



Kiely, A. P., Miners, S., Courteny, R., Strand, CV., Love, S., & Holton, J. L. (2019). Exploring the putative role of kallikrein-6, calpain-1 and cathepsin-D in the proteolytic degradation of  $\alpha$ -synuclein in multiple system atrophy. *Neuropathology and Applied Neurobiology*, 45(4), 347-360. <https://doi.org/10.1111/nan.12512>

Peer reviewed version

Link to published version (if available):  
[10.1111/nan.12512](https://doi.org/10.1111/nan.12512)

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**Exploring the putative role of kallikrein-6, calpain-1 and cathepsin-D in the proteolytic degradation of  $\alpha$ -synuclein in multiple system atrophy**

Journal:	<i>Neuropathology and Applied Neurobiology</i>
Manuscript ID	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Keywords:	multiple system atrophy, alpha-synuclein, kallikrein-6, calpain-1, cathepsin-D

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3 **Exploring the putative role of kallikrein-6, calpain-1 and cathepsin-D in the proteolytic**  
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5 **degradation of  $\alpha$ -synuclein in multiple system atrophy**  
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37 **Keywords:** multiple system atrophy; alpha-synuclein; kallikrein-6; calpain-1; cathepsin-D  
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42 **Running title:** alpha-synuclein proteolysis in multiple system atrophy  
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46 **Number of words in text:** 3228  
47  
48  
49  
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51 **Number of tables and figures:** 1 table and 7 figures  
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**Abstract**

Aims. There is evidence that accumulation of  $\alpha$ -synuclein ( $\alpha$ -syn) in Parkinson's disease (PD) and dementia with Lewy bodies (DLB) results from impaired removal of  $\alpha$ -syn rather than its overproduction. Kallikrein-6 (KLK6), calpain-1 (CAPN1) and cathepsin-D (CTSD) are among a small number of proteases that cleave  $\alpha$ -syn and are dysregulated in PD and DLB. Our aim in this study was to determine whether protease activity is altered in another  $\alpha$ -synucleinopathy, multiple system atrophy (MSA), and might thereby modulate the regional distribution of  $\alpha$ -syn accumulation.

Methods. mRNA and protein level and/or activity of KLK6, CAPN1 and CTSD were measured and assessed in relation to  $\alpha$ -syn load in multiple brain regions (posterior frontal cortex, caudate nucleus, putamen, occipital cortex, pontine base and cerebellar white matter), in MSA (n = 20) and age-matched post-mortem control tissue (n = 20).

Results. CTSD activity was elevated in MSA in the pontine base and cerebellar white matter. KLK6 and CAPN1 levels were elevated in MSA in the putamen and cerebellar white matter. However, the activity or level of these proteolytic enzymes did not correlate with the regional distribution of  $\alpha$ -syn.

Conclusions. Accumulation of  $\alpha$ -syn in MSA is not due to reduced activity of the proteases we have studied. We suggest that their upregulation is likely to be a compensatory response to increased  $\alpha$ -syn in MSA.

**Abbreviations**

AD	-	Alzheimer's disease
ALP	-	autophagy-lysosomal pathway
$\alpha$ -syn	-	$\alpha$ -synuclein
CAPN1	-	Calpain-1
CSF	-	cerebrospinal fluid
CTSD	-	Cathepsin D
DLB	-	dementia with Lewy bodies
GCI	-	glial cytoplasmic inclusion
KLK6	-	kallikrein-6
MSA	-	multiple system atrophy
NAC	-	non-amyloid- $\beta$ component
NCI	-	neuronal cytoplasmic inclusion
NNI	-	neuronal nuclear inclusion
PD	-	Parkinson's disease
<i>SNCA</i>	-	$\alpha$ -synuclein gene
UPS	-	ubiquitin-proteasomal pathway

## Introduction

Abnormal aggregates of  $\alpha$ -synuclein ( $\alpha$ -syn) within neurons, neurites or glial cells are the defining neuropathological hallmark of the  $\alpha$ -synucleinopathies. In Parkinson's disease (PD) and dementia with Lewy bodies (DLB), the aggregates form characteristic neuronal and neuritic inclusions (Lewy bodies and neurites), while multiple system atrophy (MSA) is characterised by  $\alpha$ -syn-containing glial cytoplasmic inclusions (GCIs) in oligodendrocytes. Smaller numbers of neuronal nuclear or cytoplasmic inclusions (NNI or NCI) are also a feature of the disease. In  $\alpha$ -synucleinopathies,  $\alpha$ -syn accumulates without increased *SNCA* mRNA expression (1-3), suggesting that impaired protein clearance may play a role in the pathogenesis of these disorders. The ubiquitin-proteasomal pathway (UPS) and the autophagy-lysosomal pathway (ALP) are major routes of  $\alpha$ -syn clearance and have been shown to be dysregulated in PD and DLB (reviewed in (4-6)). Cathepsin D (CTSD) is the main lysosomal protease responsible for  $\alpha$ -syn cleavage (7-9). CTSD-mediated  $\alpha$ -syn cleavage reduces aggregation and toxicity of  $\alpha$ -syn both *in vitro* and *in vivo* (7, 8), and is upregulated, probably as a compensatory response to elevated  $\alpha$ -syn, in DLB and PD (10).

