Title: An altered secretome is an early marker of the pathogenesis of CLN6 Batten disease

Order of Authors: Hannah L. Best*[1], Alison J. Clare[2], Kirstin O. McDonald, Hollie E. Wicky, & Stephanie M. Hughes**

*First Author, **Corresponding Author

**stephanie.hughes@otago.ac.nz

Affiliation Institution: Department of Biochemistry, School of Biomedical Sciences, Brain Health Research Centre, University of Otago, Dunedin, New Zealand

Abbreviations: AAV, Adeno-associated virus; AD, Alzheimer's disease; AGT, Angiotensiongen; ALS, Amyotrophic lateral sclerosis; APP, amyloid precursor protein; C3, Complement component 3; CD68, Cluster of Differentiation 68; CLN, Ceroid-lipofuscinosis neuronal protein; CNS, central nervous system; CRMP, Collapsin response mediator protein; CSF, Cerebrospinal fluid; CSPG2, Chondroitin sulfate proteoglycan 2; CTS, Cathepsin; DIV, Days in vitro; EASE, Expression Analysis Systematic Explorer; ECM, extracellular matrix; EEF1A1, Elongation factor 1-alpha 1; ER, Endoplasmic reticulum; FDR, False discovery rate; GAG, Glycosaminoglycans; GFAP, Glial fibrillary acidic protein; H4C1, Histone H4; HD, Huntington's disease; HEXB, Beta-hexosaminidase subunit beta; HNRNPA, Heterogeneous nuclear ribonucleoprotein A; HSP90, heat shock protein 90; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS, Liquid chromatography–mass spectrometry; LSD, Lysosomal storage disorder; LV, Lentivirus; M6PR, mannose 6-phosphate receptors; MAP2, Microtubule-associated protein 2; MOI, Multiplicity of infection; NCL, Neuronal ceroid lipofuscinosis; NPC1, Niemann-Pick disease, type C1; PANTHER, protein analysis through evolutionary relationships; PD, Parkinson's disease; PKM, Pyruvate kinase M1/M2; PRDX, Peroxiredoxin; PRSS1, Trypsin-1; RFU, Relative fluorescent units; RRID, Research Resource Identifiers; SEM, standard error of the mean; SPARCL1, SPARC-like protein 1; STRING, Search Tool for the Retrieval of Interacting proteins; YWHAZ, 14-3-3 protein zeta/delta

[1] Present address: School of Biosciences, Cardiff University, Cardiff, UK
[2] Present address: Translational Health Sciences, Bristol Medical School, Biomedical Science Building, University of Bristol, Bristol, UK

Abstract

Neuronal ceroid lipofuscinoses (NCLs) are a group of inherited childhood neurodegenerative disorders. In addition to the accumulation of auto-fluorescent storage material in lysosomes, NCLs are largely characterised by region-specific neuroinflammation that can predict neuron loss. These phenotypes suggest alterations in the extracellular environment - making the secretome an area of significant interest. This study investigated the secretome in the CLN6 (ceroid-lipofuscinosis neuronal protein 6) variant of NCL. To investigate the CLN6 secretome, we co-cultured neurons and glia isolated from *Cln6*^{nclf} *or Cln6*$^{+/-}$ mice, and utilised mass spectrometry to compare protein constituents of conditioned media. The significant changes noted in cathepsin enzymes, were investigated further via western blotting and enzyme activity assays. Viral-mediated gene therapy was used to try and rescue the wild-type phenotype and restore the secretome – both *in vitro* in co-cultures and *in vivo* in mouse plasma. In *Cln6*^{nclf} cells, proteomics revealed a marked increase in catabolic and cytoskeletal associated proteins – revealing new similarities between the pathogenic signatures of NCLs with other neurodegenerative disorders. These changes were, in part, corrected by gene therapy intervention, suggesting these proteins as candidate *in vitro* biomarkers. Importantly, these *in vitro* changes show promise for *in vivo* translation, with Cathepsin L (CTSL) activity reduced in both co-cultures and *Cln6*^{nclf} plasma samples post gene-therapy. This work suggests the secretome plays a role in CLN6 pathogenesis and highlights its potential use as an *in vitro* model. Proteomic changes present a list of candidate biomarkers for monitoring disease and assessing potential therapeutics in future studies.

Keywords

Batten disease, Neuronal ceroid lipofuscinosis, Gene therapy, Secretome, CLN6

Introduction

Neuronal ceroid lipofuscinoses (NCLs), or Batten disease, are a heterogeneous group of neurodegenerative lysosomal storage disorders (LSDs), typically characterized by blindness, myoclonic epilepsy, and progressive cognitive and motor decline (Schulz *et al*. 2013; Warrier *et al*. 2013; Dozieres-Puyravel *et al*. 2020). To date there are 13 genetically identified variants (Ceroid-lipofuscinosis neuronal/CLN genes), which collectively represent the most common cause of childhood dementia (Verity *et al*. 2010; Mole & Cotman 2015). Despite genetic heterogeneity, NCLs are grouped together based on two principle features; progressive neurodegeneration in the central nervous system (CNS) and retina, and the lysosomal accumulation of proteinaceous, lipofuscin-like, storage material (Cooper *et al*. 2015; Palmer *et al*. 2013).

Neurodegeneration progresses in a distinctive pattern, selectively targeting specific regions and cellular subpopulations, in both animal models (CLN1, CLN3, CLN6) (Oswald *et al*. 2008; Bible *et al*. 2004; Pontikis *et al*. 2005) and humans (CLN1, CLN2, CLN3, CLN5 & CLN8) (Anderson *et al*. 2013; Haltia *et al*. 2001; Tyynela *et al*. 2004). However, the deposition of storage material does not correspond with neuron loss, suggesting it is not directly responsible for cell death. Instead, localised microglial and astrocyte activation and are shown to both precede and predict neuronal death in cellular and animal models, as well as human patients, as reviewed by others (Palmer *et al*. 2013; Cooper *et al*. 2015).

Astrocytes and microglia are critical for maintaining an optimal cellular environment, with primary roles in synaptogenesis, synaptic transmission, and the development of neural networks (Theodosis *et al*. 2004; Mederos *et al*. 2018). Regulation and modulation of the cellular environment is achieved through direct interaction with synapses, and through release of neurotrophic growth factors into the surrounding milieu (Allen & Eroglu 2017). Neuroinflammation is characterised changes in glial morphology, alongside the release of proinflammatory mediators (Ransohoff 2016; Liddelow *et al*. 2017). This reactive neuroinflammatory morphology is commonplace in neurodegenerative disease, and has been reported to contribute to neuronal cell death in a range of disorders including Alzheimer's

disease (AD), Huntington's disease (HD), Parkinson's Disease (PD), and amyotrophic lateral sclerosis (ALS) (Pekny *et al*. 2016; Kinney *et al*. 2018; Hsiao *et al*. 2013; Perry 2012; McCauley & Baloh 2019).

The presence of both neuroinflammation and reactive glia-predicted cell death in the NCLs suggests cell secretions in the CNS have a crucial impact on disease pathogenesis - making the cell secretome an interesting area of research. In addition to gaining insight into disease processes, secreted factors are a great source of potential prognostic, diagnostic, and therapeutic biomarkers. This presents the opportunity for less invasive and continuous sampling. This was recently investigated by Sleat *et al* to identify candidate biomarkers in the cerebrospinal fluid (CSF) of CLN1, CLN2 and CLN3 mouse models (Sleat *et al*. 2019). This study investigated the CNS secretome in a CLN6 model – an NCL subtype that presents with both reactive gliosis and region-specific neuron loss in mice, sheep, and humans. CLN6 is a transmembrane endoplasmic reticulum (ER) protein that forms an obligate complex with CLN8. The CLN6-CLN8 complex recruit's lysosomal proteins at the ER and transfers them to the Golgi (Bajaj *et al*. 2020). It has also been suggested to have roles in protein trafficking, prevention of protein aggregates, bio-metal homeostasis, and neuronal maturation (Benedict *et al*. 2009; Yamashita *et al*. 2020; Kanninen *et al*. 2013).

Cellular models of NCLs are commonly used to understand cellular biology (Minnis *et al*. 2019), and as platforms for therapeutic efficacy studies (Kim *et al*. 2012; Linterman *et al*. 2011; Gavin *et al*. 2013). In this study we used mass spectrometry (MS) based secretome profiling to characterise disease specific changes in primary cortical cell cultures of neurons, microglia, and astrocytes derived from the *Cln6*^{nclf} mouse. The *Cln6*^{nclf} secretome showed significant changes when compared to controls, with an enrichment of proteins associated with catabolic processes and cytoskeletal dynamics. Gene therapy treatment of these cultures resulted in the restoration of a subset of these proteins, within 48 h, identifying candidate biomarkers with *in vitro* therapeutic testing. Furthermore, Cathepsin L enzyme activity was decreased in both *Cln6*^{nclf} primary cultures and in blood plasma obtained from the *Cln6*^{nclf} mouse after gene therapy treatment, suggesting these cellular changes reflect *in vivo* alterations.

Methods

All animal studies were conducted in strict accordance with the approval for animal husbandry, surgical procedures, and use of animal tissue granted by the University of Otago Animal Ethics Committee under the New Zealand Animal Welfare Act 1999. Mice used in these studies were either male healthy wildtype (*Cln6*$^{+/+}$), male healthy heterozygotes (*Cln6*$^{+/-}$), or male affected (*Cln6*^{nclf}; The Jackson Laboratory, B6.Cg-*Cln6*$^{nclf/J}$ 003605, RRID:5604252). Ideally future studies would benefit from the inclusion of female animals. The animals used per group is stated in the appropriate figure legend as 'n =' (biological n). All animals in the colony were maintained in Techniplast individually vented cages in groups of 4-5 male or female mice with access to food and water, and 12/12 h light/dark cycle. Animals were weighed regularly to check they remained at a healthy weight for their age, as defined by the Jackson Laboratory. A total of 24 male pups were used to generate cultures for the following: liquid chromatography (LC) MS (12 pups; three *Cln6*$^{+/-}$, nine *Cln6*^{nclf}), western blotting, & enzyme assays (12 pups; six *Cln6*$^{+/-}$, six *Cln6*^{nclf}), and 15 maintained till 12 months of age for Adeno-associated virus (AAV) study (five *Cln6*$^{+/+}$ PBS controls, five *Cln6*^{nclf} PBS controls, five *Cln6*^{nclf} AAV.CLN6 treated). A graphical timeline of the methodology used in this study is available in **Figure 1**. This study was not pre-registered.

