently.

294

295 2.9. Activity of recombinant cathepsin V in the presence of CHO cell-free lysates- Chinese 296 297 Hamster Ovary (CHO) cell lines K1 (HS positive, American Type Culture Collection: ATCC, 298 code CCL-61) and 2244 (HS negative, pgsD-677, ATCC code CRL-2244) were a kind gift 299 from Dr. Romain Vivès (Institut de Biologie Structurale, Grenoble, France). CHO cells were 300 cultured at 37°C, under 5% CO2, in EMEM supplemented with 10% fetal calf serum, 301 penicillin (50 IU/mL), and streptomycin (50 µg/mL). CHO cells were harvested at a density of approximately $2x10^6$ cells/mL, centrifuged at 200 g for 10 min, then washed once with the 302 303 preservative buffer (100 mM sodium acetate, pH 5.0, 0.5 mM PMSF, 0.5 mM EDTA, 40 µM pepstatin A, and 1 mM MMTS). The cell pellet was resuspended to achieve a density of 304 305 approximately 5x10⁸ cells/mL. Cell lines K1 and 2244 disruption was accomplished by 306 syringing the harvested cell pellet through a 20-gauge needle, and CHO cell-free supernatants 307 were recovered after centrifugation (10,000 g, 10 min). Protein concentration was determined 308 by a BCA assay, while sulfated GAGs and HS contents were measured using DMMB and 309 ELISA assays, respectively. CatV (1 nM) was incubated with each cell lysate (K1 and 2244, 310 50 µg of total protein) in 100 mM sodium acetate buffer pH 5.5 containing 2 mM DTT. The 311 residual activity was measured after addition of Z-Phe-Arg-AMC (20 µM) (λ_{ex} =350 nm, 312 λ_{em} =460 nm). Assays were repeated under the same conditions using CHO K1 cell-free 313 supernatants pretreated with a solution containing glycosidases (heparinase I, II, and III, 314 chondroitinase B, and Δ -4,5-glycuronidase) (180 mU/mL), sodium chromate (10 mM), and 315 Dnase I (100 mU/mL) for 48 h at 37°C under robust agitation. All kinetic measurements were 316 performed in triplicates and repeated three times.

317

318 2.10. Complex formation between cathepsin V and HS- Recombinant CatV (1 µg) was 319 incubated in the presence or absence of HS (0.15%, w/v, 14 kDa) in a final volume (100 µL) 320 for 10 min in 100 mM sodium acetate buffer, pH 5.5. Samples were then applied to a size 321 exclusion Superdex 200 column (AKTA purifier 900 HPLC system, Amersham, 322 GEHealthcare Bio-Sciences AB, Uppsala, Sweden), previously equilibrated in the same 323 buffer. Protein elution was monitored at 280 nm. The calibration curve was obtained using the 324 standard protein molecular mass markers (Amersham). CatV enzymatic activity was recorded 325 for each fraction (0.5 mL) using Z-Phe-Arg-AMC (20 µM) as substrate. Immunoreactive 326 CatV was detected using a polyclonal goat anti-human CatV antibody (1:1,000) as described 327 previously. HS was revealed in collected fractions using the DMMB assay. Alternatively, 328 dissociation equilibrium constant (K_D) determination between recombinant CatV and HS 329 (0.15%) was measured by surface plasmon resonance imaging (SPRi), as reported elsewhere 330 (Sage et al., 2013).

331

332 2.11. Molecular modeling of interactions between Cathepsin V and HS- Autodock 3 was first 333 used for docking HS, heparin and C4-S tetrasaccharide to CatV (PDB ID: 1FH0) (Morris et 334 al., 1998). The ligands were modeled using GLYCAM06 parameters (Kirschner et al., 2008). 335 The charges of the ligands were previously integrated into the corresponding libraries of GAG 336 monomeric units (Pichert et al., 2012). The protocols for molecular docking and clustering 337 previously optimized for protein-GAG systems were used (Panitz et al., 2016). Structures of 338 CatV/GAG complexes were then used for molecular dynamics (MD) simulations with 339 AMBER 14 (Case et al., 2017). The details for the MD protocols are detailed elsewhere (Panitz et al., 2016). Free energy calculations and per residue decomposition were performed 340 341 using Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) with gb = 2342 (Onufriev et al., 2002) implemented in AMBER for 100 frames evenly distributed in the 343 productive MD run. Analysis of the trajectories was done using the cpptraj module of 344 AMBER and VMD for visualization (Humphrey et al., 1996). Alternatively, assays were 345 performed on CatL (PDB ID: 2XU3).

346

347 2.12. Kinetic parameters of wild-type cathepsin V and K20G cathepsin V mutant with GAGs-

348 CatV (0.5 nM) was incubated 10 min in the activity buffer in the presence or absence of HS,

349 C4-S, C6-S, Hep, DS, and HA, respectively (0.15 and 0.3%, w/v) at 37°C. CatV activity was

350 then monitored using Z-Phe-Arg-AMC (20 µM) as a substrate. Experiments with HS were

- repeated in the presence of increasing amount of NaCl (0-0.5 M). Assays (triplicate) were
 achieved three times in separate experiments, and relative data are given as means±SD.
 Steady-state kinetics were assessed as previously described (Sage et al., 2013). Similar assays
 were repeated with K20G CatV (0.5 nM) and HS (0.15%, w/v).
- Further, the peptidase activity of CatV was recorded in the presence of increasing amounts of HS (0–0.01%), using Z-Phe-Arg-AMC (1–20 μ M) as substrate. Data were plotted using the equation 1/V=f(1/S) (i.e., Lineweaver-Burk plot). Inhibition data were fit by nonlinear regression analysis using the non-competitive model inhibition to determine the equilibrium dissociation constant (K_i) (GraphPad Software). All measurements were performed three times in triplicate.
- 361

362 2.13. Inhibition of cathepsin V by perlecan and HS derivatives- CatV (0.5 nM) activity was 363 measured in the presence or absence of perlecan (0.1-2 μ g/mL), aggrecan (0.1-2 μ g/mL), and 364 synthetic HS di- (dp2) and tetra-saccharide (dp4; 0.001-0.005%, w/v) (Liu et al., 2010) in the 365 activity buffer, using Z-Phe-Arg-AMC (20 μ M) as a substrate. Control experiment was 366 carried out with perlecan (2 μ g/mL) pretreated with heparinase I (2 U/mL) in the activity 367 buffer during 15 minutes at 37°C. Assays were repeated in the presence of Surfen (10 μ M). 368 All measurements were performed three times in triplicate.