A small number of non-lysosomal proteases including kallikrein-6 (KLK-6) (neurosin, zyme, protease M) (11-13) and calpain-1 (CAPN1) (14) have been shown to cleave and regulate  $\alpha$ -syn level, both *in vitro* and *in vivo*. KLK6 and CAPN1 proteases cleave monomeric  $\alpha$ -syn within the non-amyloid- $\beta$  component (NAC) region, required for  $\alpha$ -syn aggregation (15, 16), and generate cleavage fragments that are less neurotoxic and inhibit  $\alpha$ -syn aggregation *in vitro* (8, 11, 12). KLK6 is expressed in neurons and oligodendrocytes (17-19); it is found within Lewy bodies and GCIs (18, 20) and degrades extracellular  $\alpha$ -syn (13). Brain-targeted, viral vector-mediated upregulation of KLK6 protects against  $\alpha$ -syn pathology in

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3 animal models of DLB (21) and MSA (22). CAPN1 is expressed neuronally and co-localises  
4 with  $\alpha$ -syn in the cytosol (23). CAPN1 is capable of degrading both monomeric and fibrillar  
5  $\alpha$ -syn (14). Degradation of fibrillar  $\alpha$ -syn by CAPN is, however, associated with accelerated  
6  $\alpha$ -syn aggregation, suggesting a complex relationship between CAPN1 activity and  $\alpha$ -syn  
7 pathogenicity. We, and others, demonstrated that the expression and activity of KLK6 and  
8 CAPN1 are reduced in DLB and PD in human tissue and CSF (21, 23, 24) and in animal  
9 models (21), and that the extent of reduction correlates closely with  $\alpha$ -syn accumulation  
10 (23).  
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22 In this study we have explored the hypothesis that a reduction in proteolytic  
23 cleavage of  $\alpha$ -syn is responsible for the elevated  $\alpha$ -syn level in MSA. We have measured  
24 KLK6, CAPN1 and CTSD in MSA and assessed their association with  $\alpha$ -syn load in brain  
25 regions with varied predilection for MSA pathology. Unlike in DLB, our findings do not  
26 support the hypothesis that the accumulation of  $\alpha$ -syn in MSA is associated with reduced  
27 proteolytic clearance. The upregulation of the three proteases in regions with high  $\alpha$ -syn  
28 load is probably a physiological (if ineffective) response to the increase in  $\alpha$ -syn.  
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## 42 **Materials and Methods**

### 43 *Case selection*

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48 20 MSA and 20 control brains were used for biochemical assessment of enzyme  
49 protein/activity and measurement of  $\alpha$ -syn load (See Table 1). A subset of 6 control and 6  
50 mixed MSA cases were used for western immunoblotting and NanoString nCounter mRNA  
51 expression studies. Mixed MSA cases were selected, based on Ozawa criteria (25). MSA can  
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3 be subdivided according to the distribution of pathology into three subcategories:  
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5 striatonigral degeneration (SND), olivopontocerebellar atrophy (OPCA), and a combination  
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7 of the two known as mixed MSA. For the current study we chose to use mixed MSA cases  
8  
9 (hereafter referred to simply as MSA cases) to allow us to compare the findings in multiple  
10  
11 brain regions within a single dataset.  
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#### 14 15 *Human brain tissue*

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18 All brains had been donated to the Queen Square Brain Bank for Neurological Disorders,  
19  
20 UCL Institute of Neurology. The brain donation programme and protocols have ethical  
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22 approval from the NRES Committee London–Central, and tissue is stored for research under  
23  
24 a licence issued by the Human Tissue Authority (No. 12198).  
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28 The right hemisphere was sliced, and the slices flash frozen and stored at -80°C.  
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30 Tissue was dissected from the frontal lobe, caudate nucleus, putamen, pontine base,  
31  
32 occipital lobe and cerebellar white matter and homogenised in lysis buffer: 50mM Tris, 175  
33  
34 mM NaCl, protease and phosphatase inhibitor tablet and 1% Triton-X (all reagents from  
35  
36 Roche/Merck, UK), in a Precellys<sub>24</sub> homogeniser with ceramic beads (Bertin technologies,  
37  
38 France). The left half-brain was fixed in 10% buffered formalin, sliced, tissue blocks selected  
39  
40 for paraffin wax embedding, and 8 µm sections prepared for histology.  
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#### 48 *NanoString nCounter analysis*