Commented [HB1]: Editorial office 12

Commented [MOU2]: Editorial office 12

Commented [HB3]: Editorial office 12

Mouse primary cultures

Primary neural cultures were generated from P0-1 mouse pups genotyped via polymerase chain reaction (PCR). Pups were anaesthetised with pentobarbitol (150 mg/kg) and checked for complete anaesthesia using a tail-pinch reflex test. The cortex was dissected as described previously (Schildge *et al.* 2013), and finely diced in Leibovitz L-15 complete (L15 with pen/strep and glucose to a final concentration of 6g/L), and then placed in digestion media; L15 complete containing 12 U/mL papain (Worthington Biochemical Corporation, NJ, US) and 1 U/mL DNase I. Tubes were placed in a MACS-mix rotator (Miltenyi Biotec, DE) for 15 min at 37 °C. Digestion solution was discarded and replaced with 1 mL mouse blocking/ trituration solution (L15 complete containing 1U/ mL DNase I and 2% B27), and samples returned to the incubator, in the MACS-mix rotator for 10 min. The supernatant was removed

and replaced with fresh mouse blocking/trituration solution, resuspended, and triturated through a series of fire-polished Pasteur pipettes of decreasing pore size. The sample was then passed through a 100 µm strainer and centrifuged (1000 x g for 5 min). The supernatant was discarded, and the cells were plated at 1 x 10^6 cells per T25 in DMEM/F12 containing 2 mM L-Glutamine, 50 U/mL pen-strep, 2% B27 (GIBCO, Life technologies, NZ), 4 mM KCl. Each pup yielded one T25 flask (n = 1). Three pups were used per group (*Cln6*+/-, *Cln6*^{nclf}, *Cln6*$^{nclf\ LV.CLN5}$, *Cln6*$^{nclf.LV.CLN6}$) for MS, and six per group (*Cln6*$^{+/-}$, *Cln6*^{nclf}) for western blotting and enzyme assays No sample calculation was performed to determine sample size, instead numbers were determined by unpublished pilot studies using these animals. Unless stated, experimenters were not blinded to experiments.

Commented [HB4]: Editorial office 11

Immunocytochemistry

Immunocytochemistry was used for characterization of mouse primary cell cultures. Cells were blocked for 1 h at room temperature in phosphate buffered saline (PBS) containing 0.2% triton (PBS-T) and 10% normal goat serum (NGS). Cells were incubated overnight in primary antibodies in PBS-T (0.1%) and 2% NGS; Glial fibrillary acidic protein (GFAP,1:1000, Sigma-Aldrich G3893, RRID:AB_477010), Microtubule-associated protein 2 (MAP2, 1:1000, Synaptic Systems 188004, RRID:AB_2138181), or Cluster of Differentiation 68 (CD68,1:1000, Bio-Rad MCA1957, RRID:AB_322219) at 4 °C. Primary antibodies were removed, and cells washed with PBS prior to incubating with the appropriate secondary; MAP2 (Alexa fluor 488 Goat anti-guinea pig; Invitrogen A11073, RRID:AB_2534117), GFAP (Alexa fluor 594 goat anti-mouse; Invitrogen A11005, RRID:AB_141372), CD68 (Alexa fluor 555 goat anti-rat, Invitrogen A21434, RRID:AB_2535855) for 2 h at room temperature, followed by DAPI counterstaining. Cells were imaged using a 40x objective on an Olympus IX71 fluorescent microscope with an Olympus DP71 camera attachment.

Mass spectrometry and relative expression data analysis

Liquid chromatography-mass spectrometry (LC-MS) was used to identify proteins that were differentially expressed in the media obtained from *Cln6*$^{+/-}$,*Cln6*^{nclf}, or *Cln6*^{nclfLV} primary cultures. For mass spectrometry analysis, experimenters were blinded to the groups with samples provided as either

Commented [HB5]: Editorial office 11

group A or group B. Media was changed at 7 days *in vitro* (DIV) to 5 mL of B27-free media, collected after 48 h then centrifuged at 10,000 x g for 10 min to remove cells and debris. Samples were concentrated using filter-aided sample preparation (FASP) (Wisniewski *et al.* 2009). Briefly, 100 µg of sample was depleted of detrimental compounds in urea-containing buffer, followed by carboamindomethylation of thiol groups to prevent disulphide bond formation. Protein digestion was performed using sequencing grade modified trypsin overnight at 37 °C, followed by peptide elution for analysis by liquid chromatography-coupled LTQ-Orbitrap MS (Thermo Scientific, CA). Raw data was processed through Proteome Discoverer software v1.3 and searched against the Swiss-Prot mouse protein sequencing database to generate a list of proteins.

Bioinformatics

To initially visualize the differentially expressed proteins, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to generate a functional protein association network based on protein-protein interactions and shared functions (Szklarczyk *et al.* 2017). Protein analysis through an evolutionary conservation classification system (PANTHER) was used to classify and identify the function of gene products. Differentially expressed protein IDs were imported into PANTHER and *Mus Musculus* used as the organism for comparison (Mi *et al.* 2010; Thomas *et al.* 2003). To determine pathways of interest, term enrichment analysis was performed using Gene Ontology pathway analysis on the online Database for Annotation Visualisation and Integrated Discovery (DAVID v6.8) tool (Huang da *et al.* 2009b; Huang da *et al.* 2009a). All identified proteins were uploaded as a background list and the differentially expressed genes uploaded as a target gene list for analysis of enrichment. The databases included for enrichment were; InterPro and Protein information resource (PIR) superfamily, gene ontology (biological processes, cellular components and molecular function, Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway and BioCarta. The classification stringency was set to high and the Expression Analysis Systematic Explorer (EASE) score set to 1.0. Genes were given annotation clusters with an assigned EASE score (-log10 of the p-value), meaning an EASE score of 1.3 is equal to $p \leq 0.05$.

Western blotting

For western blotting and cathepsin activity assays, B27 depleted media was collected and centrifuged to remove cellular debris. The supernatant was concentrated using an Amicon Ultra-15 centrifugal Filter Device – Ultracel 30 K (Merck, UFC9030) at 4000 x g for 10 min in a swinging bucket rotor. Solute was recovered from the bottom of the filter device and stored at -80 ^{o}C for western blotting or used fresh for activity assays. Protein concentrations were determined by bicinchoninic acid assay (BCA), and 20 μg protein per lane was resolved on a 10% SDS-page gel before semi-dry transfer to 0.45 μm nitrocellulose membrane (Amersham Protran; GE Healthcare Life Sciences). Membranes were immunoblotted with primary antibodies either; Cathepsin D (1:1000, Abcam ab75852, RRID:AB_1523267), and β-actin (1:2000, Sigma Aldrich A2228, RRID:AB_476697). For detection, blots were incubated with LiCor IRDye 680RD goat-anti-rabbit (92568071, RRID:AB_2721181) or IRDye 800RD goat-anti-mouse (92532210, RRID:AB_2687825) secondary antibodies and imaged using an Odyssey® Fc Imaging System (LI-COR Biosciences, NE). Densiometric analysis was achieved using Image Studio^{TM} Lite quantification software, and protein of interest divided by housekeeping gene to achieve relative protein expression.

Cellular cathepsin enzyme activity assays

Fluorometric activity assay kits were used to determine relative activities of Cathepsin B (Abcam, ab65300), D/E (Abcam, ab65302) and L (Abcam, ab65306), in either the cellular or media fraction, as per the manufacturer's instructions. Briefly, cells were washed in dPBS and harvested using trypsin. Cells collected from each sample were counted and 1 x 10^6 cells used per sample, per assay. After appropriate lysis and supernatant collection steps, 50 μL of cell lysate was added to a 96-well black sided plate well in triplicate for each sample, alongside the appropriate controls as described in the kit. For assessing cathepsin activity in media samples, 5 μL of concentrated sample (5x) was added to the plate in triplicate. Appropriate volumes of buffer were added to each well, followed by addition of the relevant enzyme substrate, and the plate sealed and incubated at 37 ^{o}C protected from light. Activity was quantified using a CLARIOstar microplate reader (BMG LABTECH, Ortenberg Germany) at the relevant Ex/Em. Endpoint readings were taken at 90 min (in the window recommended by

manufacturer) with preliminary studies ensuring readings fell within the linear range. Fluorescence readings at endpoint were averaged across technical triplicates per sample, and *Cln6*^{nclf} and *Cln6*$^{nclf.AAV}$ samples normalized to controls in the same run, resulting in data presented as a relative fold change. For plasma samples, protein concentration was determined by BCA and 50 µg of sample used per well. Endpoint readings were taken, again with preliminary studies ensuring readings fell within the linear range. All samples were tested in the same plate negating the need for standardisation; therefore, plasma data is presented as endpoint relative fluorescent units (RFU).

Lentivirus and Adeno-associated virus generation, packaging and use

Lentiviral (LV) vectors were generated and used with approval from the Environmental Protection Authority (EPA), New Zealand, under approval number GMD002849. Two HIV-1 derived LV vectors were generated expressing either murine *Cln6* (LV.CLN6) or *Cln5* (LV.CLN5) under the myeloid proliferative U5 enhancer element (MND). Constructs were packaged using a second-generation packaging system as described previously (Best *et al.* 2017; Zufferey *et al.* 1997). For transduction of primary neural cultures, concentrated virus was thawed and added directly to the culture medium at 5 DIV at a multiplicity of infection (MOI) of 10. Post-transduction cells were maintained for 48 h prior to addition of B27-free media. B27-free media was 'conditioned' for 48 h prior to collection for LC-MS. Vector expression was confirmed via western blotting and qPCR (data not shown). AAV was packaged and used to inject new-born mouse pups expressing CLN6 cDNA under the hybrid chicken β-actin promoter (scAAV9.CB.CLN6), as described previously (Cain *et al.* 2019). Animals were assigned to their treatment group (*Cln6*^{nclf} + AAV.CLN6, or *Cln6*^{nclf} untreated) by arbitrary assignment of treatments to each litter born to avoid individual pups within a litter being misclassified for treatment. Injections were performed by personnel who were not subsequently involved in the analysis shown in this study. The mice are part of a wider study in preparation including both male and female mice, however for the analysis shown here we focused on male mice to be consistent with the pup neural culture data. Group size was determined based on similar studies (Cain *et al.* 2019).

Commented [HB6]: Editorial office 10

Plasma isolation from Cln6$^{+/+}$, Cln6^{nclf} and Cln6$^{nclf.AAV}$ mice

Twelve-month old male mice (*Cln6*$^{+/+}$, *Cln6*^{nclf} and *Cln6*$^{nclf.AAV}$; n = 5/group) were anaesthetised with pentobarbitol (150 mg/kg) and monitored until unconscious. Pentobarbitol was chosen in order to minimize animal suffering, as studies have indicated euthanasia is rapid and does not result in overt pain signs when compared with saline administration (Dutton *et al.* 2019). Between 0.2 mL - 1 mL of whole blood was extracted from the right ventricle of the heart, with a 27 G needle and 1 mL syringe, and incubated at room temperature for 30 min. Whole blood was fractionated at 440 x g for 15 min and the plasma fraction collected. Plasma samples were centrifuged for a further 5 minutes and kept on dry ice until being stored at -80 °C. Animals were identified by ear tag and experimenters were blinded to the groupings (A, B, C) during collection, enzyme assays, and input of data for analysis. An arbitrary selection of mouse bloods from the larger study (5/20) were analyzed here with no prior knowledge of gene therapy efficacy, or vector expression. The only inclusion/exclusion criteria were that the mice survived for the 12 months before study.