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370 2.14. Inhibition of the elastolytic activity of cathepsin V by HS- Elastin-Congo Red conjugate 371 (10 mg/mL) was incubated at 37°C overnight under vigorous agitation with MPS samples (50 372 µg of total protein) in 100 mM sodium acetate buffer, pH 5.5 containing 10 mM DTT, in the 373 presence or absence of Surfen (10 µM). Controls were performed by preincubating previous 374 mixtures with cathepsin inhibitors: E-64 (100 µM), CA-074 (1 µM), CatL inh.7 (1 µM), 375 LHVS (10 nM) and Odanacatib (1 µM). In another set of experiments, aliquots of 10 mg/mL 376 of elastin-Congo Red conjugate were incubated with wild-type CatV (1 µM) or K20G CatV 377 $(1 \ \mu M)$ in the presence or absence of HS (0.15 %) in 100 mM sodium acetate buffer, pH 5.5 378 containing 2 mM DTT, and the mixture was vigorously vortexed at 37°C overnight. All 379 assays were stopped by centrifugation (13,500 g, 4°C) to remove the supernatant from the 380 insoluble contents (pellet). The absorbance of the supernatant was measured at 490 nm by 381 spectrophotometry (Cary 100 spectrophotometer, Agilent). All measurements were performed 382 three independent times in triplicate. Similar experiments were repeated under the same 383 conditions with insoluble elastin from bovine neck (10 mg/mL) in the absence and presence 384 of CatV (1 μ M) for scanning electron microscopy. Pellets were then fixed by incubation for

385 24 h in 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). 386 Afterwards, samples were then washed in phosphate-buffered saline (PBS), post-fixed by 387 incubation with 2% osmium tetroxide for 1 h, then dehydrated by ethanol and dried in 388 hexamethyldisilazane (HMDS, Sigma-Aldrich). Finally, samples were coated with 40 Å 389 platinum, using a GATAN PECS 682 apparatus (Pleasanton, CA, USA), before observation 390 under a Zeiss Ultra plus FEG-SEM scanning electron microscope (Oberkochen, Germany).

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392 2.15. Statistical analysis- Data were expressed as mean \pm SD unless indicated. Statistical 393 significance between the different values was analyzed by non-parametric Mann-Whitney U 394 test and patients groups comparison were performed with non-parametric Wilcoxon test. 395 Statistical analysis was performed using GraphPad Prism (GraphPad software, San Diego, 396 CA, USA). Differences at a *P*-value < 0.05 were considered significant.

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3. Results

3.1. Cathepsin V and heparan sulfate colocalize in MPS-I lung biopsy

The expression of CatV in lung tissue from a 2-year old female with MPS-I (Hurler syndrome) was investigated by IHC (Figure 1). Main clinical and pathological details have been described previously (Gupta et al., 2013). Briefly, the stained lung tissue sections showed pulmonary parenchyma and bronchial structures modified by autolysis lesions due to the autopsy delay. Malformation or dystrophic arteriolar vascular lesions were observed with edematous and concentric wall fibrosis (Fig. 1A), harboring well-formed granulomas with resorptive multinucleated giant cells (MNGCs) containing few lipid crystals deposits (Fig. 1B). Well-formed granulomas were less present in the alveolar walls. Numerous plump alveolar macrophages with vacuolated cytoplasm were identified in alveolar spaces (Fig. 1C), including cases engorged with Periodic Acid Schiff positive material (Fig. 1D, e.g., glycoprotein, carbohydrate and mucins-like structures). Cells, especially endothelial and bronchial epithelial cells, ubiquitously expressed significant amount of CatV (Fig. 1E, F). Moreover, a fluorescence overlay of CatV and HS probes was observed predominantly in bronchial epithelial cells in MPS-I lung tissue, suggesting a possible co-localization (Fig. 1G).

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478 Figure 1: Immunodetection of cathepsin V in MPS-I lung tissue.

Hematoxylin eosin saffron (HES) staining (panels A and B), CD68 IHC (panel C), PAS staining
(panel D), CatV IHC (panels E and F), and CatV/HS IF (panel G) were performed on lung tissue
obtained from a post-mortem 2-year-old female with MPS-I. A) Mild concentric edematous fibrosis of
blood vessels (x10). B) Granuloma exhibiting multinucleated giant cells in venular walls (x20). C)
Alveolar macrophages with numerous vacuoles (white arrows) in the cytoplasm (inset) (x40). D)
Eosinophils and mucopolysaccharides deposits mainly in alveolar macrophages (x40). E) CatV in

485 alveolar macrophages (x300) and F) in bronchial cells (x150), as indicated by black arrows. G) 486 Representative IF images (of two independent experiments performed) showing CatV stained in red 487 and HS in green. Merged panel revealed the co-localization of both antigens in bronchial cells. Cell 488 nuclei were stained in blue (DAPI staining). Scale bar is 50 μ m (panels C, D), 100 μ m (panels B, E, 489 G), and 250 μ m (panels A, F).

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491 **3.2.** Cathepsin V and heparan sulfate levels in MPS-I, II, and III respiratory samples

492 Given the scarcity of lung biopsies from young MPS patients, we decided to use as biological 493 specimen sputum and tracheal aspirates. Eleven MPS patients with a history 494 of respiratory complaints, and nine non-MPS patients with no respiratory symptoms were 495 recruited. MPS patient characteristics were summarized in Table 1. There were more males, 496 which reflects the disease population (in particular MPS-II, an X-linked disease affecting 497 mainly males). The mean age, size and weight of the MPS patients (10± 6 years; 112.3±18 498 cm; 26.5 ± 11 kg) were not statistically different than control groups (9±6 years; 133 ± 41 ; 499 34±22 kg).