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51 We used NanoString nCounter Human Inflammation panel v2 mRNA Expression Assay  
52  
53 (NanoString Technologies, Seattle, WA) to quantify transcripts of the three  $\alpha$ -syn-degrading  
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55 enzymes. Total mRNA was isolated from frontal lobe and cerebellar white matter of 6  
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3 control and 6 MSA cases (Table 1). 150 ng of total RNA from each sample was analysed.  
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5 Probes were designed according to the manufacturer's design principles (26), including  
6  
7 screening for inter- and intra-reporter and capture probe interactions, and selection for  
8  
9 probes with optimal melting temperature (26). The laboratory running the assay was  
10  
11 blinded to the diagnosis. To avoid run-order bias, samples of cases or controls were  
12  
13 randomly assigned to plates. Raw counts were subjected to a technical normalisation and  
14  
15 normalized to the geometric mean using nSolver Analysis Software v2.0 (NanoString). For  
16  
17 biological normalization we used reference genes (*CLTC*, *GAPDH*, *GUSB*, *HPRT1*, *PGK1*, *TUBB*)  
18  
19 included in the CodeSet. The NanoString data were analysed using NanoString nSolver  
20  
21 software. Data from NanoString nCounter expression counts were inspected and any  
22  
23 samples determined to be outliers relative to negative controls were excluded from further  
24  
25 analysis. The count data were normalised to negative controls and to positive controls  
26  
27 according to the manufacturer's instructions, correcting for differences in hybridization  
28  
29 efficiency and processing variables including purification and RNA/reporter complex  
30  
31 immobilisation. Two-tailed Student's *t* tests were used to test for significant difference in  
32  
33 gene expression between MSA and control frontal lobe and cerebellar white matter. The  
34  
35 Benjamini-Yekutieli method was used to exclude false positive results.  
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#### 42 *Immunohistochemistry*

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44  
45 Immunohistochemistry was performed with anti- $\alpha$ -syn (Catalogue number ab15530 at  
46  
47 1:1300) (Abcam, UK) for 1 h and used a modified method for automated Intellipath FLX  
48  
49 staining system (Menarini Diagnostics, Wokingham, UK) as per the manufacturer's  
50  
51 instructions. Sections were pre-treated in the Menarini unit with an excess of super buffer  
52  
53 and mounted in DPX with glass coverslips. Immunolabelled sections were processed by  
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3 digital slide scanner and images acquired using a Leica Aperio Imagescope. The percentage  
4  
5 section area immunopositive for  $\alpha$ -syn was determined by applying an Image J colour-  
6  
7 thresholding macro.  
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#### 10 *Western immunoblotting*

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13 Protein determination was performed by BCA assay (Pierce) as per the manufacturer's  
14  
15 instructions. For SDS-PAGE and western immunoblotting we used the XCell surelock blot  
16  
17 system (Thermo Fisher Scientific). Samples diluted 20  $\mu$ g/20  $\mu$ l in MES buffer (Thermo Fisher  
18  
19 Scientific) were run at 120 V. Proteins were transferred from the gel to nitrocellulose  
20  
21 membrane (GE healthcare) in 1X transfer buffer (Thermo Fisher Scientific) at 37 V for 80 min  
22  
23 on ice. The blots were analysed using the Odyssey system. The integrated area-density of  
24  
25 immunoreactive bands at the predicted molecular weight for each protein of interest was  
26  
27 quantified using Image J. Statistical analysis was performed using a two-tailed student *t* test  
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29 in which significance was set at  $p < 0.05$ .  
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#### 34 *Kallikrein-6 (KLK6) sandwich ELISA*

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38 KLK6 level was determined in brain tissue homogenates by sandwich ELISA that was  
39  
40 developed in-house, as previously described (23). Mouse monoclonal anti-KLK6 antibody  
41  
42 (Sigma Aldrich, Dorset, UK) diluted in PBS (1:200) was used to coat wells in clear Costar™  
43  
44 high-binding 96-well microplates (R&D systems, Abingdown, UK) and was left overnight at  
45  
46 room temperature (RT). The plates were washed in PBS 0.01% tween-20 five times and  
47  
48 blocked for 3 h in 1%BSA/PBS at 26°C. Following a further wash step, tissue homogenates  
49  
50 (6.5  $\mu$ g total protein) and human recombinant standards (R&D systems, UK) diluted in PBS  
51  
52 containing 1% BSA were added overnight at 4°C. After another wash, biotinylated goat  
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3 polyclonal anti-KLK6 antibody (1 µg/ml) (R&D systems, Cambridge, UK) diluted in 1 %  
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5 BSA/PBS, was added for 2 h at 26°C. After further washes, streptavidin:HRP (1:200) (R&D  
6  
7 systems, UK) diluted in PBS/0.01% tween-20 was added for 1 h at 26°C, the plate was  
8  
9 washed, and chromogenic substrate (TMBS, R&D systems, UK) added for 20 min in the dark.  
10  
11 The reaction was stopped with 2 N sulphuric acid, and absorbance read at 450 nM in a  
12  
13 FLUOstar Optima plate reader. KLK6 concentration was determined by interpolation against  
14  
15 measurements on serial dilutions of recombinant human KLK6 (720-11.25 ng/ml) included  
16  
17 on each plate. All sample measurements were made in duplicate.  
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#### 22 *Calpain-1 (CAPN1) fluorogenic activity assay*