Statistical analysis

For immunocytochemistry analysis: GFAP, MAP2 and CD68 staining area was averaged per positive cell (e.g GFAP positive pixels per GFAP positive cell) using Image j (Rueden *et al.* 2017). Ten images were acquired per 'n' (biological replicates) and unpaired t tests used to compare markers in *Cln6*$^{+/-}$ vs *Cln6*^{nclf}. For mass spec analysis: semi-quantitative comparison was performed, normalizing the amount of protein per sample, back to that of the healthy control *Cln6*$^{+/-}$ samples. Using Microsoft excel for Mac (version 16.38, **Supplementary B**), relative amounts of individual proteins were averaged across the three animals per group, and t-tests used to compare the amount between the pooled sample groups ($p \leq 0.05$). Multiple comparisons *p* values were corrected for using the false discovery rate approach (FDR, Q = 5%, Benjamini, Krieger and Yekutieli method). When analyzing the effects of treatment, a ratio t-test was used to provide a more consistent measure of effect. Heat maps were generated in GraphPad Prism using the fold changes to express data as a % of control. For western blot analysis: unpaired t-tests were used to compare Cathepsin D expression in the media, and two-way ANOVA followed by Sidak's multiple comparisons test used to compare pro and active forms in total cell lysate.

Commented [HB7]: Editorial office 7

Commented [HB8]: Editorial office 10

Commented [HB9]: Editorial office 13

For enzyme assay analysis: all data was analysed using unpaired t tests (*Cln6*$^{+/-}$ vs *Cln6*^{nclf}), or one-way ANOVA (*Cln6*$^{+/-}$ vs *Cln6*^{nclf} vs *Cln6*$^{nclf.AAV}$) followed by Tukey's multiple comparisons test. For both western blotting and enzyme assay statistical analyses - including normality (D'Agostino-Pearson) and testing for outliers (Grubb's test, no exclusions) - were carried out using GraphPad Prism 8 for macOS (version 8.2.0). Data is presented as the mean +/- SEM.

Deleted: ¶

Results

Neural cell cultures derived from Cln6$^{+/-}$ *and Cln6*^{nclf} *mice have different secretome profiles at 9 days in vitro*

Mixed cortical neural cultures (containing neurons, astrocytes and microglia; **Supplementary A; Fig. A1**) were used to generate an *in vitro* model of the CLN6 secretome. A substantial body of previous work has shown astrocytes and microglia are essential for neuronal function and survival (Pekny *et al.* 2016; Benarroch 2013; Eroglu & Barres 2010; Pfrieger & Barres 1997), and are key players in CNS disorders (Ransohoff 2016). By culturing these cells together, we aimed to better simulate the interplay between glial cells and neurons during CLN6 Batten disease. Cerebrocortical cultures were chosen to assess the effects of CLN6 loss as the *Cln6*^{nclf} cortex is one of the first regions to display overt cellular phenotypes *in vivo* (Morgan *et al.* 2013), and has a 2.4 - 4 fold increase in *Cln6* mRNA in the first 28 days of life (Thelen *et al.* 2012). To better understand the 'disease stage' of these cells at the point which we are investigating the secretome we looked for three common phenotypes associated with CLN6 pathogenic onset; accumulation of autofluorescent storage material and altered glial cell activation via immunoblotting for GFAP (astrocytes) or CD68 (microglia) (Thelen *et al.* 2012). No change in the ratio of cell types (**Supplementary A; Fig. A1 & Fig. A2)** or autofluorescent storage material was observed at 9 DIV (**Supplementary A; Fig. A3**). This suggests that the changes reported below are very early disease phenotypes.

Conditioned media was collected from both *Cln6*$^{+/-}$ and *Cln6*^{nclf} samples at 9 DIV, and analysed by LC-MS. A total of 173 proteins were detected, with 169 identified in *Cln6*^{nclf} samples and 152 in *Cln6*$^{+/-}$ (**Supplementary B1**), a similar number of proteins to that found in other studies looking at media

fraction constituent (Loov *et al.* 2013). SignalP 5.0 (Almagro Armenteros *et al.* 2019) predicted 47% of identified proteins to contain signal peptides for the classical secretion pathway, with a further 22% identified by SecretomeP 2.0 (Bendtsen *et al.* 2004) as likely to be secreted through non-classical means. The other 31% may be secreted via unconventional secretion pathways which are often increased in the presence of cellular stressors such as inflammation (Kim *et al.* 2018). In order to begin validation, we compared all identified proteins to previously published datasets obtained from murine astrocyte or primary culture conditioned media. We identified a 69% (Dowell *et al.* 2009), 56% (Moore *et al.* 2009) and 71% (Thouvenot *et al.* 2012) overlap of our proteins with similarly derived datasets (**Supplementary B2**).

We focused in on the 37 proteins identified after applying a false discovery rate to the data set (Q = 5%, ; **Supplementary B3**). Markedly, all 37 were significantly increased in the *Cln6*^{nclf} samples suggestive of a compensatory response or, as CLN6 is located in the ER and known to partake in the trafficking of lysosomal enzymes (Bajaj *et al.* 2020), aberrations in protein trafficking/processing.

Term enrichment analysis of differentially expressed proteins shows an increase in the secretion of catabolic proteins in Cln6^{nclf} cells

To visualise any common protein networks, we applied STRING analysis to the top 37 differentially expressed proteins ($p \leq 0.05$; **Fig. 2A**). Among the clusters in the network with the highest confidence associations were proteins required for proteolytic degradation and processing (**Fig. 2A**, red), and proteins associated with nervous system development (**Fig. 2A**, yellow). Of particular note, the latter group includes Collapsin Response Mediator Protein 2 (CRMP2), a protein previously demonstrated to be an interaction partner of CLN6 (Benedict *et al.* 2009).

Analysis of differentially expressed proteins could give important clues to the pathways and components altered as a result of CLN6 dysfunction. To classify and identify functions of proteins that showed significantly increased expression in the *Cln6*^{nclf} cultures, we utilised PANTHER to assign functional classifications to proteins, by using comprehensive gene ontology (GO) annotations (Mi *et al.* 2010; Thomas *et al.* 2003). The majority of significantly altered proteins were related to catalytic

activity (52.2%; GO – molecular function; **Fig. 2B)** and metabolic processes (26.3%; GO – biological process; **Supplementary C; Fig. C.1**) with the largest protein class being metabolic enzymes (28%; GO – protein class; **Supplementary C; Fig. C.2**).

To look for specific enrichment terms, all proteins identified in the secretome dataset were used as a background gene list. Functional annotation clustering revealed seven clusters with significant enrichment (EASE = 1.59), including protein localisation, neuronal cell body, nucleotide metabolism, RNA binding, transport, peptidase activity and cell adhesion (**Fig. 2C; Supplementary C; Table C.1**). Collectively, these analyses robustly demonstrate enrichment of secreted catabolic processing and protein localisation components in *Cln6*^{nclf} neural cultures This is perhaps not surprising given the role of CLN6 in the transport of lysosomal enzymes (Bajaj *et al.* 2020).

Validation of LC-MS shows increased Cathepsin D secretion and increased Cathepsin L enzyme activity

In order to validate the quantitative proteomic data, we chose to investigate the presence of cathepsin enzymes in the secretome. Three cathepsin enzymes Cathepsin D (aspartyl protease; CTSD), Cathepsin L, and Cathepsin B (cysteine proteases; CTSL, CTSB), are in the 37 proteins overexpressed ($p \leq 0.05$) in *Cln6*^{nclf} conditioned media, compared to healthy *Cln6*$^{+/-}$. These were highly abundant in the secretome and, as lysosomal proteins, are of particular interest to LSDs. In fact, the CLN10 NCL variant is caused by mutations in *CTSD* (Mole & Cotman 2015; Koike *et al.* 2000).

Cathepsins can be present as enzymatically inactive proforms or smaller active mature variants (Turk *et al.* 2012; Lowry & Klegeris 2018). By looking at the peptide sequences identified with MS, it is clear a proportion of all over-secreted cathepsin variants are proforms. Immunoblotting conditioned media for CTSD showed a single proform band at ~50 kDa in both the *Cln6*$^{+/-}$ and *Cln6*^{nclf} cultures, which was significantly increased in the *Cln6*^{nclf} conditioned media samples, validating the CTSD increase seen via LC-MS (**Fig. 3A; Supplementary D**). In the corresponding cell lysates - which produced the secreted CTSD - immunoblotting showed presence of proforms (~48 kDa) and a mature active variant

(~25 kDa). The quantity of the proform was significantly reduced in *Cln6*^{nclf} cells compared to healthy *Cln6*$^{+/-}$ (**Fig. 3B**), suggesting it could be mistrafficked and secreted in the absence of CLN6.

To corroborate whether changes in cathepsin expression led to measurable changes in enzyme activity we assessed relative enzyme activity of CTSD, CTSL and CTSB in both the conditioned media (**Fig. 3C**), and whole cell lysates (**Fig. 3D**). We chose to perform enzyme assays, as they provide a faster and cheaper high-throughput option, opposed to comparative proteomics, making them more appropriate for future *en masse* analysis of biomarkers.

CTSL activity was significantly increased in the conditioned media, but not CTSD or CTSB (**Fig. 3C**). CTSL, CTSD and CTSB enzyme activities were unchanged in the cell lysates (**Fig. 3D**). A caveat of measuring enzyme activity using these assays is that the assay buffer artificially modulates the environment - and subsequently the degree of enzyme activation - preventing direct correlation between the level of enzyme activity reported and the maturation state of the enzyme in *in vitro* cultures. However, comparison to controls demonstrated a genotype and disease-specific change in CTSL enzyme activity, indicating more abundant CTSL. This result corroborates the high fold-change of CTSL expression in the secretome data set (Log2 fold change in expression = 24; **Supplementary B3**).