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501	Table I: Clin	ical character	ristics of MPS	patients.
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#	Age	Sex	weight	Height	Diagnosis	Enzyme	Treat-	GK22
	(yr)		(kg)	(cm)	(MPS type, subtype)	deficiency	ment	score
1	11	М	22.3	104	Hurler (I)	IDUA	ERT	4
2	14	F	24.8	106	Hurler (I)	"	ERT	4
3	2	Μ	14.4	85	Hunter (II)	IDS	ERT	2
4	8	Μ	49.5	125	Hunter (II)	"	ERT	3
5	4	Μ	20.5	105	Hunter (II)	"	ERT	2
6	2	Μ	14.1	90	Hunter (II)	"	ERT	2
7	19	Μ	22.9	114	Hunter (II)	"	ERT	4
8	7	Μ	29.4	118	San Filippo (III, A)	HNS	no	3
9	18	F	33.4	127	San Filippo (III, B)	NAG	no	2
10	15	Μ	40.0	149	San Filippo (III, B)	"	no	4
11	6	Μ	20.8	112	San Filippo (III, C)	HGSNAT	no	1

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504 IDUA: α -L-iduronidase; IDS: iduronate-2-sulfatase; HNS: heparan-N-sulfatase; NAG: N-acetyl-505 glucosaminidase; HGSNAT: heparan α -glucosaminide-N-acetyltransferase; ERT: enzyme 506 replacement therapy; no: no specific treatment. MPS patients were assigned with a number (#).

507 The global respiratory symptoms severity (GRSS) score was assessed for each patient on the basis of 508 four sub-type evaluations: ear-nose-throat symptoms (chronic rhinitis or sinusitis, otitis, adeno-509 tonsillar hypertrophy, hearing loss, macroglossia, stridor), pulmonary symptoms (dyspnea, wheezing, 510 cough, sputum, asthma, bronchitis, pneumonia), clinical symptoms of obstructive sleep apnea, and 511 skeletal abnormalities causing restrictive lung disease (scoliosis, kyphosis, ribcage narrowing, chest 512 wall deformity). The GRSS was graduated from 0 to 4, corresponding to the number of sub-types 513 symptoms reported for each patient.

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516 The expression levels of CatV in cell-free supernatants of induced sputum and tracheal 517 aspirates of MPS patients was evaluated by Western-blot and ELISA (Figure 2). Both pro-518 CatV (as full or partially processed proforms) and mature CatV were detected by Western 519 blots analysis (Fig. 2A). We did not find a significant difference in expression level of 520 immunoreactive CatV (ELISA assay) between MPS patients and non-MPS patients (379±181 521 pg/mL vs. 439±256 pg/mL, normalized to total homogenate protein content of 100 µg in each sample) (Fig. 2B). Also, there was no difference in CatV levels between MPS types 522 523 (supplementary Fig. 1A). Conversely, ELISA analysis demonstrated that all MPS patients 524 displayed a 4-fold increase of HS levels (19.3±10 ng/mL) compared to controls (3.9±4 525 ng/mL) (P<0.0001) (Fig. 2C). Nevertheless, no significant difference of HS levels was 526 observed between MPS types (supplementary Fig. 1B). DS levels were lower (5±8 ng/mL) 527 than HS in MPS patients, but higher compared to controls $(1\pm 1 \text{ ng/mL})$ (P<0.05, Fig. 2D). No 528 significant difference was observed for chondroitin sulfate (CS) between MPS and non-MPS 529 patients (supplementary Fig. 1C). Present data suggest that, among major lung sulfated 530 GAGs, HS concentration is increased substantially in respiratory samples from MPS types I, 531 II, and III. Of note, a significant increase of total sulfated GAGs content (DMMB test) was 532 measured in MPS patients vs. controls (P < 0.0001) (supplementary Fig. 1D). Nevertheless, the 533 total sulfated GAGs levels (µg/mL) was higher than HS, DS, and CS levels (pg/mL), this 534 difference probably depending on the presence of other sulfated GAGs, proteoglycans or 535 DNA fragments, which may interact with DMMB as reported by Barbosa et al. (Barbosa et 536 al., 2003). We expanded our observations by comparing HS levels in respiratory samples 537 from the cohort of MPS patients on the basis of the global respiratory symptoms severity 538 (GRSS) (Fig. 2E). Interestingly, the GRSS scores correlated positively ($r_s=0.91$, P<0.0001) 539 with HS concentration, suggesting that an elevated HS level is associated with the onset of 540 respiratory-related disorders. In addition, the GRSS scores correlated positively with the age 541 of MPS patients (r_s=0.6515, P<0.0354, supplementary Fig 1.E), supporting that accumulation 542 of GAGs leads to diverse clinical manifestations including respiratory problems that worsen 543 with age.

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573 Figure 2: Cathepsin V and heparan sulfate levels in respiratory secretions from MPS-I, 574 II, and III patients.

575 A) Representative western blot (of two independent experiments) reflecting CatV protein expression 576 (white and black arrows: pro- and mature form, respectively) in cell-free supernatants from MPS-I, II, 577 and III respiratory secretions (total protein deposited: 50 µg/well). Number (#) assigned to each MPS 578 patient refers to Table 1. Two MPS (type II and IIIB) samples were not shown due to their limited 579 availability for western blot. B) ELISA protein levels of CatV in MPS (N=11) and non-MPS patients 580 (control, N=9). C) HS concentration determined by ELISA. D) DS concentration determined by 581 ELISA. E) Correlation between HS levels and global respiratory symptoms severity (GRSS) score. 582 The GRSS score was graduated from 0 to 4, corresponding to the number of major clinical respiratory 583 signs/symptoms diagnosed for each patient. Study was completed by linear regression. Spearman 584 coefficient (r_s) and level of significance (P) were reported. Statistical analyses were performed using 585 Mann-Whitney U test, (**: P < 0.01; ****: P < 0.001). Results are expressed as mean±standard 586 deviation (SD) of three independent experiments.