23  
24  
25 CAPN1 activity was measured in brain tissue homogenates by use of an internally quenched  
26  
27 fluorogenic substrate for CAPN1, as previously described (23). The substrate included the  
28  
29 CAPN1-specific cleavage site in  $\alpha$ -spectrin (1 µM, H-K(FAM)-EYY ~ GMMK(DABCYL)-OH  
30  
31 (Millipore, Hertfordshire, UK)) and the assays were performed with or without the addition  
32  
33 of a calpain-1-specific inhibitor (10 µM Tocris, Bristol, UK). Brain tissue homogenates and  
34  
35 purified human CAPN1 (Sigma Aldrich, Dorset, UK) were diluted in assay buffer (10 µl of  
36  
37 homogenate in 40 ul assay buffer, containing 50 mM Tris-HCL, 50 mM NaCl, 5 mM  $\beta$ -  
38  
39 mercaptoethanol, 5 mM CaCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA) pre-warmed to 37°C as described  
40  
41 by Mittoo et al (27). Each sample and standard was added in duplicate to wells, with or  
42  
43 without inhibitor, and left for 10 min at 37°C, prior to addition of the fluorogenic substrate  
44  
45 which was also diluted in assay buffer. The reaction was left to proceed at 37°C for 3 h in the  
46  
47 dark after which fluorescence was read in a FLUOstar Optima plate reader with excitation at  
48  
49 490 nm and emission at 518 nm. For each sample the fluorescent signal in the inhibited  
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51 wells was subtracted from that in the uninhibited wells. Measurements were repeated on  
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3 two separate occasions. The calpain-1 activity of each sample was interpolated from a  
4  
5 standard curve derived from measurements on serial dilutions of calpain-1 (20-1.25 µg/ml);  
6  
7 these were included in each plate to control for variation across plates.  
8  
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#### 10 *Cathepsin D (CTSD) fluorogenic activity assay*

11  
12  
13 CTSD enzyme activity was measured in brain tissue homogenates by adding a fluorogenic  
14  
15 peptide substrate, Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH<sub>2</sub> [Mca= (7-  
16  
17 methoxycoumarin-4-yl)acetyl; Dnp=dinitrophenyl] (BML-P-145-001) at 20 µM (Enzo Life  
18  
19 Sciences, Exeter, UK ) in the presence or absence of a specific cathepsin-D inhibitor  
20  
21 (Pepstatin-A at 4µM). Brain tissue homogenates (1.25 µl in 1 ml assay buffer) or purified  
22  
23 cathepsin-D from liver (BML-SE199, Enzo Life Sciences, Exeter, UK) was diluted in assay  
24  
25 buffer made up of 0.1 M sodium acetate and 0.1 M sodium chloride at pH 3.5. Each sample  
26  
27 or standard was added in duplicate and incubated with or without pepstatin-A for 10 min at  
28  
29 37°C prior to addition of the fluorogenic substrate. All components were diluted in assay  
30  
31 buffer. The reaction was left to proceed at 37°C for 3 h in the dark after which fluorescence  
32  
33 was read using a FLUOstar Optima plate reader with excitation at 320-340 nm and emission  
34  
35 at 393-420 nm. For each sample the fluorescent signal in the inhibited wells was subtracted  
36  
37 from that in the uninhibited wells. Each sample was measured in duplicate and  
38  
39 measurements were repeated on two separate occasions. CTSD activity was interpolated  
40  
41 from measurements on serial dilutions of serially recombinant CTSD that were included on  
42  
43 each plate.  
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#### 50 *Statistical analysis*

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3 Parametric statistical tests were used for comparisons between groups, and ANOVA with  
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5 Bonferroni post-hoc test was used for multiple-group comparisons. In some cases the data  
6  
7 required logarithmic transformation to obtain a normal distribution. For variables that were  
8  
9 not normally distributed even after transformation, Kruskal-Wallis test was used, followed  
10  
11 by Dunn's test for pairwise intergroup comparisons. Pearson analysis was used to assess the  
12  
13 correlation between pairs of variables. Statistical tests were performed using SPSS version  
14  
15  
16  
17 16. P-values < 0.05 were considered statistically significant.