Cathepsin L activity is decreased after CLN6 gene therapy treatment in both primary cultures and plasma from the Cln6^{nclf} mouse

We next investigated whether these cathepsin changes are altered with gene therapy treatment. To achieve this, primary cultures were treated with a lentivirus expressing murine CLN6 (LV.CLN6). Conditioned media was collected 48 h post gene therapy treatment. CLN6 gene therapy has been shown to prevent, or drastically reduce, all pathological disease hallmarks, and improve behaviour and prolong lifespan *in vivo*, making it a good positive control for biomarker investigation (Cain *et al.* 2019; Kleine Holthaus *et al.* 2019). Primary culture treatment with LV.CLN6 for 48 h resulted in a decrease in secreted CTSL activity, with activity in *Cln6*$^{nclf.LV}$ cultures no longer different to that of the control *Cln6*$^{+/-}$ (**Fig. 4A**). Consistent with no observable difference between *Cln6*$^{+/-}$ and *Cln6*^{nclf} cells, CTSD activity was not affected by treatment with LV.CLN6.

To see if CTSL has potential for *in vivo* use, we monitored cathepsin activity in plasma samples isolated from mice. Three cohorts of 12-month old mice were investigated; *Cln6*$^{+/-}$, *Cln6*^{nclf}, and *Cln6*^{nclf} treated with CLN6 gene therapy (*Cln6*$^{nclf:AAV}$). Gene therapy animals were treated with AAV.CLN6 at P1-3 via direct injection into the lateral ventricles. We chose to look at plasma as it is more relevant in terms of identifying a useful *in vivo* biomarker, due to the ease of collection, in comparison to the intrusive nature of CSF collection. Similar to the primary cultures, there was no change in CTSD enzyme activity between the three groups. There was also no significant difference in CTSL activity between *Cln6*$^{+/-}$ and *Cln6*^{nclf} (**Fig. 4B**). However, treatment with AAV9.CLN6 significantly reduced plasma CTSL activity, in comparison to untreated *Cln6*^{nclf}. Additionally, there is no significant difference between *Cln6*$^{nclf:LV}$ and healthy *Cln6*$^{+/-}$, suggesting that a reduction in CTSL in *Cln6*^{nclf} could be part of a battery of indicators for successful treatment.

Overexpression of wild-type murine CLN6 begins to restore the altered Cln6^{nclf} secretome

In order to further characterise the *in vitro* secretome model and identify potential biomarkers for use in monitoring therapeutic potential *in vitro*, we sought to identify which secreted proteins respond quickly to gene therapy treatment. As mentioned previously, CLN6 gene therapy has been shown to drastically, and in some cases completely, reduce pathological hallmarks, making it a good positive control for pathological 'correction'.

Treatment of *Cln6*^{nclf} cells with LV.CLN6 for four days resulted in a significant decrease in 17 secreted proteins compared to *Cln6*^{nclf} untreated, the majority of which were classified as having catalytic activity. Five of these proteins were amongst those increased ($p \leq 0.05$) in the *Cln6*^{nclf} compared to healthy *Cln6*$^{+/-}$ cells (AGT, SPARCL1, YWHAZ, PRSS1, HSP90). The reduction of these proteins towards levels observed in healthy *Cln6*$^{+/-}$ cultures highlights them as potential candidate biomarkers for short-term therapeutic studies (**Fig. 5A, bold**). Whilst Cathepsin L was reduced with LV.CLN6, this was not quite significant ($p = 0.0579$).

Interestingly, 11 proteins were also increased in the secreted fraction of *Cln6*$^{nclf:LV}$, the majority of these having a catalytic molecular function. Five of these proteins were also significantly increased in the

Cln6^{nclf} cells in comparison to *Cln6*$^{+/-}$ (PRDX1, C3, PKM, EEF1A1, H4C1). This further increase after treatment suggests they may be part of a compensatory or protective response.

Overexpression of wild-type murine CLN6 and CLN5 in combination further restores the altered Cln6^{nclf} secretome

In addition to gene therapy treatment with CLN6, we also decided to look at the ability of CLN5 and CLN6 as a combination therapeutic. CLN5 has been implicated as central to a common NCL pathway with reported molecular interactions with CLN1, CLN2, CLN3, CLN6 and CLN8 (Vesa *et al.* 2002; Lyly *et al.* 2009; Huber *et al.* 2020). Additionally, CLN5 is depleted in CLN7 mouse embryonic fibroblast models (Danyukova *et al.* 2018), and we have observed altered *Cln5* transcript levels in adult *Cln6*^{nclf} mice (**Supplementary E)**.

Treatment with LV.CLN6 and LV.CLN5 in combination for 48 hours resulted in a significant decrease of 28 secreted proteins, significantly more than LV.CLN6 alone, indicative of an enhanced short-term, therapeutic effect. Eight of the decreased proteins are increased in the *Cln6*^{nclf} cultures, thereby restored towards physiological levels with combined gene therapy (AGT, CTSD, CTSB, APOE, HEXB, SPARCL1, CRMP2, HNRNPA; **Fig. 5A**; **Supplementary B5**). The larger number of downregulated proteins in the combination therapy, compared to LV.CLN6 only, made it possible to look for enrichment clusters. Ontology analysis showed 3 clusters of significant enrichment; podosome/actin binding, lipoprotein and cytoskeleton regulation (**Fig. 5C; Supplementary C; Table C.2**). Ten proteins showed increased secretion after CLN6/CLN5 gene therapy (**Fig. 5B**), one of which was CLN5 itself, supporting the gene therapy strategies that rely on cells secreting overexpressed soluble therapeutic proteins to 'cross-correct' other cells.

Collectively, this shows that treatment can significantly restore expression of some secreted proteins in the *Cln6*^{nclf} mouse model towards physiological levels seen in the healthy *Cln6*$^{+/-}$ mouse (**Fig. 5D**). Proteins that are reduced in response to both therapeutic strategies may represent the most promising candidates (SPARCL1, AGT). Of note, Cystatin C (an endogenous cathepsin inhibitor) was reduced after both treatments, potentially in response to the reduction of cathepsins as a result of treatment.

Discussion

Our exploratory interrogation of the *Cln6*^{nclf} secretome has revealed distinct changes that reflect and - based off other neurodegenerative disorders with increased cathepsin secretion - potentially propagate pathogenesis in CLN6 disease. By monitoring *Cln6*^{nclf} mixed neural culture secretions, we have a better understanding of the environment in which neurons mature during disease. These secretome changes are occurring before marked glial activation, one of the earliest reported phenotypes *in vivo* in the *Cln6*^{nclf} model (Danyukova *et al*. 2018). Specifically, we observed enrichment of catabolic proteins and proteins involved in cytoskeletal dynamics - highlighting new similarities between the NCLs and a host of other neurological disorders such as AD and PD (Yoshiyama *et al*. 2000; McMurray 2000).

Of course, we must be cautious when correlating what we see *in vitro* to what is occurring *in vivo* – as some of these phenotypes may be present due to the unique conditions of tissue culture, or the additional intrinsic stress on disease cells. However, cultured cells from CLN6-deficient models are known to recapitulate key phenotypic markers of CLN6 disease, such as lysosomal storage (Cao *et al*. 2011), making them suitable for furthering our understanding of disease, or the preliminary investigation of therapeutics. Indeed, in this study we observed that gene therapy restored a subset of these disease-associated secreted proteins indicating these secretome alterations are due to a lack of CLN6 (or in the case of CLN5 treatment, CLN6 interacting partners). We also highlighted the potential for translation of these secreted biomarkers for *in vivo* use, as seen by a reduction in CTSL activity in *Cln6*^{nclf} plasma post gene therapy. Taken together, this data provides novel information on the roles the extracellular milieu is playing in CLN6 disease pathogenesis and provides a candidate list of potential biomarkers for the study of disease progression *in vitro*. It is yet to be determined whether these secretome alterations are neurotoxic, neuroprotective, or both.

Increased cathepsin secretion and activity mirrors that seen in other neuroinflammatory disorders

Cathepsins are present in the milieu under normal physiological conditions where they are associated with the remodelling of the extracellular matrix (ECM) and processing of prohormones (Repnik *et al*. 2012; Turk *et al*. 2012). However, the over-secretion of these enzymes, and other lysosomal hydrolases,

is frequently associated with pathological conditions including a range of neurodegenerative disorders (Hook 2006; Lowry & Klegeris 2018; Balducci *et al*. 2007; Sleat *et al*. 2012). Cathepsin over-secretion has been associated with degradation of the ECM, proteolysis of toxic aggregates such as β-amyloid (Wang *et al*. 2012), α-synuclein (McGlinchey & Lee 2015) and huntingtin (Kim *et al*. 2006), and positive promotion of axonal growth (Tohda & Tohda 2017). In this study, we showed an increase in the secretion of CSTD, CSTB and CTSL; the first evidence that an increase in extracellular proteases could be a disease component of an NCL. Cathepsin alteration has been implicated in other neurological LSDs. For example, increased expression/activity of CTSD, and altered localisation of CTSB, has been reported in an NPC1$^{-/-}$ mouse model (Amritraj *et al*. 2009). Elevated mRNA expression of cathepsin D, Z, S, B, L & O has been reported in a mouse model of neuronopathic Gaucher disease (Vitner *et al*. 2010). Additionally, increased proCTSD is observed in a combined CTSB$^{-/-}$ and CTSL$^{-/-}$ knock-out mouse model which presents with 'NCL-like' phenotype (Koike *et al*. 2005; Felbor *et al*. 2002). Interestingly, a recent study investigating NCL protein biomarkers present in mouse brain samples noted an increase in activity of CTSB in CLN3, and CTSL in CLN3 and CLN2, although these changes were not replicated in CSF samples. Although they did note elevated PRDX6 in CSF from CLN1, CLN2 and CLN3 – a marker we note elevated in the *Cln6*^{nclf} secretome (Sleat *et al*. 2019). Comparisons with the aberrant secretomes of other neurological disorders may help delineate what is a generalised response to neuronal stress, and what is specific to CLN6$^{-/-}$ deficiency (Song *et al*. 2019).

Where these extracellular cathepsins originate from is unclear. As lysosomal instability is a distinct feature of LSDs, extracellular cathepsin expression has been proposed as a consequence of ruptured lysosomes, leading to cathepsin leakage and induction of apoptotic pathways (de Castro *et al*. 2016; Zhang *et al*. 2009; Artal-Sanz & Tavernarakis 2005). Additionally, increased lysosomal exocytosis is prominently associated with LSDs and neurodegenerative disease (Tsunemi *et al*. 2019; Samie & Xu 2014; Lloyd-Evans & Waller-Evans 2020) and may explain the increase in extracellular proteases noted in this study. Although, not all lysosomal enzymes reported in the secreted media data set (e.g CTSC and CTSZ) showed increased secretion, suggesting it is not as simple as increased lysosomal exocytosis alone. Additionally, we saw CTSD to be predominantly secreted as a ~50 kDa proform indicating that

it has not been catalytically processed in the lysosome suggestive of mistrafficking. Disease-associated changes in the lysosome - such as altered constituents or pH - could be hindering the processing and activation of CTSD. Indeed, reduced lysotracker staining (indicative of an increase in pH or reduced lysosomes) has been reported in cerebellar neuronal precursor lines derived from *Cln6*^{nclf} mice (Cao *et al*. 2011), and primary cultures derived from a CLN6 ovine model (Best *et al*. 2017), and, an increase in intralysosomal pH has been reported in a patient fibroblast line (Holopainen *et al*. 2001).

