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Abstract: Background: Cathepsin C (CatC) is a lysosomal enzyme involved in the degradation of intracellular proteins and associated with the autophagic process. Several mutations with loss of function in the Cathepsin C (CatC) gene have been shown to be the genetic mark of Papillon-Lefèvre syndrome (PLS), a rare autosomal recessive disease characterized by severe early-onset periodontitis, palmoplantar hyperkeratosis and increased susceptibility to infections. Deficiencies or dysfunction in other cathepsins such as B or D have been associated with autophagic and lysosomal disorders.
Objectives: Here, we characterized the basis for autophagic dysfunction in PLS by analyzing skin fibroblasts from PLS patients with several mutations in CatC and reduced enzymatic activity.
Methods: Skin fibroblasts were isolated from patients and characterized by genetic study. Authophagy flux was evaluated by accumulation of p62/SQSTM1 and bafilomycin assay and confirmed by visible autophagosome accumulation by Transmision Electron Microscopy. A recombinant CatC was produced by produced by produced by baculovirus system in insect cell cultures.
Results: Our data showed metabolic alterations in the oxidative/antioxidative status, reduced oxygen consumption and a marked autophagic dysfunction with autophagosome accumulation. This was associated with lysosomal permeabilization, CatB release and NLRP3-inflammasome activation. A treatment with recombinant CatC (rCatC) improved cell growth, autophagic flux and partially restored lysosomal permeabilization.

Conclusions: Our data provide a novel molecular mechanism of PLS. Dysfunctional autophagy as a secondary event resulting from insufficient lysosomal function in PLS could show a new therapeutic target.

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CEGIR (Consortium of Eosinophilic Gastrointestinal Disease Researchers) (Ongoing): Principal Investigator, International Eosinophil Society (Ongoing): Steering Committee TIGERS (Ongoing): Steering Committee. **Conflict Resolution:** I present unbiased information in my activities for the AAAAI, and I am not currently studying any product produced by these companies.

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Employer: Christian-Albrechts-University of Kiel and University Hospital Schleswig-Holstein. **Competing Relationships:** Speaker: Sanofi-Aventis, Novartis, Galderma. Adivsory Boards: Astellas, Novartis, Sanofi-Aventis. Research Grants: Sanofi-Aventis (ongoing), La Roche Posay (ongoing), Novartis (finished), Pfizer (finished), Biogen (finished).
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FACULTY DISCLOSURES
Please refer to the opening pages of the assigned manuscript for the authors' relevant funding and employment information.

Cover Letter to Editor only

Cezmi A. Akdis, Editor-in-Chief
Journal of Allergy and Clinical Immunology

September 06, 2017

Dear Editor,

We would like to submit our manuscript: "**Autophagic dysfunction in Papillon Lefèvre is restored by recombinant Cathepsin C treatment**" to be considered for publication in *Trends in Immunology*, in form of an original contribution.

Papillon-Lefèvre syndrome (PLS) is a rare autosomal recessive condition characterized by severe early-onset periodontitis and palmoplantar hyperkeratosis and where dermatological symptoms begin prior to 2 years of age and continue throughout life 2 accompanied by increased susceptibility to infections, furunculosis and pyoderma, or pyogenic liver abscess, among others. PLS results from mutations that inactivate or introduce loss of function in the Cathepsin C (CatC) gene.
In this study, we have described for first time the implication of the authophagic dysfunction and lysosomal permeabilization in the pathophysiology of skin fibroblasts from PLS. Furthermore, we show a partial reconstitution of the pathological process after treatment with a recombinant CatC produced by produced by baculovirus system in insect cell cultures.
In summary, we believe that our study considerably enriches the perception of pathogenic mechanisms of the dermatological alterations of PLS and propose new pharmacological targets.
I hereby state:

1. That the submitting author has the written consent from all authors to submit the manuscript and that all authors accept complete responsibility for the contents of the manuscript

2. That the manuscript is not currently under consideration elsewhere and the work reported will not be submitted for publication elsewhere until a final decision has been made as to its acceptability by the Journal

3. That the manuscript is truthful original work

Yours sincerely

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Autophagic dysfunction in Papillon Lefèvre is restored by recombinant Cathepsin C treatment

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Running Title: Autophagic dysfunction in Papillon Lefèvre

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Abstract

Background: Cathepsin C (CatC) is a lysosomal enzyme involved in the degradation of intracellular proteins and associated with the autophagic process. Several mutations with loss of function in the Cathepsin C (CatC) gene have been shown to be the genetic mark of Papillon-Lefèvre syndrome (PLS), a rare autosomal recessive disease characterized by severe early-onset periodontitis, palmoplantar hyperkeratosis and increased susceptibility to infections. Deficiencies or dysfunction in other cathepsins such as B or D have been associated with autophagic and lysosomal disorders.