## 18 19 20 **Results**

### 21 22 23 *KLK6, CTSD and CAPN1 mRNA*

24  
25 We examined KLK6, CAPN1 and CTSD mRNA level in frontal lobe and cerebellar white  
26  
27 matter from MSA and control brains. KLK6 mRNA level was significantly elevated in frontal  
28  
29 lobe in MSA (4.53-fold increase) ( $P < 0.01$ ). KLK6 mRNA level in the cerebellar white matter  
30  
31 did not differ between MSA and control brains. CTSD and CAPN1 mRNA were unchanged in  
32  
33 MSA compared to controls in any of the regions studied (Fig. 1). As in previous studies ((2,  
34  
35 3),  $\alpha$ -syn (*SNCA*) mRNA expression was not altered in MSA.

### 36 37 38 39 40 41 *KLK6, CTSD and CAPN1 protein*

42  
43 We assessed KLK6, CAPN1 and CTSD protein level in a subset of brains, by western blot, in  
44  
45 MSA and controls. KLK6 protein level was significantly elevated in the putamen ( $P < 0.05$ )  
46  
47 but was unchanged in all other regions examined (Fig. 2). CAPN1 level, measured by western  
48  
49 blot, was significantly increased in MSA in the putamen ( $P < 0.05$ ) (Fig. 3) and cerebellar  
50  
51 white matter ( $P < 0.01$ ) (Fig. 3). CTSD level was unchanged in MSA in all regions (Fig. 4).

### 52 53 54 55 56 57 58 59 60 *Regional distribution of $\alpha$ -syn load in MSA*

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3 We determined the  $\alpha$ -syn load (percentage section area immunopositive for  $\alpha$ -syn) as an  
4  
5 indicator of regional variation in severity of GCI pathology in MSA. The level of  $\alpha$ -syn was  
6  
7 highest in the pontine base and putamen, intermediate in the cerebellar white matter,  
8  
9 frontal cortex, and caudate nucleus, and lowest in the occipital cortex (Fig. 5).

#### 12 13 *Regional variation in KLK6, CAPN1 and CTSD level or activity in MSA*

14  
15  
16 We measured KLK6 protein level by ELISA, and CAPN1 and CTSD enzyme activities, in all  
17  
18 regions, in the complete MSA and control cohorts. KLK6 level was significantly elevated in  
19  
20 MSA in the putamen ( $P < 0.05$ ) and cerebellar white matter ( $P < 0.0001$ ) (Fig. 6A). CAPN1  
21  
22 enzyme activity was significantly elevated in the putamen ( $P < 0.0001$ ) and cerebellar white  
23  
24 matter ( $P < 0.001$ ) (Fig. 6B). CTSD activity was significantly elevated in the pontine base and  
25  
26 cerebellar white matter in MSA (Fig. 6C).

#### 29 30 *Regional variation in KLK6, CAPN1 and CTSD in relation to $\alpha$ -syn load in MSA*

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32  
33 Lastly, we assessed whether regional differences in  $\alpha$ -syn load were related to KLK6, CAPN1  
34  
35 or CTSD level or activity. Across the six regions examined,  $\alpha$ -syn load did not correlate  
36  
37 significantly with KLK6 level, or with CAPN1 or CTSD level or activity. KLK6 was generally  
38  
39 reduced, and CAPN1 and CTSD activity tended to be elevated, in regions with higher  $\alpha$ -syn  
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41 load but none of these trends reached statistical significance (Fig 7)  
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## 49 **Discussion**