An alternative explanation is activated microglia, which are shown to release cathepsin proforms (including B, D & L) in response to inflammatory cues (Kim *et al*. 2007; Yoshiyama *et al*. 2000; Lively & Schlichter 2013). CTSB is a major causative factor in microglial-induced inflammation via the maturation of interleukin 1ß (Kingham & Pocock 2001). Indeed, glial cells in a primed 'pro-inflammatory' state is commonplace in animal and cellular models of NCL (Bosch & Kielian 2015; Palmer *et al*. 2013; Cooper *et al*. 2015), including the CLN6 sheep (Kay *et al*. 2006). However, we do not see obvious changes in glial morphology, GFAP or CD68 expression, and no specific enrichment of inflammatory associated proteins in our cultures, suggesting secretome changes occur before the onset of overt neuroinflammation.

A recent study has identified CLN6 as an obligate component of a CLN6-CLN8 complex that recruits lysosomal enzymes for ER to Golgi delivery (Bajaj *et al*. 2020). Therefore, we could be seeing over-secretion as a result of mistargeting. This study showed a depletion of lysosomal enzymes in enriched lysosomal fractions isolated from *Cln6*^{nclf} mice, including reduced CTSD, as we also noted in our cell lysates (**Fig. 2B**). They also noted reduced CTSB, but no change in other investigated cathepsins (CTSZ and CTSC). It is pleasing to note the continuity with this study and helps validate *Cln6*^{nclf} cultures as an *in vitro* model. CTSD CTSB and CTSL can be trafficked from the Golgi to the lysosome via mannose-6-phospate receptors (M6PRs) (Bannoud *et al*. 2018; Otomo *et al*. 2015). A disruption of M6P-containing-protein to M6PR ratios, such as that seen in some models of PD (Matrone *et al*. 2016) and CLN3 (Metcalf *et al*. 2008), can result in the proteins escaping binding in the trans-Golgi network, and instead being directed towards the cell surface where they are secreted into the extracellular space (Coutinho *et al*. 2012). As far as the authors are aware, there are no studies looking at M6PR expression

in CLN6, although one study has shown no difference in the M6P proteome between brain tissue from CLN6 patients and healthy controls (Sleat *et al*. 2005).

Although the acidic environment of the lysosome provides the optimum pH for cathepsin activity, roles have been identified at more neutral pH points. For example, CTSD has been shown to cleave Tau at pH 7.0 (Kenessey *et al*. 1997), and CTSB can swap from an exopeptidase to an endopeptidase at a neutral pH (Turk *et al*. 2001). Additionally, cathepsin activity can be altered by other components of the extracellular space - for example, glycosaminoglycans (GAGs) can facilitate CTSB stabilisation and activation at a more neutral pH (Bojarski *et al*. 2020). Interestingly, a previous study has shown an increase in a GAG derived from Chondroitin Sulfate Proteoglycan 2 (CSPG2; a major component of the brain ECM) in CLN6 patient urine (Teixeira *et al*. 2006). Other potential modulating factors include cathepsin inhibitors, namely cystatins. Indeed, cystatin C (an inhibitor of CTSD, CTSL and CTSB) (Soond *et al.* 2019) was elevated in the *Cln6*^{nclf} secretome, albeit not significantly ($p = 0.06$), and after 48 hours of CLN6 gene therapy there was a significant decrease ($p < 0.05$) - suggesting corrective treatment modulates cathepsin activity early-on.

In this study, increased cathepsin protein expression correlates with an increase in *in vitro* activity of CTSL, which was restored towards normal levels after CLN6 gene therapy treatment. Plasma samples isolated from mice *in vivo* also showed a reduction in CTSL activity post gene therapy, although there was no significant difference between the control (*Cln6*$^{+/-}$) and the affected (*Cln6*^{nclf}). This is potentially due to a less pervasive phenotype in the periphery or the age of the animals (12 months) at the time of sample collection. By 12 months of age, the *Cln6*^{nclf} mouse displays significantly reduced cortical thickness and neuronal counts (Kleine Holthaus *et al*. 2019), reducing the number of potential cells secreting protein into the extracellular space. Nevertheless, a robust reduction in plasma CTSL activity, from treated animals compared to *Cln6*^{nclf} untreated, suggests potential for CTSL as a biomarker for monitoring therapeutic intervention *in vivo*, and is worth investigating further in younger animals

It must be acknowledged these activity assays are performed at the pH optima of the enzyme, meaning this artificial environment can induce cathepsin processing either via auto-activation, or processing via

other cathepsins. Additionally, in the case of CTSD, it could be due to the non-specific nature of this widely used assay kit, where the substrate is also cleaved by Cathepsin E (Yasuda *et al.* 1999). Although the enzyme activity assays are not reflective of the activity *in vitro* or *in vivo*, this does not negate the potential use as biomarkers. Cathepsins as both biomarkers and drug targets has been suggested in other neurodegenerative disorders (Hook *et al*. 2015). For example, AD patients have a 50% higher CTSB than age-matched controls (Sun *et al*. 2010), and deleting CTSB in the transgenic amyloid precursor protein (APP) mouse model is shown to improve memory and reduce amyloid plaque accumulation (Kindy *et al*. 2012).

Cytoskeletal proteins, including the CLN6 interaction partner CRMP2, are altered in the disease secretome

Another interesting area highlighted here is the over-secretion of proteins associated with the KEGG terms "nervous system components" and "cytoskeletal dynamics" - another group of proteins that are frequently dysregulated in neurodegenerative disorders (McMurray 2000). Neurons are especially vulnerable to a disordered cytoskeleton due to their long axons, making them extra reliant upon vesicular transport.

Of particular interest we saw an increase in CRMP2 secretion, a known molecular interacting partner of CLN6 (Benedict *et al*. 2009). The CRMPs are a family of proteins which are highly expressed throughout brain development; with roles in cytoskeletal formation, synapse dynamics, and migration (Charrier *et al*. 2003). CRMP2 specifically is a positive regulator of axonal branching and polarity (Khanna *et al*. 2012). Alongside CRMP2, we also saw increased secretion of CRMP5, and narrowly cut off after FDR were CRMP1 ($p = 0.058$) & CRMP4 ($p = 0.058$). Reduced CRMP2 expression was reported in the brains of *Cln6*^{nclf} mice, particularly in the thalamus (Benedict *et al*. 2009). *In vitro*, decreased branching of 4 DIV *Cln6*^{nclf} hippocampal neurons was correlated with reduced interaction between CLN6 and CRMP2 (Benedict *et al*. 2009). It is possible the reduced expression described in the *Cln6*^{nclf} brain is due to aberrant secretion as a result of mis trafficking from a lack of interaction with CLN6 in the ER.

After gene therapy treatment with CLN6 we also saw a reduction of CRMP4, and with combination gene therapy (CLN5 & CLN6) a reduction in CRMP1 & CRMP2. In fact, with combination therapy, the downregulation of proteins associated with actin binding and the cytoskeleton was significantly enriched (**Fig. 5C**). Targeting CRMPs may be of therapeutic interest in NCL, with modulation of CRMP2 by Lacosamide (an inhibitor of CRMP2 phosphorylation) demonstrating neuroprotective effects, such as reduced glial cell activation, in a mouse model of AD (Ahn *et al*. 2015). Indeed, therapeutic modulation of CRMP2 using Lacosamide resulted in moderate reduction in astrocyte activation in 11-month old *Cln6*^{nclf} mice, but ultimately this did not translate to a functional benefit (White *et al*. 2019).

Short-term gene therapy with a combination of CLN5 & CLN6 restores more secretome markers than CLN6 alone

In this study gene therapy treatment with CLN6, or a combination of CLN5 and CLN6, both begin to restore the secretome to that of the healthy *Cln6*$^{+/-}$ cells within 48 hours. In terms of CLN6 gene therapy, which we know corrects disease phenotypes *in vivo (Cain et al. 2019; Kleine Holthaus et al. 2019)*, our study provides a number of additional non-invasive biomarkers for assessing therapeutic correction. A combination therapy of CLN6 and CLN5 had a greater effect on reducing overexpressed secreted proteins back to healthy levels, including the downregulation of CLN6 interaction partner CRMP2 and reduction in the aberrant lysosomal enzyme secretion of CTSB, CTSD and HEXB. This could be due to the fact CLN5 is also reduced in CLN6 disease, or that combined overexpression means the known interaction between the two proteins is facilitated more rapidly (Lyly *et al*. 2009). Additionally, the overexpression of a lysosomal enzyme could compensate for the reduction in ER-Golgi lysosomal enzyme transport that has been reported in CLN6 (Bajaj *et al*. 2020). Collectively, this suggests combination therapeutics, or the presence of additional lysosomal enzymes, may provide an early boost - an important consideration when investigating therapeutics for an early-onset, fast progressing disease.

The quantity of lentivirus given to cells in this study has likely resulted in overexpression of both genes – highlighted by the increase in the secretion of CLN5 after treatment. This overexpression could

account for some of the noted 'off-target' effects, although as overexpression is commonplace in NCL gene therapy trials *in vivo* (including with CLN6), we would postulate these additional changes are not preventing a beneficial effect (Cain *et al*. 2019). However, these off-target changes could be reducing the full beneficial potential. Alternatively, these changes are part of a compensatory response and may revert to physiological levels over a longer time course.

Conclusions

This study implicates an altered secretome as part of the pathogenesis of CLN6 disease. This is highlighted by the phenotypic overlap with other neurodegenerative disorders, such as the increased secretion of catabolic and cytoskeletal-associated proteins. These changes occur before glial cell activation, providing novel biomarkers for the tracking of early CLN6 disease progression *in vitro*. As such, these markers provide a powerful tool for investigating therapeutics at the earliest stages of disease progression *in vitro*, potentially before the occurrence of irreversible damage. Furthermore, biomarkers - such as Cathepsin L - show promise for *in vivo* translation to the *Cln6*^{nclf} mouse. Access to secreted biomarkers facilitates repeated biochemical monitoring of the same cell population/ animal overtime, making it extremely advantageous for continual therapeutic assessment in future studies.