3.3. Cathepsin V activity in MPS-I, II, and III samples

A former report showed that elevated HS concentrations impair CatV activity in vitro (Yasuda et al., 2004). Accordingly, we evaluated the ability of HS-enriched MPS samples to inhibit CatV (Figure 3). We first measured the total endopeptidase activity of cysteine cathepsins in cell-free supernatant from both MPS and non-MPS samples, using Z-Phe-Arg-AMC as a broad-spectrum substrate, and E-64 as titration reagent. A ~2.5-fold decrease of active cathepsins was measured in all MPS types (259±74 nM) compared with controls (616±296 nM) (Fig. 3A; P < 0.05). Also, this drop of active forms of cathepsins correlated negatively (Fig. 3B; r_s =-0.66; P=0.0015) with HS levels and sulfated GAG content (r_s =-0.79; P<0.0001, supplementary Fig. 1F), and with the GRSS scores (Fig. 3C; r_s=-0.7052; P=0.0005). Due to a lack of both selective substrate and inhibitor of CatV, an indirect method was devised to uncover the influence of GAG on CatV activity. Three serial dilutions of cell-free supernatants (corresponding to 300, 30, and 3 ng of total protein, respectively) were mixed with a constant amount of exogenous recombinant CatV (0.5 nM), before monitoring the residual activity by Z-Phe-Arg-AMC (Fig. 3D-G).



Figure 3: Inhibition of cathepsin V in respiratory secretions from MPS-I, II, and III
patients.

660 A) Active cathepsins concentration was determined by E-64 titration (MPS and non-MPS samples; 30 µg of total protein). B) Correlation between concentrations of active cysteine cathepsins and HS level. 661 662 C) Correlation between concentrations of active cysteine cathepsins and GRSS score. D) Recombinant 663 CatV (0.5 nM) was incubated for 10 min in the activity buffer alone (control: C) or with different 664 dilutions of cell-free supernatants from individual respiratory secretion of MPS-I (N=2), E) MPS-II (N=5), F) MPS-III (N=4), and G) non-MPS (N=9). The same experiment was carried out with samples 665 666 pre-treated with glycosidases (heparinases I, II, III, and Δ -4, 5-glycuronidase). Residual activity 667 (tripicate) was measured with Z-Phe-Arg-AMC (20 µM). The relative activity was determined based 668 on the residual protease activity of CatV after incubation (control). Results are expressed as mean±SD 669 of three independent experiments. Statistical analyses were performed using Mann-Whitney U test 670 (**: *P*<0.01; ***: *P*<0.001; ****: *P*<0.0001).

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672 No endogenous peptidase activity toward Z-Phe-Arg-AMC was detected in the diluted 673 samples prior addition of exogenous CatV. CatV activity was significantly reduced in 1:20 674 dilution samples of all MPS types (P < 0.0001) and rose progressively along the increasing 675 serial dilutions (Fig. 3D-F). In contrast, no significant difference in the activity of CatV was detectable in non-MPS individuals (Fig. 3G). Pre-treatment of MPS samples with 676 677 glycosidases (heparinase I, II, and III, chondroitinase B and Δ -4,5-glycuronidase) yielded to a 678 significant retrieval of CatV activity in the presence of MPS-I (Fig. 3D) and MPS-II samples 679 (Fig. 3E). CatV activity was fully recovered in the presence of MPS-III samples (Fig. 3F). 680 Taken together, present results support that accumulation of GAG, including HS, could 681 potently impair lung CatV activity during MPS.

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683 **3.4. Inhibition of cathepsin V by heparan sulfate**

684 Cell-free lysates from wild-type CHO (HS positive strain) and HS-deficient CHO (HS 685 negative strain) cultures were incubated with recombinant CatV (Figure 4). A striking 686 difference was observed between HS positive and HS negative CHO samples, since WT 687 lysates but not HS deficient lysates, impaired peptidase CatV activity (P<0.0001) (Fig. 4A). 688 Conversely, CatV activity was fully recovered when HS positive CHO lysates were treated 689 beforehand with heparinases I, II, III, and Δ -4,5-glycuronidase (P<0.001), and thus confirmed 690 that HS is a potent inhibitor of CatV. Moreover, this finding was supported by testing various 691 GAGs at concentrations that mimic those found in liver, lungs and ovaries in MPS models 692 (Chung et al., 2007; Haskins et al., 1992). Indeed, among GAGs tested, only HS and DS 693 reduced significantly, in a dose dependent manner the peptidase activity of CatV (P < 0.05) 694 (Fig. 4B). HS (0.15%) produced a ~5-fold drop of the specificity constant (k_{cat}/K_m), which 695 mostly affected the catalytic activity of CatV (decrease of the k_{cat} value) (Table 2). Increase 696 of the ionic strength by addition of NaCl restored CatV peptidase activity in a dose-dependent

697	manner, suggesting that electrostatic interactions mainly governed binding of CatV to
698	negatively charged HS (Fig. 4C). We next investigated the mechanism of inhibition of CatV
699	by HS. HS inhibited peptidase CatV activity according to a non-competitive inhibition
700	mechanism (K _i estimated = 11.4 \pm 3 µM) (Fig. 4D). Then, we incubated CatV with insoluble
701	Congo Red Elastin to determine whether HS could also alter the proteolytic activity of CatV.
702	Figure 4E revealed that HS (0.15%) compromised the elastolytic activity of CatV (90 \pm 2%
703	inhibition, $P < 0.001$). This was further corroborated by SEM analysis. While long elastin
704	fibers (from bovine neck) were broken up into shorter fibrils by CatV, presence of HS
705	impaired fragmentation of elastin fibers (Fig. 4F).
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758 Figure 4: Inhibition of cathepsin V by heparan sulfate.