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52 We have examined the expression of three proteases, previously shown to regulate  
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54  $\alpha$ -syn homeostasis, in MSA, in relation to the  $\alpha$ -syn load in different regions of brain. KLK6,  
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3 CAPN1 and CTSD were increased in MSA within the putamen, pontine base and cerebellar  
4  
5 white matter – regions of high GCI burden and  $\alpha$ -syn load in MSA. However, differences in  
6  
7  $\alpha$ -syn load between regions were not correlated with the level or activity of KLK6, CAPN1 or  
8  
9 CTSD. The findings contrast with our previous observations in PD and DLB (23), and indicate  
10  
11 that accumulation of  $\alpha$ -syn in MSA is unlikely to result from reduced activity of the  
12  
13 proteases we have tested. The upregulation of these  $\alpha$ -syn degrading enzymes in regions of  
14  
15 high GCI burden and  $\alpha$ -syn load is likely to be a compensatory response to elevated  $\alpha$ -syn. It  
16  
17 should be noted, of course, that we have only tested a small number of potential  $\alpha$ -syn  
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19 proteases, and it remains possible that others may be downregulated in MSA.  
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24 Abnormal accumulation of  $\alpha$ -syn in neurons and glial cells is a characteristic hallmark  
25  
26 of PD, DLB and MSA. There is no evidence that  $\alpha$ -syn mRNA level is increased in  $\alpha$ -  
27  
28 synucleinopathies (1-3) suggesting that failure of proteolysis or removal by other means  
29  
30 may contribute to the pathogenesis of MSA. The endosomal-lysosomal and autophagic  
31  
32 pathways are major routes of  $\alpha$ -syn clearance and have been shown to be dysregulated in  
33  
34 PD and DLB (4-6, 28) and MSA (29-32). A small number of proteases have been identified  
35  
36 that cleave and degrade intracellular and extracellular pools of  $\alpha$ -syn *in vitro* and are down-  
37  
38 regulated in PD and DLB (23). KLK6, CAPN1 and CTSD are expressed neuronally, and within  
39  
40 oligodendrocytes and are likely to impact on  $\alpha$ -syn load in PD, DLB and MSA. KLK6, CAPN1  
41  
42 and CTSD all have major cleavage sites within the non-A $\beta$  component (NAC) region which is  
43  
44 required for  $\alpha$ -syn aggregation, and cleavage was shown to inhibit aggregation of full-length  
45  
46 monomeric  $\alpha$ -syn (15, 16). Brain-specific induction of KLK6 is protective against  $\alpha$ -syn in  
47  
48 mouse models of DLB (21) and MSA (22), and there is evidence that CTSD has protective  
49  
50 effects *in vivo* (8). We, and others, showed previously that KLK6 and CAPN1 are reduced in  
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3 human brain tissue in PD and DLB and that the reduction is most marked in regions of  
4  
5 cerebral cortex that contain the highest levels of  $\alpha$ -syn (23). It seemed therefore reasonable  
6  
7 to hypothesise that a reduction in proteolytic cleavage might contribute to  $\alpha$ -syn  
8  
9 accumulation in MSA.  
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12  
13 In the present study, we have explored the hypothesis that  $\alpha$ -syn accumulation in  
14  
15 MSA results, at least in part, from defective proteolytic clearance. We have chosen to study  
16  
17 CTSD, CAPN1 and KLK6 in MSA because these enzymes are expressed within neurons and  
18  
19 oligodendrocytes (17-19), are found within Lewy body inclusions and GICs (11), are partly  
20  
21 co-localised with  $\alpha$ -syn in SH-SY5Y cells, and were shown to regulate  $\alpha$ -syn levels *in vivo* and  
22  
23 *in vitro* (33-37). CTSD is the primary enzyme responsible for lysosomal  $\alpha$ -syn degradation (9)  
24  
25 whereas KLK6 interacts with and degrades  $\alpha$ -syn both intracellularly (11) and extracellularly  
26  
27 (13, 34). A reduction in proteolytic activity of KLK6 has been proposed to play a role in the  
28  
29 pathogenesis of  $\alpha$ -synucleinopathies, including MSA (21-24).  
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34  
35 However, the present findings do not support our hypothesis. Instead, KLK6, CAPN1  
36  
37 and CTSD were increased in MSA in regions that have a high  $\alpha$ -syn load and pronounced  
38  
39 MSA pathology: KLK6 and CAPN1 were elevated in the putamen and cerebellar white  
40  
41 matter, and CTSD in the pontine base and cerebellar white matter. We failed to detect an  
42  
43 association between regional  $\alpha$ -syn load and the level or activity of any of the proteases.  
44  
45 The most marked up-regulation of KLK6, CAPN1 and CTSD in regions with the highest  $\alpha$ -syn  
46  
47 burden is in keeping with previous observations suggesting that  $\alpha$ -syn accumulation can  
48  
49 upregulate degradative proteases in PD and DLB (10). Whether this is a compensatory  
50  
51 response that affords any protection against  $\alpha$ -syn accumulation but eventually becomes  
52  
53 overwhelmed or even contributes to disease pathology (see below) is unknown.  
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3 Assessing the potential role of these enzymes in the pathogenesis of  $\alpha$ -  
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5 synucleinopathies is complicated. Although cleavage of  $\alpha$ -syn might be expected to have a  
6  
7 protective role, some of these enzymes, such as CAPN1, generate C-terminally truncated  
8  
9 fragments that seed the propagation of full-length  $\alpha$ -syn following incomplete degradation  
10  
11 of fibrillar  $\alpha$ -syn (14, 38, 39). CAPN1 is increased in PD (40-42) and CAPN1 inhibitors are  
12  
13 protective in an experimental mouse models of PD (41, 43). Most evidence for KLK6  
14  
15 suggests that this enzyme has a protective function: i.e. proteolytic cleavage reduces  $\alpha$ -syn  
16  
17  $\alpha$ -syn aggregation and toxicity and facilitates clearance. The potential for CTSD cleavage  
18  
19 fragments to promote aggregation remains to be determined. Further research is needed to  
20  
21 define the role of these enzymes in the pathogenesis of MSA.  
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## 26 **Conclusion.**