Declarations

Conflicts of interest: none

Acknowledgements: This study was made possible by funding from the Charlotte and Gwenyth Gray Foundation to Cure Batten Disease, USA. The authors would like to thank Torsten Kleffmann and Abishek Kumar at the University of Otago Centre for Protein Research for performing and preparing samples for the LC-MS. We would also like to thank Kathryn Meyer for the gift of the AAV-Cln6 viral particles. Lastly, the authors would like to thank Indranil Basak for his comments on the manuscript.
Author contributions: **Hannah L Best**: Conceptualization, Investigation, Funding acquisition, Writing – original draft preparation. **Alison J Clare**: Investigation, Writing – Review & Editing. **Kirstin O McDonald:** Investigation, Writing – Review & Editing. **Hollie E Wicky:** Conceptualization, Investigation, Writing – Review & Editing. **Stephanie M Hughes:** Conceptualization, Funding acquisition, Writing – Review & Editing.

Availability of data and materials: A summary of proteomic data sets generated and analysed during this study are including in this published article (and its supplementary information files). Any additional data from the current study are available from the corresponding author on reasonable request.

Funding: **HB** was funded by a University of Otago PhD scholarship and Hanns Mohler Roche scholarship through the Brain Health Research Centre, Otago. Support for this research was also supplied by the Charlotte and Gwenyth Gray Foundation to Cure Batten Disease, USA.

References

Ahn, J. Y., Yan, B. C., Park, J. H. et al. (2015) Novel antiepileptic drug lacosamide exerts neuroprotective effects by decreasing glial activation in the hippocampus of a gerbil model of ischemic stroke. *Exp Ther Med* **10,** 2007-2014.

Allen, N. J. and Eroglu, C. (2017) Cell Biology of Astrocyte-Synapse Interactions. *Neuron* **96,** 697-708.

Almagro Armenteros, J. J., Tsirigos, K. D., Sonderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G. and Nielsen, H. (2019) SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat Biotechnol* **37,** 420-423.

Amritraj, A., Peake, K., Kodam, A., Salio, C., Merighi, A., Vance, J. E. and Kar, S. (2009) Increased activity and altered subcellular distribution of lysosomal enzymes determine neuronal vulnerability in Niemann-Pick type C1-deficient mice. *Am J Pathol* **175,** 2540-2556.

Anderson, G. W., Goebel, H. H. and Simonati, A. (2013) Human pathology in NCL. *Biochim Biophys Acta* **1832,** 1807-1826.

Artal-Sanz, M. and Tavernarakis, N. (2005) Proteolytic mechanisms in necrotic cell death and neurodegeneration. *FEBS Lett* **579,** 3287-3296.

Bajaj, L., Sharma, J., di Ronza, A. et al. (2020) A CLN6-CLN8 complex recruits lysosomal enzymes at the ER for Golgi transfer. *J Clin Invest*.

Balducci, C., Pierguidi, L., Persichetti, E. et al. (2007) Lysosomal hydrolases in cerebrospinal fluid from subjects with Parkinson's disease. *Mov Disord* **22,** 1481-1484.

Bannoud, N., Carvelli, F. L., Troncoso, M., Sartor, T., Vargas-Roig, L. M. and Sosa, M. (2018) Cation-dependent mannose-6-phosphate receptor expression and distribution are influenced by estradiol in MCF-7 breast cancer cells. *PLoS One* **13,** e0201844.

Benarroch, E. E. (2013) Microglia: Multiple roles in surveillance, circuit shaping, and response to injury. *Neurology* **81,** 1079-1088.

Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G. and Brunak, S. (2004) Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng Des Sel* **17,** 349-356.

Benedict, J. W., Getty, A. L., Wishart, T. M., Gillingwater, T. H. and Pearce, D. A. (2009) Protein product of CLN6 gene responsible for variant late-onset infantile neuronal ceroid lipofuscinosis interacts with CRMP-2. *J Neurosci Res* **87,** 2157-2166.

Best, H. L., Neverman, N. J., Wicky, H. E., Mitchell, N. L., Leitch, B. and Hughes, S. M. (2017) Characterisation of early changes in ovine CLN5 and CLN6 Batten disease neural cultures for the rapid screening of therapeutics. *Neurobiol Dis* **100,** 62-74.

Bible, E., Gupta, P., Hofmann, S. L. and Cooper, J. D. (2004) Regional and cellular neuropathology in the palmitoyl protein thioesterase-1 null mutant mouse model of infantile neuronal ceroid lipofuscinosis. *Neurobiol Dis* **16,** 346-359.

Bojarski, K. K., Karczynska, A. S. and Samsonov, S. A. (2020) Role of Glycosaminoglycans in Procathepsin B Maturation: Molecular Mechanism Elucidated by a Computational Study. *J Chem Inf Model*.

Bosch, M. E. and Kielian, T. (2015) Neuroinflammatory paradigms in lysosomal storage diseases. *Front Neurosci* **9,** 417.

Cain, J. T., Likhite, S., White, K. A. et al. (2019) Gene Therapy Corrects Brain and Behavioral Pathologies in CLN6-Batten Disease. *Mol Ther* **27,** 1836-1847.

Cao, Y., Staropoli, J. F., Biswas, S., Espinola, J. A., MacDonald, M. E., Lee, J. M. and Cotman, S. L. (2011) Distinct early molecular responses to mutations causing vLINCL and JNCL presage ATP synthase subunit C accumulation in cerebellar cells. *PLoS One* **6,** e17118.

Charrier, E., Reibel, S., Rogemond, V., Aguera, M., Thomasset, N. and Honnorat, J. (2003) Collapsin response mediator proteins (CRMPs): involvement in nervous system development and adult neurodegenerative disorders. *Mol Neurobiol* **28,** 51-64.

Cooper, J. D., Tarczyluk, M. A. and Nelvagal, H. R. (2015) Towards a new understanding of NCL pathogenesis. *Biochim Biophys Acta* **1852,** 2256-2261.

Coutinho, M. F., Prata, M. J. and Alves, S. (2012) A shortcut to the lysosome: the mannose-6-phosphate-independent pathway. *Mol Genet Metab* **107,** 257-266.

Danyukova, T., Ariunbat, K., Thelen, M., Brocke-Ahmadinejad, N., Mole, S. E. and Storch, S. (2018) Loss of CLN7 results in depletion of soluble lysosomal proteins and impaired mTOR reactivation. *Hum Mol Genet* **27,** 1711-1722.

de Castro, M. A., Bunt, G. and Wouters, F. S. (2016) Cathepsin B launches an apoptotic exit effort upon cell death-associated disruption of lysosomes. *Cell Death Discov* **2,** 16012.

Dowell, J. A., Johnson, J. A. and Li, L. (2009) Identification of astrocyte secreted proteins with a combination of shotgun proteomics and bioinformatics. *J Proteome Res* **8,** 4135-4143.

Dozieres-Puyravel, B., Nasser, H., Elmaleh-Berges, M. et al. (2020) Paediatric-onset neuronal ceroid lipofuscinosis: first symptoms and presentation at diagnosis. *Dev Med Child Neurol* **62,** 528-530.

Dutton, J. W., 3rd, Artwohl, J. E., Huang, X. and Fortman, J. D. (2019) Assessment of Pain Associated with the Injection of Sodium Pentobarbital in Laboratory Mice (Mus musculus). *J Am Assoc Lab Anim Sci* **58,** 373-379.

Eroglu, C. and Barres, B. A. (2010) Regulation of synaptic connectivity by glia. *Nature* **468,** 223-231.

Felbor, U., Kessler, B., Mothes, W., Goebel, H. H., Ploegh, H. L., Bronson, R. T. and Olsen, B. R. (2002) Neuronal loss and brain atrophy in mice lacking cathepsins B and L. *Proc Natl Acad Sci U S A* **99,** 7883-7888.