759 A) Recombinant CatV (0.5 nM) was incubated for 10 min in the activity buffer with cell culture media 760 alone (control), CHO cells lysate (-), CHO cell lysate pre-treated with glycosidases (+), and CHO cells 761 lysate lacking HS (KO). B) CatV (0.5 nM) was incubated in the absence (control: C) or in the presence 762 of different GAGs (Hep: low molecular weight heparin; HS: heparan sulfate; C4-S/C6-S: chondroitin 763 4/6-sulfate; DS: dermatan sulfate; HA: hyaluronic acid) at 0.15% (weight/volume, w/v) (white bar) 764 and 0.3% (black bar) in the activity buffer. C) CatV (0.5 nM) activity in the presence of HS (0.1%) is 765 restored by NaCl. Hydrolysis of Z-Phe-Arg-AMC (20 µM) was monitored with a spectrofluorometer. 766 The mean values±SD of three triplicate independent assays are expressed as relative value for the 767 hydrolysis of Z-Phe-Arg-AMC in each sample (normalized to control=1). D) Lineweaver-Burk plot 768 analysis of CatV (0.5 nM) velocity (Vi) with varying Z-Phe-Arg-AMC concentrations (1-20 µM) at a 769 HS concentration of 0-0.01% (0 % : • , 0.001 % : ■, 0.005% : ▲, 0.01% : ▼). E) Elastolytic activity 770 of CatV (1 μ M) in the absence (white bar) or presence of HS (0.15%, black bar) was measured by the 771 insoluble Congo Red elastin (10 mg/mL) spectrophotometry assay and was expressed as relative value 772 (normalized to control=1) at 490 nm. F) Representative electron micrographs (SEM) showing the 773 effect of HS (0.3%) on CatV (1 µM) elastolytic activity toward bovine neck elastin powder (10 774 mg/mL) after overnight incubation. Magnification bars, 10 μ m (upper panel) and 1 μ m (lower panel). Results were analyzed using Mann-Whitney U test (P-value *: P<0.05; ***: P<0.001; ****: 775 776 *P*<0.0001).

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GAG	Sulfatation	[Disaccharide]n	k _{cat}	K _m	k _{cat} /K _m
(0.15%)	degree		(s^{-1})	(µM)	$(x10^{6} M^{-1}.s^{-1})$
	(number of				
	SO4 ²⁻ by				
	disaccharide)				
-	-	-	7.6±0.5	6.9±1.6	11.0±0.9
+ Hep	2.5	L-IdoA2S- $\alpha(1\rightarrow 4)$ -D-GlcNS6S-	5.3±0.3	5.7±1.7	9.0±1.0
. 110	0.0	$\alpha(1 \rightarrow 4)$ D GleA $\beta(1 \rightarrow 4)$ D GleNAc	1 4 0 2	57110	24106
+ HS	0.8	$\alpha(1\rightarrow 4)$	1.4 ± 0.2	5.7 ± 1.0	2.4±0.6
+ C4-S	0.9	D-GlcA- $\beta(1\rightarrow 3)$ -D-GalNAc4S-	5.4 ± 0.2	4.5 ± 0.6	12.0±1.1
	0.0	$\beta(1\rightarrow 4)$	0.0.0	60.10	11.0.00
+C6-S	0.9	D-GICA-p(1 \rightarrow 5)-D-GaINAcoS- $\beta(1\rightarrow 4)$	8.2±0.9	6.9±1.2	11.8±0.9
+ DS	1.1	L-IdoA- $\alpha(1\rightarrow 3)$ -D-GalNAc4S-	1.3±0.2	4.8±1.2	2.7±0.1
		$B(1 \rightarrow 4)$			

779 **Table 2:** Kinetic parameters for the hydrolysis of Z-Phe-Arg-AMC by CatV in the presence

of GAGs of GAGs

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783 **3.5. Binding of heparan sulfate to cathepsin V**

784 CatV (25 kDa) and HS (14 kDa) were eluted at 19.5 mL and at >25 mL, respectively (size 785 exclusion chromatography, Superdex 200 column). Analysis of a mixture of CatV and HS 786 revealed the presence of an additional peak at 200-250 kDa (elution volume: 5-8 mL), which 787 was specifically detected by DMMB assay (Figure 5A). Also, peptidase CatV activity was 788 detected in both 25 kDa and 200-250 kDa peaks, endorsing that HS bound to CatV in 789 solution. The binding affinity between HS and CatV was calculated by surface plasmon 790 resonance imaging (Fig. 5B). The dissociation equilibrium constant value (K_D) of the 791 HS/CatV complex was 55±5 nM. This high affinity binding value was in accordance with 792 previous finding above. Molecular modeling studies were further performed to tentatively 793 understand the basis of CatV inhibition and identify putative HS binding sites on CatV (Fig. 794 5C). According to HS chains vary widely in extent in epimerization and sulfation, we 795 conducted computational experiments using two well-defined HS tetrasaccharide models: a

796	highly sulfated variant corresponding to Hep, which has α -L-iduronic acid-2-sulfate-N-sulfo-
797	α -D-glucosamine-6-sulfate (IdoA2S-GlcNS6S) disaccharide units and an unsulfated variant
798	with β -D-glucuronic acid- <i>N</i> -acetyl- <i>a</i> -D-glucosamine (GlcA-GlcNAc) disaccharide unit.
799	Docking scores were obtained for tetrasaccharides as the smallest unit required for specific
800	protein-GAG interactions based on the data available in the PDB (Samsonov & Pisabarro,
801	2016). Among the 50 top scoring complexes obtained, two major binding sites of unsulfated
802	HS variant were identified on CatV. The most populated cluster (21 poses) was located within
803	the active site cleft (Q19, K20, Q21, G23, S24, C25, N66, G67, and W190) while the second
804	one (15 poses) (V169, G170, Y171, G172, K182, Y183, W184, L185, V186, V200, K201,
805	and I202) located on the backside of the enzyme, away from the catalytic cleft. By contrast,
806	no clusters of Hep were observed in the catalytic domain of CatV (supplementary Figure 2A).
807	Heparin formed multiple contacts with CatV residues scattered on the backside of the
808	molecule, in the vicinity to the second binding site of unsulfated HS. Furthermore, no binding
809	poses of C4-S tetrasaccharide (β -D-GlcA-N-acetyl- β -D-galactosamine-4-sulfate (GalNAc4S)
810	disaccharide units) were predicted in the active site of CatV (supplementary Fig. 2B),
811	suggesting that unsulfated HS accommodated preferentially the active cleft of the enzyme.
812	These results corroborated the difference in the inhibition of CatV peptidase activity
813	measured by HS, Hep, and C4-S, as previously reported.
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857 A) Size-exclusion chromatography profiles of CatV in the absence (solid line) or presence of HS 858 (dashed line). The elution volumes of size standards under identical conditions are shown above the 859 graph. B) CatV-HS binding analyzed by surface plasmon resonance imaging. Compiled sensorgrams 860 of the kinetics of the interactions between CatV (6.25-200 nM) with HS-bearing biochips. C) Clusters 861 from 50 top-scored docking solutions of tetrameric HS (unsulfated) binding to CatV. A "top view" of 862 CatV and "back view" following a 90° rotation around the horizontal axis is shown. The most 863 populated cluster (blue) is located within the active cleft of the enzyme, while the second major cluster 864 (red) is on the backside. D) Docking of tetrameric unsulfated HS within the active site of CatV