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29 We have shown that KLK6, CAPN1 and CTSD proteases are upregulated in MSA, particularly  
30  
31 in regions with a high  $\alpha$ -syn burden. The findings indicate that  $\alpha$ -syn is unlikely to  
32  
33 accumulate in MSA due to reduced activity of these enzymes. The upregulation of KLK6,  
34  
35 CAPN1 and CTSD in MSA may be a compensatory response to elevated  $\alpha$ -syn load. Further  
36  
37 studies are required to determine the precise role of these enzymes in the pathogenesis of  
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39 MSA.  
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### 8 **Acknowledgements**

9  
10  
11 This work was funded by the Multiple System Atrophy (MSA) trust (Grant number 1402  
12  
13 HoLo) and the MSA coalition. JLH is supported by the Multiple System Atrophy Trust; the  
14  
15 Multiple System Atrophy Coalition; Fund Sophia, managed by the King Baudouin  
16  
17 Foundation; Alzheimer's Research UK and CBD Solutions. AK is supported by the Multiple  
18  
19 System Atrophy Trust. Queen Square Brain Bank is supported by the Reta Lila Weston  
20  
21 Institute for Neurological Studies and the Medical Research Council UK. This research was  
22  
23 supported by the National Institute for Health Research University College London Hospitals  
24  
25 Biomedical Research Centre.  
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### 30 **Author contributions**

31  
32  
33 SL and JH were responsible for the conception and design of experiments; JSM and AK were  
34  
35 responsible for acquisition of data; JSM analysed and interpreted the data; all authors  
36  
37 contributed to drafting the manuscript; SL and JH revised and reviewed the final article for  
38  
39 intellectual content and final approval.  
40  
41  
42

### 43 **Ethical approval**

44  
45  
46 We used brain tissue from cases donated to the Queen Square Brain Bank for Neurological  
47  
48 Disorders, UCL Institute of Neurology. The brain donation programme and protocols have  
49  
50 received ethical approval for research by the NRES Committee London – Central and tissue  
51  
52 is stored for research under a license issued by the Human Tissue Authority (No. 12198).  
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**Conflict of interest**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Figure Legends:**

Figure 1. Graphical representation of fold change in mRNA expression in MSA compared to control. Significantly greater KLK6 mRNA is detected in MSA frontal lobe compared to control (A). No significant difference in CTSD (B) or SNCA (C) mRNA expression is detected in MSA frontal lobe. No significant difference is detected in the mRNA expression of KLK6 (D), CTSD (E) or SNCA (F) in the cerebellar white matter of MSA cases compared to control.

Figure 2. Graphical representation of levels of kallikrein-6 (KLK6) in control (C) and MSA (M) following western blot analysis. Densitometry analysis of immunoblots were performed in triplicate and normalised to  $\beta$ -actin for frontal lobe, putamen, caudate nucleus, pontine base, cerebellar white matter and occipital lobe.

Figure 3. Graphical representation of levels of calpain-1 (CAPN1) in control (C) and MSA (M) following western blot analysis. Densitometry analysis of immunoblots were performed in triplicate and normalised to  $\beta$ -actin for frontal lobe, putamen, caudate nucleus, pontine base, cerebellar white matter and occipital lobe.

Figure 4. Graphical representation of levels of cathepsin-D (CTSD) in control (C) and MSA (M) following western blot analysis. Densitometry analysis of immunoblots were performed in triplicate and normalised to  $\beta$ -actin for frontal lobe, putamen, caudate nucleus, pontine base, cerebellar white matter and occipital lobe.



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3 Figure 5. Bar chart showing regional  $\alpha$ -syn load in multiple system atrophy.  $\alpha$ -syn load  
4 represents the percentage section area immunopositive for  $\alpha$ -syn determined in putamen  
5 (PUT) caudate nucleus (caudate), pontine base (PNS), cerebellar white matter (CBM),  
6 occipital lobe (OCC) and frontal lobe (FCX)). Bars indicate the mean and SEM. \*\*P < 0.01  
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12 \*\*\*P < 0.001 \*\*\*\*P < 0.0001.