Gavin, M., Wen, G. Y., Messing, J., Adelman, S., Logush, A., Jenkins, E. C., Brown, W. T. and Velinov, M. (2013) Substrate Reduction Therapy in Four Patients with Milder CLN1 Mutations and Juvenile-Onset Batten Disease Using Cysteamine Bitartrate. *JIMD Rep* **11,** 87-92.
Haltia, M., Herva, R., Suopanki, J., Baumann, M. and Tyynela, J. (2001) Hippocampal lesions in the neuronal ceroid lipofuscinoses. *Eur J Paediatr Neurol* **5 Suppl A,** 209-211.
Holopainen, J. M., Saarikoski, J., Kinnunen, P. K. and Jarvela, I. (2001) Elevated lysosomal pH in neuronal ceroid lipofuscinoses (NCLs). *Eur J Biochem* **268,** 5851-5856.
Hook, G., Jacobsen, J. S., Grabstein, K., Kindy, M. and Hook, V. (2015) Cathepsin B is a New Drug Target for Traumatic Brain Injury Therapeutics: Evidence for E64d as a Promising Lead Drug Candidate. *Front Neurol* **6,** 178.
Hook, V. Y. (2006) Unique neuronal functions of cathepsin L and cathepsin B in secretory vesicles: biosynthesis of peptides in neurotransmission and neurodegenerative disease. *Biol Chem* **387,** 1429-1439.
Hsiao, H. Y., Chen, Y. C., Chen, H. M., Tu, P. H. and Chern, Y. (2013) A critical role of astrocyte-mediated nuclear factor-kappaB-dependent inflammation in Huntington's disease. *Hum Mol Genet* **22,** 1826-1842.
Huang da, W., Sherman, B. T. and Lempicki, R. A. (2009a) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37,** 1-13.
Huang da, W., Sherman, B. T. and Lempicki, R. A. (2009b) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4,** 44-57.
Huber, R. J., Mathavarajah, S. and Yap, S. Q. (2020) Mfsd8 localizes to endocytic compartments and influences the secretion of Cln5 and cathepsin D in Dictyostelium. *Cell Signal* **70,** 109572.
Kanninen, K. M., Grubman, A., Caragounis, A. et al. (2013) Altered biometal homeostasis is associated with CLN6 mRNA loss in mouse neuronal ceroid lipofuscinosis. *Biol Open* **2,** 635-646.
Kay, G. W., Palmer, D. N., Rezaie, P. and Cooper, J. D. (2006) Activation of non-neuronal cells within the prenatal developing brain of sheep with neuronal ceroid lipofuscinosis. *Brain Pathol* **16,** 110-116.
Kenessey, A., Nacharaju, P., Ko, L. W. and Yen, S. H. (1997) Degradation of tau by lysosomal enzyme cathepsin D: implication for Alzheimer neurofibrillary degeneration. *J Neurochem* **69,** 2026-2038.
Khanna, R., Wilson, S. M., Brittain, J. M., Weimer, J., Sultana, R., Butterfield, A. and Hensley, K. (2012) Opening Pandora's jar: a primer on the putative roles of CRMP2 in a panoply of neurodegenerative, sensory and motor neuron, and central disorders. *Future Neurol* **7,** 749-771.
Kim, J., Gee, H. Y. and Lee, M. G. (2018) Unconventional protein secretion - new insights into the pathogenesis and therapeutic targets of human diseases. *J Cell Sci* **131**.
Kim, J. B., Lim, N., Kim, S. J. and Heo, T. H. (2012) N-acetylcysteine normalizes the urea cycle and DNA repair in cells from patients with Batten disease. *Cell Biochem Funct* **30,** 677-682.
Kim, S., Ock, J., Kim, A. K., Lee, H. W., Cho, J. Y., Kim, D. R., Park, J. Y. and Suk, K. (2007) Neurotoxicity of microglial cathepsin D revealed by secretome analysis. *J Neurochem* **103,** 2640-2650.
Kim, Y. J., Sapp, E., Cuiffo, B. G. et al. (2006) Lysosomal proteases are involved in generation of N-terminal huntingtin fragments. *Neurobiol Dis* **22,** 346-356.
Kindy, M. S., Yu, J., Zhu, H., El-Amouri, S. S., Hook, V. and Hook, G. R. (2012) Deletion of the cathepsin B gene improves memory deficits in a transgenic ALZHeimer's disease mouse model expressing AbetaPP containing the wild-type beta-secretase site sequence. *J Alzheimers Dis* **29,** 827-840.
Kingham, P. J. and Pocock, J. M. (2001) Microglial secreted cathepsin B induces neuronal apoptosis. *J Neurochem* **76,** 1475-1484.
Kinney, J. W., Bemiller, S. M., Murtishaw, A. S., Leisgang, A. M., Salazar, A. M. and Lamb, B. T. (2018) Inflammation as a central mechanism in Alzheimer's disease. *Alzheimers Dement (N Y)* **4,** 575-590.
Kleine Holthaus, S. M., Herranz-Martin, S., Massaro, G. et al. (2019) Neonatal brain-directed gene therapy rescues a mouse model of neurodegenerative CLN6 Batten disease. *Hum Mol Genet* **28,** 3867-3879.
Koike, M., Nakanishi, H., Saftig, P. et al. (2000) Cathepsin D deficiency induces lysosomal storage with ceroid lipofuscin in mouse CNS neurons. *J Neurosci* **20,** 6898-6906.
Koike, M., Shibata, M., Waguri, S. et al. (2005) Participation of autophagy in storage of lysosomes in neurons from mouse models of neuronal ceroid-lipofuscinoses (Batten disease). *Am J Pathol* **167,** 1713-1728.
Liddelow, S. A., Guttenplan, K. A., Clarke, L. E. et al. (2017) Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541,** 481-487.
Linterman, K. S., Palmer, D. N., Kay, G. W., Barry, L. A., Mitchell, N. L., McFarlane, R. G., Black, M. A., Sands, M. S. and Hughes, S. M. (2011) Lentiviral-mediated gene transfer to the sheep brain: implications for gene therapy in Batten disease. *Hum Gene Ther* **22,** 1011-1020.
Lively, S. and Schlichter, L. C. (2013) The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *J Neuroinflammation* **10,** 75.
Lloyd-Evans, E. and Waller-Evans, H. (2020) Lysosomal Ca(2+) Homeostasis and Signaling in Health and Disease. *Cold Spring Harb Perspect Biol* **12**.
Loov, C., Shevchenko, G., Geeyarpuram Nadadhur, A., Clausen, F., Hillered, L., Wetterhall, M. and Erlandsson, A. (2013) Identification of injury specific proteins in a cell culture model of traumatic brain injury. *PLoS One* **8,** e55983.
Lowry, J. R. and Klegeris, A. (2018) Emerging roles of microglial cathepsins in neurodegenerative disease. *Brain Res Bull* **139,** 144-156.
Lyly, A., von Schantz, C., Heine, C., Schmiedt, M. L., Sipila, T., Jalanko, A. and Kyttala, A. (2009) Novel interactions of CLN5 support molecular networking between Neuronal Ceroid Lipofuscinosis proteins. *BMC Cell Biol* **10,** 83.
Matrone, C., Dzamko, N., Madsen, P. et al. (2016) Mannose 6-Phosphate Receptor Is Reduced in -Synuclein Overexpressing Models of Parkinsons Disease. *PLoS One* **11,** e0160501.
McCauley, M. E. and Baloh, R. H. (2019) Inflammation in ALS/FTD pathogenesis. *Acta Neuropathol* **137,** 715-730.
McGlinchey, R. P. and Lee, J. C. (2015) Cysteine cathepsins are essential in lysosomal degradation of alpha-synuclein. *Proc Natl Acad Sci U S A* **112,** 9322-9327.
McMurray, C. T. (2000) Neurodegeneration: diseases of the cytoskeleton? *Cell Death Differ* **7,** 861-865.
Mederos, S., Gonzalez-Arias, C. and Perea, G. (2018) Astrocyte-Neuron Networks: A Multilane Highway of Signaling for Homeostatic Brain Function. *Front Synaptic Neurosci* **10,** 45.
Metcalf, D. J., Calvi, A. A., Seaman, M., Mitchison, H. M. and Cutler, D. F. (2008) Loss of the Batten disease gene CLN3 prevents exit from the TGN of the mannose 6-phosphate receptor. *Traffic* **9,** 1905-1914.
Mi, H., Dong, Q., Muruganujan, A., Gaudet, P., Lewis, S. and Thomas, P. D. (2010) PANTHER version 7: improved phylogenetic trees, orthologs and collaboration with the Gene Ontology Consortium. *Nucleic Acids Res* **38,** D204-210.

Minnis, C. J., Thornton, C. D., FitzPatrick, L. M. and McKay, T. R. (2019) Cellular models of Batten disease. *Biochim Biophys Acta Mol Basis Dis***,** 165559.
Mole, S. E. and Cotman, S. L. (2015) Genetics of the neuronal ceroid lipofuscinoses (Batten disease). *Biochim Biophys Acta* **1852,** 2237-2241.
Moore, N. H., Costa, L. G., Shaffer, S. A., Goodlett, D. R. and Guizzetti, M. (2009) Shotgun proteomics implicates extracellular matrix proteins and protease systems in neuronal development induced by astrocyte cholinergic stimulation. *J Neurochem* **108,** 891-908.
Morgan, J. P., Magee, H., Wong, A., Nelson, T., Koch, B., Cooper, J. D. and Weimer, J. M. (2013) A murine model of variant late infantile ceroid lipofuscinosis recapitulates behavioral and pathological phenotypes of human disease. *PLoS One* **8,** e78694.
Oswald, M. J., Palmer, D. N., Kay, G. W., Barwell, K. J. and Cooper, J. D. (2008) Location and connectivity determine GABAergic interneuron survival in the brains of South Hampshire sheep with CLN6 neuronal ceroid lipofuscinosis. *Neurobiol Dis* **32,** 50-65.
Otomo, T., Schweizer, M., Kollmann, K., Schumacher, V., Muschol, N., Tolosa, E., Mittrucker, H. W. and Braulke, T. (2015) Mannose 6 phosphorylation of lysosomal enzymes controls B cell functions. *J Cell Biol* **208,** 171-180.
Palmer, D. N., Barry, L. A., Tyynela, J. and Cooper, J. D. (2013) NCL disease mechanisms. *Biochim Biophys Acta* **1832,** 1882-1893.
Pekny, M., Pekna, M., Messing, A., Steinhauser, C., Lee, J. M., Parpura, V., Hol, E. M., Sofroniew, M. V. and Verkhratsky, A. (2016) Astrocytes: a central element in neurological diseases. *Acta Neuropathol* **131,** 323-345.
Perry, V. H. (2012) Innate inflammation in Parkinson's disease. *Cold Spring Harb Perspect Med* **2,** a009373.
Pfrieger, F. W. and Barres, B. A. (1997) Synaptic efficacy enhanced by glial cells in vitro. *Science* **277,** 1684-1687.
Pontikis, C. C., Cotman, S. L., MacDonald, M. E. and Cooper, J. D. (2005) Thalamocortical neuron loss and localized astrocytosis in the Cln3Deltaex7/8 knock-in mouse model of Batten disease. *Neurobiol Dis* **20,** 823-836.
Ransohoff, R. M. (2016) How neuroinflammation contributes to neurodegeneration. *Science* **353,** 777-783.
Repnik, U., Stoka, V., Turk, V. and Turk, B. (2012) Lysosomes and lysosomal cathepsins in cell death. *Biochim Biophys Acta* **1824,** 22-33.
Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T. and Eliceiri, K. W. (2017) ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* **18,** 529.
Samie, M. A. and Xu, H. (2014) Lysosomal exocytosis and lipid storage disorders. *J Lipid Res* **55,** 995-1009.
Schildge, S., Bohrer, C., Beck, K. and Schachtrup, C. (2013) Isolation and culture of mouse cortical astrocytes. *J Vis Exp*.
Schulz, A., Kohlschutter, A., Mink, J., Simonati, A. and Williams, R. (2013) NCL diseases - clinical perspectives. *Biochim Biophys Acta* **1832,** 1801-1806.
Sleat, D. E., Lackland, H., Wang, Y., Sohar, I., Xiao, G., Li, H. and Lobel, P. (2005) The human brain mannose 6-phosphate glycoproteome: a complex mixture composed of multiple isoforms of many soluble lysosomal proteins. *Proteomics* **5,** 1520-1532.
Sleat, D. E., Wiseman, J. A., El-Banna, M., Zheng, H., Zhao, C., Soherwardy, A., Moore, D. F. and Lobel, P. (2019) Analysis of Brain and Cerebrospinal Fluid from Mouse Models of the Three Major Forms of Neuronal Ceroid Lipofuscinosis Reveals Changes in the Lysosomal Proteome. *Mol Cell Proteomics* **18,** 2244-2261.
Sleat, D. E., Wiseman, J. A., Sohar, I., El-Banna, M., Zheng, H., Moore, D. F. and Lobel, P. (2012) Proteomic analysis of mouse models of Niemann-Pick C disease reveals alterations in the steady-state levels of lysosomal proteins within the brain. *Proteomics* **12,** 3499-3509.
Song, P., Kwon, Y., Joo, J. Y., Kim, D. G. and Yoon, J. H. (2019) Secretomics to Discover Regulators in Diseases. *Int J Mol Sci* **20**.
Soond, S. M., Kozhevnikova, M. V., Townsend, P. A. and Zamyatnin, A. A., Jr. (2019) Cysteine Cathepsin Protease Inhibition: An update on its Diagnostic, Prognostic and Therapeutic Potential in Cancer. *Pharmaceuticals (Basel)* **12**.
Sun, L., Wu, Z., Baba, M., Peters, C., Uchiyama, Y. and Nakanishi, H. (2010) Cathepsin B-dependent motor neuron death after nerve injury in the adult mouse. *Biochem Biophys Res Commun* **399,** 391-395.
Szklarczyk, D., Morris, J. H., Cook, H. et al. (2017) The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res* **45,** D362-D368.
Teixeira, C. A., Lin, S., Mangas, M., Quinta, R., Bessa, C. J., Ferreira, C., Sa Miranda, M. C., Boustany, R. M. and Ribeiro, M. G. (2006) Gene expression profiling in vLINCL CLN6-deficient fibroblasts: Insights into pathobiology. *Biochim Biophys Acta* **1762,** 637-646.
Thelen, M., Damme, M., Schweizer, M., Hagel, C., Wong, A. M., Cooper, J. D., Braulke, T. and Galliciotti, G. (2012) Disruption of the autophagy-lysosome pathway is involved in neuropathology of the nclf mouse model of neuronal ceroid lipofuscinosis. *PLoS One* **7,** e35493.
Theodosis, D. T., Piet, R., Poulain, D. A. and Oliet, S. H. (2004) Neuronal, glial and synaptic remodeling in the adult hypothalamus: functional consequences and role of cell surface and extracellular matrix adhesion molecules. *Neurochem Int* **45,** 491-501.
Thomas, P. D., Campbell, M. J., Kejariwal, A., Mi, H., Karlak, B., Daverman, R., Diemer, K., Muruganujan, A. and Narechania, A. (2003) PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* **13,** 2129-2141.
Thouvenot, E., Urbach, S., Vigy, O., Seveno, M., Galeotti, N., Nguyen, G., Bockaert, J. and Marin, P. (2012) Quantitative proteomic analysis reveals protein expression changes in the murine neuronal secretome during apoptosis. *J Proteomics* **77,** 394-405.
Tohda, C. and Tohda, M. (2017) Extracellular cathepsin L stimulates axonal growth in neurons. *BMC Res Notes* **10,** 613.
Tsunemi, T., Perez-Rosello, T., Ishiguro, Y. et al. (2019) Increased Lysosomal Exocytosis Induced by Lysosomal Ca(2+) Channel Agonists Protects Human Dopaminergic Neurons from alpha-Synuclein Toxicity. *J Neurosci* **39,** 5760-5772.
Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B. and Turk, D. (2012) Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochim Biophys Acta* **1824,** 68-88.
Turk, V., Turk, B. and Turk, D. (2001) Lysosomal cysteine proteases: facts and opportunities. *EMBO J* **20,** 4629-4633.
Tyynela, J., Cooper, J. D., Khan, M. N., Shemilts, S. J. and Haltia, M. (2004) Hippocampal pathology in the human neuronal ceroid-lipofuscinoses: distinct patterns of storage deposition, neurodegeneration and glial activation. *Brain Pathol* **14,** 349-357.
Verity, C., Winstone, A. M., Stellitano, L., Will, R. and Nicoll, A. (2010) The epidemiology of progressive intellectual and neurological deterioration in childhood. *Arch Dis Child* **95,** 361-364.
Vesa, J., Chin, M. H., Oelgeschlager, K., Isosomppi, J., DellAngelica, E. C., Jalanko, A. and Peltonen, L. (2002) Neuronal ceroid lipofuscinoses are connected at molecular level: interaction of CLN5 protein with CLN2 and CLN3. *Mol Biol Cell* **13,** 2410-2420.
Vitner, E. B., Dekel, H., Zigdon, H., Shachar, T., Farfel-Becker, T., Eilam, R., Karlsson, S. and Futerman, A. H. (2010) Altered expression and distribution of cathepsins in neuronopathic forms of Gaucher disease and in other sphingolipidoses. *Hum Mol Genet* **19,** 3583-3590.
Wang, C., Sun, B., Zhou, Y., Grubb, A. and Gan, L. (2012) Cathepsin B degrades amyloid-beta in mice expressing wild-type human amyloid precursor protein. *J Biol Chem* **287,** 39834-39841.
Warrier, V., Vieira, M. and Mole, S. E. (2013) Genetic basis and phenotypic correlations of the neuronal ceroid lipofusinoses. *Biochim Biophys Acta* **1832,** 1827-1830.