865 (PDB:1FH0) and CatL (PDB:3HHA). MD-based free energy calculations (ΔG) for the obtained 866 binding poses of unsulfated HS were evaluated in the active site (ΔG_{enz}), outside the active site 867 (ΔG_{out}) . Electrostatic component of binding energy was reported $(\Delta G_{enz el})$ $(\Delta G_{out el})$. Surface electrostatic potential of both enzymes is shown. Blue areas correspond to positive charge and red 868 869 areas to negative charge. Among residues of the active cleft of CatV that are predicted to interact with 870 structural model of HS, K20 was a target residue for mutation to generate a cathepsin L-like mutant, 871 K20G, of CatV. K20 and G20 residues location is depicted by a circle. E) A bar chart analysis 872 showing the effect of HS (0.15%, black bar) on the elastolytic activity (Congo Red elastin assay) of 873 K20G CatV mutant compared with wild-type CatV. Results were analyzed using Mann-Whitney U 874 test (*P*-value *: *P*<0.05; ***: *P*<0.001).

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876 3.6. Lys20 is an essential residue within the active-site cleft of cathepsin V for interaction 877 with HS

878 Human CatV and CatL that share 78% identity and 85% homology of protein sequences 879 display similar three-dimensional structures (Fig. 5D). Although both enzymes disclose 880 similar subsite specificities (Choe et al., 2006), high concentration (0.15%) of cartilage and 881 bone-resident GAGs did not inhibit CatL peptidase activity (Li et al., 2000). Docking and MD 882 analysis of unsulfated HS tetrasaccharide on the active site of CatL revealed striking 883 differences with CatV (Fig. 5D). Binding free energy (ΔG_{enz}) calculated by MM-GBSA 884 approach was about twice less favorable for CatL compared to CatV. In addition, electrostatic 885 potential surface of residues bordering the active site of CatL is highly negative, thus less 886 energetically favorable to bind to unsulfated HS ($\Delta G_{enz el}=439\pm24$ kcal/mol) compared to 887 CatV ($\Delta G_{enz el}$ =-54±18 kcal/mol).

888 Next, we analyzed the residues in the CatV cleft that can theorically interact with unsulfated 889 HS and were different than their counterpart in CatL sequence. The only noticeable difference 890 between CatV and CatL was observed at the position 20 within the active-site cleft. CatV 891 possesses a positively charged lysine residue at position 20, which is replaced by a glycine 892 residue in CatL. Hence, we generated and expressed in P. pastoris a K20G CatV mutant. 893 Specificity constant values for Z-Phe-Arg-AMC hydrolysis by wild-type CatV $(k_{cat}/K_m=11\pm0.9x10^6 \text{ M}^{-1})$ and by K20G CatV mutant $(k_{cat}/K_m=8.3\pm1x10^6 \text{ M}^{-1}.\text{s}^{-1})$ were 894 895 comparable. This indicates that the overall peptidase activity of the CatV mutant was not 896 affected by this single mutation. Contrary to CatV, addition of HS (0.15%) did not change significantly the specificity constant of K20G CatV mutant ($k_{cat}/K_m=9.63\pm1\times10^6$ M⁻¹.s⁻¹). 897 898 Although a slight but significant impairment of the elastolytic activity of K20G CatV mutant 899 was observed in the presence of HS (P<0.05), K20G CatV still retained a greater elastolytic 900 activity than HS-bound wild-type CatV (P=0.0006) toward Congo Red Elastin (Fig. 4E). This

- 901 result supported that positively charged Lys20 played a critical role in the binding of HS chain902 within the active site-cleft of CatV.
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3.7. Surfen is a potent antagonist of cathepsin V inhibition by HS and HS-relatedglycosaminoglycans

906 In MPS, abnormal levels of heparan sulfate proteoglycans (HSPGs) and accumulation of HS 907 chains (both native or partially degraded forms) are found (De Pasquale & Pavone, 2019; Pan 908 et al., 2005). To address the biological significance of HS attached to a core protein and HS 909 fragments in the inhibition of CatV, the effect of perlecan (a.k.a. heparan sulfate proteoglycan 910 2) and synthetic HS di- and tetra-saccharides were examined. Perlecan inhibited CatV in a 911 concentration-dependent manner (Figure 6A), with 80±5% inhibition at a concentration of 2 912 µg/mL, contrary to aggrecan, a KS/CS-containing proteoglycan (Fig. 6B). To ensure that the 913 observed inhibition was mediated by HS, perlecan was treated with heparinase I. Cleavage of 914 HS chains in perlecan (2 μ g/mL) fully restored the peptidase activity of CatV (P<0.0001) 915 (Fig. 6A). Similarly, a consistent decrease in CatV activity was observed with di- and tetra-916 saccharides of HS (dp2 and dp4, dp: degree of polymerization) (Fig. 6C). Equal percentage 917 (0.001-0.005%, m/v) was used to allow direct comparison between dp2 and dp4. Our results 918 showed that dp4 was significantly more effective than dp2, suggesting that the tetrasaccharide 919 with the repeating unit of *N*-acetyl- α -D-glucosamine/ β -D-glucuronic acid (GlcNAc-GlcA) 920 was the minimal length required to inhibit CatV, which was in agreement with in silico 921 studies.

922 Several HS competitors/antagonists were reported to regulate the expression and/or reduce 923 binding of HS. Of particular interest is Surfen (bis-2-methyl-4-amino-quinolyl-6-carbamide, 924 supplementary Figure 3A), a chemical compound that binds tightly to HS and was reported to 925 antagonize HS-protein interactions (Schuksz et al., 2008). Therefore, we first investigated the 926 effect of Surfen on the inhibition of CatV in vitro by HS and derivatives. We chose 927 concentration of HS, perlecan and dp4 eliciting ~50% inhibition of CatV activity using Z-928 Phe-Arg-AMC as a substrate. First, we checked that Surfen, which exhibits weak fluorescence (λ_{ex} =350 nm, λ_{em} =460 nm) in neutral solution (Schuksz et al., 2008), did not overlap with the 929 930 fluorescence signal of 7-amido-4-methylcoumarin (AMC) group (supplementary Fig. 3B). 931 Addition of Surfen (10 µM) was sufficient to abolish completely the inhibition of CatV by HS 932 (Fig. 6D), HS-containing proteoglycan perlecan (Fig. 6E), and HS dp4 (Fig. 6F).