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15 Figure 6. Bar charts showing differences in (A) KLK6 (B) CAPN1 and (C) CTSD in MSA across  
16 regions with different predilection for pathology. KLK6 protein levels, measured by sandwich  
17 ELISA and CAPN1 and CTSD enzyme activities using fluorogenic peptide substrate activity  
18 assays were measured in MSA (n=20) and controls (n=20) in putamen (PUT) caudate nucleus  
19 (caudate), pontine base (PNS), cerebellar white matter (CBM), occipital lobe (OCC) and  
20 frontal lobe (FCX)). Bars indicate the mean and SEM. \*P < 0.05 \*\*\*P < 0.001 \*\*\*\*P < 0.0001.  
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30 Figure 7. Regional association between (A) KLK6, (B) CAPN1, (C) CTSD and  $\alpha$ -syn load in MSA  
31 and controls. The protein level or activity of each protease was plotted against  $\alpha$ -syn load in  
32 putamen (PUT) caudate nucleus (caudate), pontine base (PNS), cerebellar white matter  
33 (CBM), occipital lobe (OCC) and frontal lobe (FCX)) in MSA cases. The solid circles and thin  
34 bars indicate the mean values and SEM for  $\alpha$ -syn load (horizontal bars) and protease  
35 (vertical bars). The thick solid and dotted lines indicate the best-fit linear regression and  
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60 95% confidence intervals.

Figure 2

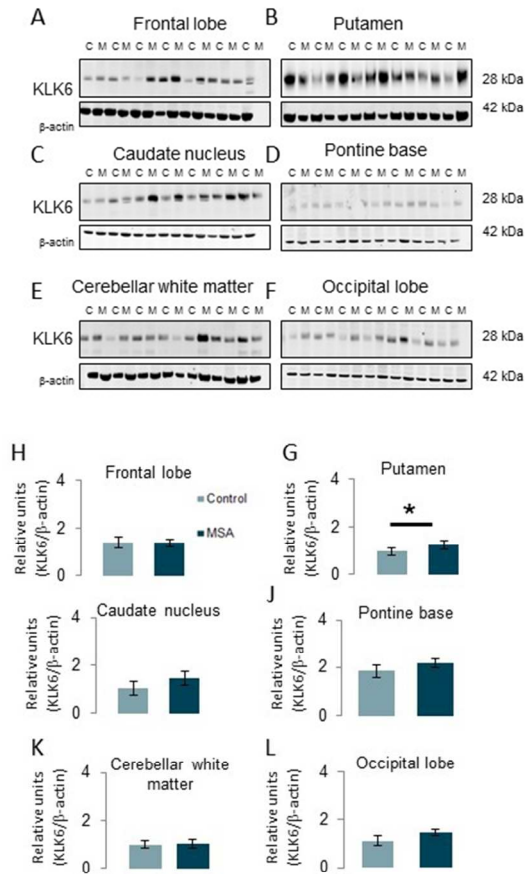


Figure 2. Kallikrein-6 (KLK6) protein level in MSA

190x254mm (96 x 96 DPI)

Patient	Application used for	Gender	Age	Post Mortem delay (h:m)
Control 1	WB, IHC, A	F	83	99:00
Control 2	WB, IHC, A	F	94	89.25
Control 3	WB, IHC, A	F	79	88:50
Control 4	NS, CA, WB, IHC, A	F	80	49:10
Control 5	WB, IHC, A	F	86	120:00
Control 6	NS, CA, WB, IHC, A	F	93	29:40
Control 7	WB, IHC, A	M	69	168.00
Control 8	IHC, A	F	87	51.45
Control 9	NS, CA, IHC, A	M	87	57.00
Control 10	IHC, A	F	92	63.5
Control 11	IHC, A	F	87	84.15
Control 12	IHC, A	F	91	53:25
Control 13	IHC, A	F	86	119:05
Control 14	IHC, A	M	88	97:30
Control 15	IHC, A	M	84	76.5
Control 16	IHC, A	M	95	89.2
Control 17	NS, CA, IHC, A	M	88	16:15
Control 18	IHC, A	M	83	60:00
Control 19	CA, IHC, A		38	80.35
Control 20	CA, IHC, A	M	89	38:30
MSA 1	WB, IHC, A	F	68	36:35
MSA 2	NS, CA, WB, IHC, A	F	66	23:20
MSA 3	WB, IHC, A	F	62	118:35
MSA 4	WB, IHC, A	F	59	48:15
MSA 5	WB, IHC, A	M	69	43:15
MSA 6	WB, IHC, A	M	78	100:15
MSA 7	WB, IHC, A	F	71	80:00
MSA 8	IHC, A	F	70	83:15
MSA 9	IHC, A	F	70	64:45
MSA 10	IHC, A	M	72	89:49
MSA 11	NS, CA, IHC, A	F	60	35:30
MSA 12	IHC, A	M	66	107:20
MSA 13	IHC, A	M	51	56:10
MSA 14	IHC, A	F	52	72:30
MSA 15	IHC, A	F	68	107:00
MSA 16	IHC, A	M	54	25:35
MSA 17	NS, CA, IHC, A	M	63	20:40
MSA 18	IHC, A	F	55	29:02
MSA 19	IHC, A	M	63	26:55
MSA 20	CA, IHC, A	F	85	89:00