White, K. A., Cain, J. T., Magee, H., Yeon, S. K., Park, K. D., Khanna, R. and Weimer, J. M. (2019) Modulation of CRMP2 via (S)-Lacosamide shows therapeutic promise but is ultimately ineffective in a mouse model of CLN6-Batten disease. *Health Psychol Behav Med* **3,** NS20190001.
Wisniewski, J. R., Zougman, A., Nagaraj, N. and Mann, M. (2009) Universal sample preparation method for proteome analysis. *Nat Methods* **6,** 359-362.
Yamashita, A., Shiro, Y., Hiraki, Y., Yujiri, T. and Yamazaki, T. (2020) Implications of graded reductions in CLN6's anti-aggregate activity for the development of the neuronal ceroid lipofuscinoses. *Biochem Biophys Res Commun*.
Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y. and Yamamoto, K. (1999) Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. *J Biochem* **125,** 1137-1143.
Yoshiyama, Y., Arai, K., Oki, T. and Hattori, T. (2000) Expression of invariant chain and pro-cathepsin L in Alzheimer's brain. *Neurosci Lett* **290,** 125-128.
Zhang, H., Zhong, C., Shi, L., Guo, Y. and Fan, Z. (2009) Granulysin induces cathepsin B release from lysosomes of target tumor cells to attack mitochondria through processing of bid leading to Necroptosis. *J Immunol* **182,** 6993-7000.
Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L. and Trono, D. (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* **15,** 871-875.

Figure Legends

Figure 1. Graphical overview of experimental methodology. A. Methods for obtaining conditioned media for LC-MS, western blotting and enzyme assays. **B.** Methods for obtaining blood plasma for enzyme assays. Figure created with BioRender.com

Figure 2. Differential expression analysis shows increased secretion of proteins involved in 'catabolic processes' from *Cln6*^{nclf} cultures. Conditioned media was collected from *Cln6*$^{+/-}$ and *Cln6*^{nclf} mixed neural cell cultures generated from P0-1 pups (n = 3 pups). Proteins and their relative expression of were analysed by LC-MS. **A.** Significantly increased expression ($p \leq 0.05$) of 37 proteins was observed in *Cln6*^{nclf} media, visualised in an interaction network via STRING. The thickness of connecting lines indicates the confidence score of functional interactions. Red nodes indicate proteins associated with cellular catabolic processes (top GO term, biological process), yellow nodes are proteins involved in myelin sheath (top GO term, cellular component), and green nodes are lysosomal proteins (top KEGG pathway) **B.** Functional classification of differentially expressed proteins indicates the largest group have catalytic activity. **C.** Annotation clusters for significantly enriched proteins, with a dotted line indicating the significance cut off point ≥ 1.3, equivalent to $p \leq 0.05$).

Figure 3. Validation of LC-MS changes in the *Cln6*^{nclf} mouse confirms altered cathepsin expression and activity. Primary mixed neural cultures derived from either *Cln6*$^{+/-}$ (+/-) or *Cln6*^{nclf} mice pups (*nclf*) were grown for 9 DIV, and protein harvested from the cell lysate and conditioned media. The presence of Cathepsin D (CTSD) was evaluated by immunoblotting and quantified relative to β-

actin. **A.** Blotting for CTSD in conditioned media showed a single proform at (~50 kDa). Increased CTSD profrom was observed in the *nclf* conditioned media when normalised to β-actin in cells (***p = 0.0003, unpaired t test, n = 4). **B.** Blotting for CTSD in cell lysates showed two bands, a proform at ~50 kDa and a mature form at ~25 kDa. Significantly reduced CTSD proform was present in the *nclf* lysate (**p = 0.0011, two-way ANOVA with Sidak's multiple comparisons test, n = 4). Fluorometric activity assays were used to compare relative enzyme activities of CTSD, Cathepsin L (CTSL) and Cathepsin B (CTSB) activity, in both **C.** conditioned media and **D.** cell lysate (unpaired t tests, n = 4 – 6). Increased CTSL activity was seen in *Cln6*^{nclf} conditioned media (***p ≤ 0.0001).

Figure 4. CLN6 gene therapy treatment reduces Cathepsin L activity both *in vivo* and *in vitro*. A. Primary cultures were treated with lentivirus expressing murine CLN6 (+LV) for 4 *DIV*. Enzyme activity assays showed no effect on CTSD activity. CTSL activity was again elevated in *Cln6*^{nclf} (*nclf*; **p = 0.0028) compared to controls (+/-). CTSL activity was not significantly different between *Cln6*^{nclf} + LV and healthy *Cln6*$^{+/-}$ controls (p = 0.0652). Data was analysed by one-way ANOVA and presented normalised to the control (+/-) in relative fluorescent units (RFU), n = 2 - 4. **B.** Plasma was isolated from 12 month old healthy male mice (+/-), *Cln6*^{nclf} mice (*nclf*) and *Cln6*^{nclf} mice treated with AAV.CLN6 gene therapy since P1 (AAV). At assay endpoint there was no significant difference in CTSD activity between any groups. At assay endpoint there was a significant decrease in CTSL activity in *nclf* mice treated with AAV compared to untreated (*p = 0.0256). Data is presented as a Box and Whisker plot (min to max) to aid visualisation of skewness in the data set, with the box containing the interquartile range and the horizontal line contained within the box denoting the median. Data was analysed via one-way ANOVA and presented as RFU at endpoint, n = 4 - 5. All data are displayed as the mean +/- SEM.

Figure 5. Gene therapy with CLN6, or combined CLN6 and CLN5, reduces a subset of proteins that are 'over-secreted' in *Cln6*^{nclf} conditioned media. *Cln6*^{nclf} mixed neural cultures were treated with either lentivirus expressing murine CLN6 (+LV.CLN6), or in combination with CLN5 (+LV.CLN5) for 4 DIV (n = 3). Relative protein expression in conditioned media was analysed via LC-MS. **A.** Proteins with a decreased expression post *Cln6*^{nclf} after gene therapy (+LV), compared to

proteins shown to be increased in disease (*Cln6*^{nclf}; $p \leq 0.05$). **B.** Proteins increased in expression after gene therapy (+LV), compared to proteins increased in disease (*Cln6*^{nclf}; $p \leq 0.05$) **C.** Annotation clusters for significantly enriched genes, with a dotted line indicating the significance cut off point ≥ 1.3 (equivalent to $p \leq 0.05$). **D.** Heat map summarising any proteins identified as increased in *Cln6*^{nclf} that were altered by at least one of the gene therapies. Data is represented at percentage of control (*Cln6*$^{+/-}$).

Supplementary Material

Supplementary A (pdf): Supporting information on mixed cell cultures used in this study.

Supplementary B (xlsx): Supporting information on proteins identified by MS in this study.

Supplementary C (pdf): Supporting information on PANTHER and DAVID ontology analysis.

Supplementary D (pdf): Original western blot images.

Supplementary E (pdf): altered *Cln5* transcript in *Cln6*^{nclf} mice