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967 Figure 6: Inhibition of cathepsin V with HS-like derivatives and influence of Surfen.

968 A) CatV (0.5 nM) was incubated for 10 min the activity buffer in the absence (control) or in the 969 presence of increasing concentrations of perlecan (0-2 µg/mL). Assays were also performed with 970 heparinase I-treated perlecan (2 µg/mL, black bar). B) Effect of aggrecan (0-2 µg/mL) and C) 971 synthetic di- and tetrameric heparan sulfate derivative (dp2, dp4; 0.001-0.005%, m/v) on CatV 972 activity. Residual activity was measured with fluorogenic substrate Z-Phe-Arg-AMC (20 µM) and 973 expressed as relative value (normalized to control=1). D-F) CatV (0.5 nM) was previously incubated 974 in the activity buffer for 10 min with HS from bovine kidney (0.05%) or perlecan (2000 ng/mL) or 975 dp4 (0.01%) before adding Surfen (10 μ M). Residual activity was measured with the fluorogenic 976 substrate Z-Phe-Arg-AMC (20 μ M). G) Surfen enhanced cysteine cathepsins-mediated elastin 977 degradation in cell-free supernatants from MPS-I, II, and III respiratory secretions. Assays were 978 performed with Congo Red elastin (10 mg/mL) incubated overnight under agitation with individual 979 MPS sample (total protein = 30 μ g) in the absence and presence of Surfer (10 μ M) in the activity 980 buffer at 37°C. Controls were performed by adding different cathepsin inhibitors : E-64 (100 µM), 981 CA-074 (1 μ M), CatL inhibitor (1 μ M), LHVS (10 nM) and ODN (1 μ M) in the activity buffer at 982 37°C. Results were expressed as mean±SD and were analyzed with GraphPad Prism software using 983 Mann-Whitney test, *P*-value **: *P*<0.01; ***: *P*<0.001; ****: *P*<0.0001.

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985 We next assessed the effect of Surfen on endogenous cathepsins-mediated elastolytic activity 986 in MPS samples, using Congo Red assays. Interestingly, pretreatment of soluble cell-free 987 supernatants of respiratory secretions with Surfen induced a significant ~6-fold increase of the 988 elastolytic activity compared to untreated MPS samples (MPS-I, II, III specimens) (Fig. 6G). 989 E-64, the broad-spectrum inhibitor of cysteine cathepsins, fully impaired elastolytic activity 990 (P<0.0001), while both odanacatib (ODN), a nitrile-based inhibitor of CatK and CA-074, a 991 selective CatB inhibitor led to a moderate but significant reduction of 23% and 20%, 992 respectively. Results endorsed that the elastin degrading activity of MPS samples depends on 993 cysteine cathepsins. Conversely, LHVS, a selective CatS inhibitor, and CatL inhibitor (N-(4-994 biphenylacetyl)-*S*-methylcysteine-(D)-Arg-Phe-β-phenethylamide) did impair not 995 elastinolysis, supporting that the elastolytic activity measured in MPS samples was mainly 996 credited to CatV-related activity.

997

998 **4. Discussion**

999 Respiratory complications (i.e. upper and lower airway obstruction and restrictive pulmonary 1000 disease) are typical irreversible features that affect early MPS patients and contribute often 1001 with cardiovascular abnormalities to death or disability associated with disease evolution. 1002 Current treatments such as hematopoietic stem cell transplantation (HSCT) or enzyme 1003 replacement therapy (ERT) are ineffective or insufficiently effective, when they are available 1004 (MPS-I, II, IV-A, and VI). Difficulties related to ERT is the need for a weekly highly 1005 expensive infusion (~\$130,000/year for a child; twice for an adult). Therefore, there is an 1006 imperative need to better understand pathophysiological mechanisms occurring during MPS

1007 disease to tentatively prevent its progression. It was reported that an accumulation of ECM 1008 components in adeno-tonsillar tissue samples of young MPS patients may contribute to the 1009 obstructive phenotype of airway disease (Pal et al., 2018). Nevertheless, the etiopathogenesis 1010 of airway deposits is yet to be elucidated. Few members of the cysteine cathepsin family were 1011 examined in the pathophysiology of MPS during the past years (for review: De Pasquale et 1012 al., 2020). Abnormal expression of cathepsins B, K, and S were reported in tissues of different 1013 MPS animal models (Baldo et al., 2017; Gonzalez et al., 2018; Ohmi et al., 2003; Viana et al., 1014 2020; Wilson et al., 2009). Their dysregulation may alter both ECM remodeling and 1015 homeostasis of skeletal, cardiac muscles and heart valves. Also, their overexpression was 1016 correlated with growth impairment, joint contractures and cardiovascular disorders found in 1017 animal models. As CatV exhibits in vitro the highest elastolytic activity yet described among 1018 mammalian proteases (Yasuda et al., 2004), we focused on CatV expression and activity in 1019 the respiratory secretions of young patients with MPS-I, II and III. CatV was mostly 1020 expressed in alveolar macrophages and bronchial epithelial cells of a MPS-I patient. CatV 1021 was found both as its proform and its mature form in respiratory specimens (sputum and 1022 tracheal aspirates) of MPS patients. However, no significant difference in the expression level 1023 of CatV was observed between MPS and non-MPS groups. Also, CatV levels were similar in 1024 MPS patients irrespective of a weekly ERT (MPS-I and II) or not (MPS-III). Direction for 1025 future research will therefore need to address whether CatV expression is regulated in the 1026 course of progressive lung disorders in a larger cohort of untreated patients with MPS. We 1027 identified HS as the major sulfated GAG ubiquitously found in MPS-I, II, and III specimen 1028 and we established a strong correlation between HS levels and the severity of respiratory-1029 related disorders. Concomitant to our observations, significant correlations were reported 1030 between the type of accumulated GAGs, including HS, and the relative involvement of 1031 neurological and visceral manifestations in MPS patients (for review: Whitelock & Iozzo, 1032 2005). Thus, high levels of stored HS may be also a useful indicator to predict respiratory 1033 disease severity in young patients with MPS. It should be noticed that despite patients with 1034 MPS-I and II of the present cohort underwent ERT treatment, high HS levels were still 1035 detected compared with healthy controls. ERT had a negligible impact on pulmonary function 1036 in younger children, probably due to limited penetration in the lungs as observed in other 1037 organs (Concolino et al., 2018). On the other hand, the activity of cathepsins is tightly 1038 regulated by linear, negatively charged sulfated Hep, HS, C4-S/C6-S, and DS, unveiling a 1039 pattern of repeating disaccharide units, GlcA/IdoA and a hexosamine, GlcNAc/GalNAc (for 1040 review: Novinec et al., 2014). The overall activity of cathepsins in MPS specimens was

1041 significantly reduced related to *in situ* HS levels, and was negatively correlated with GRSS.

- 1042 Lack of cathepsin-mediated matrix-degrading activity may affect remodeling of lung tissue in
- 1043 MPS, as reported either in a mouse model of bleomycin-induced lung fibrosis or sarcoidosis
 - 1044 (Bühling et al., 2004; Samokhin et al., 2011).
 - 1045 Here, we demonstrated that HS-rich MPS sputum and tracheal aspirates inhibited efficiently 1046 CatV activity and that HS could form a stable complex with CatV ($K_D=55\pm 5$ nM). Moreover, DS that also accumulated in MPS-I and II inhibited CatV in vitro, but not C4-S nor C6-S, 1047 1048 neither of which contains any IdoA in their disaccharide unit. IdoA is the major uronic acid 1049 component of DS and Hep, and in a lesser extent of HS, compared with its epimer β -D-1050 glucuronic acid (GlcA). However, Hep from porcine intestinal mucosal did not hinder the 1051 peptidase activity of CatV. It is difficult to explain the ineffectiveness of Hep, with a high 1052 level of both sulfation and IdoA, to hinder CatV activity. The low levels of IdoA in the 1053 context of domains with a low level sulfation, common to both HS and DS, may explain their 1054 CatV inhibitory activity.
- 1055 Numerous reports suggested a role of HS proteoglycans (HSPGs) in the pathogenesis of MPS 1056 (for review: De Pasquale & Pavone, 2019). Here, we used as model the large HSPG perlecan 1057 (~500 kDa), which possesses N-terminal ~65 kDa HS side chains (for review: (Gubbiotti et 1058 al., 2017)). We demonstrated that the inhibition of CatV by perlecan is mediated by its 1059 extended HS chains. Conversely, aggrecan, which mainly possesses CS and KS chains but not 1060 HS, did not impair the peptidase activity of CatV, sustaining the critical role of the HS 1061 moiety. Moreover, low-molecular-weight GAG fragments have been shown to accumulate in 1062 MPS (Fuller et al., 2004). We identified a synthetic HS-derived tetrasaccharide (dp4) that 1063 inhibited potently CatV, suggesting that short oligosaccharides could bind to CatV and impair 1064 its activity. These results are in good agreement with molecular modeling, advocating that dp4 1065 binds within the CatV active site while heparin and C4-S are docked in a region distant from 1066 the active cleft. Furthermore, it was shown that elastolytic activity of CatV was inhibited in 1067 vitro by the presence of 0.15% DS, but also heparin and C4-S/6-S chains (Yasuda et al., 1068 2004). This apparent discrepancy is likely due to the fact that elastin degradation by CatV 1069 requires two exosites, which are distant from its active site, and which contribute to elastin 1070 binding and its subsequent cleavage (Du et al., 2013). We found that binding sites of heparin 1071 and C4-S tetrasaccharides are located on a region nearby to one of the identified elastinbinding exosite (residues Val⁹²-Asn¹⁰⁴) and involved in the elastolytic activity of CatV. It can 1072 1073 be speculated that elongation of GAG chains length of heparin and C4-S may hinder the 1074 docking of elastin on this CatV exosite, thus suppressing its enzymatic degradation.

1075 Although human cathepsins V and L encompass a close substrate specificity, CatL activity 1076 towards peptidyl substrates was unchanged in the presence and the absence of 0.15% GAGs 1077 (Li et al., 2000). Docking studies revealed that among residues involved in HS binding, Lys20 1078 of CatV was of peculiar interest. Analysis of human cysteine cathepsin sequences revealed 1079 that a glycine residue is highly conserved in position 20, except for CatW and CatV. 1080 Replacement of the positively charged Lys20 by its counterpart in CatL (Gly20) significantly 1081 decreased the inhibition of the elastolytic activity by HS, supporting that this basic residue is 1082 critical for CatV/HS interactions.

- 1083 To further address the possibility of an HS antagonist to restore the elastin-degrading activity 1084 of CatV in respiratory specimen of patients with MPS, Surfen was chosen based on its ability 1085 to block HS-dependent activities (Schuksz et al., 2008). Despite the exact mechanism by 1086 which Surfen interacts with HS is unclear, Surfen restored endogenous cathepsin-mediated 1087 elastolytic activity in MPS types I, II and III. Using a series of selective inhibitors of human 1088 cathepsins, we showed that ~40-45% of the total elastolytic activity against Congo Red elastin 1089 can be attributed to both CatB (20%) and CatK (23%). Although CatS was reported to 1090 degrade substantially elastin in monocyte-derived macrophages (Yasuda et al., 2004), 1091 treatment of MPS samples with LHVS did not impair the elastolytic activity. Despite further 1092 studies are needed to investigate the role of CatV in MPS pathogenesis, we may assume that 1093 CatV mostly contributed to the degradation of elastin, which in turn is impaired by the 1094 accumulation of HS in patients with MPS. Accordingly, selective and safe agents (i.e. Surfen 1095 and other HS antagonists) (De Pasquale et al., 2018) preventing HS binding to its molecular 1096 partners could represent an attractive therapeutic option in MPS patients with lung disorders.
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1122

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1128 **Competing interests:** The authors declare that they have no competing interests.

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