Objectives: Here, we characterized the basis for autophagic dysfunction in PLS by analyzing skin fibroblasts from PLS patients with several mutations in CatC and reduced enzymatic activity.

Methods: Skin fibroblasts were isolated from patients and characterized by genetic study. Authophagy flux was evaluated by accumulation of p62/SQSTM1 and bafilomycin assay and confirmed by visible autophagosome accumulation by Transmision Electron Microscopy. A recombinant CatC was produced by produced by produced by baculovirus system in insect cell cultures.

Results: Our data showed metabolic alterations in the oxidative/antioxidative status, reduced oxygen consumption and a marked autophagic dysfunction with autophagosome accumulation. This was associated with lysosomal permeabilization, CatB release and NLRP3-inflammasome activation. A treatment with recombinant CatC (rCatC) improved cell growth, autophagic flux and partially restored lysosomal permeabilization.

Conclusions: Our data provide a novel molecular mechanism of PLS. Dysfunctional autophagy as a secondary event resulting from insufficient lysosomal function in PLS could show a new therapeutic target.

Keywords: Papillon-Lefèvre, Cathepsin C, autophagy, lysosomal permeabilization

Introduction

Papillon-Lefèvre syndrome (PLS) is a rare autosomal recessive condition characterized by severe early-onset periodontitis and palmoplantar hyperkeratosis resulting in a premature loss of both deciduous and permanent dentitions [1]. Dermatological symptoms in PLS begin prior to 2 years of age and continue throughout life [2]. About 20–25% of PLS patients report an increased susceptibility to infections, such as furunculosis and pyoderma, or pyogenic liver abscess, among others [2]. PLS results from mutations that inactivate or introduce loss of function in the Cathepsin C (CatC) gene, which is associated with lack of immune cell serine protease activities [3], hyperactive neutrophils [4], increased oxidative stress [2] and decreased neutrophil extracellular traps capacity as a defensive form againt microorganisms [5].

CatC is a lysosomal enzyme involved in the degradation of intracellular proteins [6]. This process is mediated by autophagy, a critical mechanism devoted to preserving cellular homeostasis. The cellular turnover of proteins and organelles requires the cooperation between autophagic and lysosomal degradation pathways where the fusion of the autophagosome with the lysosome has a relevant role [7]. As a pathological process, it is known that accumulation of undegraded substrates in lysosomes, due to a deficiency of lysosomal enzymes, impairs the fusion between autophagosomes and lysosomes. This is the molecular base of a set of pathological conditions, known as lysosomal storage diseases like Gaucher disease or Niemann pick disease [8].

To date, the autophagy pathway has never been studied in PLS. However, reduced activity of CatC could induce accumulation of undegraded substrates dysfunctional organelles. In the present study, we show an autophagic dysfunction in fibroblasts from PLS patients with accumulation of p62/SQSTM1 and visible autophagosome

accumulation by TEM (Transmision Electron Microscopy) proposing an impaired autophagic flux, which was confirmed by bafilomycin assay. An important increment of another cathepsin enzyme, cathepsin B (CatB), was observed and associated with lysosomal permeabilization. Treatment with recombinant CatC in the fibroblasts from PLS patients showed a partial recovery.

MATERIAL AND METHODS

Ethical Statements

Approval of the ethical committee of the University of Seville was obtained, according to the principles of the Declaration of Helsinki and all the International Conferences on Harmonization and Good Clinical Practice Guidelines. All the participants in the study gave their written informed consent before initiating it.

Reagents.

Trypsin and bafilomycin A1 were purchased from Sigma Chemical Co., (St. Louis, Missouri). Anti-GAPDH monoclonal antibody from Calbiochem-Merck Chemicals Ltd. (Nottingham, UK). Anti-NLRP3 antibody from Adipogen (San Diego, USA) from Santa Cruz Biotechnology. Anti-active caspase-1, LC3, ATG-12, p62, Lamp-I Cathepsin B and galectin-3 were obtained from Cell Signaling Technology. A cocktail of protease inhibitors (complete cocktail) was purchased from Boehringer Mannheim (Indianapolis, IN). Grace's insect medium was purchased from Gibco. The Immun Star HRP substrate kit was from Bio-Rad Laboratories Inc. (Hercules, CA).

Fibroblast cultures

Patients and control fibroblasts were obtained according to the Helsinki Declarations of 1964, as revised in 2001. Fibroblasts were cultured in high glucose DMEM (Dulbecco’s

modified media) (Gibco, Invitrogen, Eugene, OR, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Eugene, OR, USA) and antibiotics (Sigma Chemical Co., St. Louis, MO, USA). Cells were incubated at 37ºC in a 5% CO_2 atmosphere.

Structure of mutation analysis

The 3D structure of CatC (UniProt:P53634) was obtained from the PDB database using the identifier 2DJG, which has the native structure of the human cathepsin C at high resolution and include only a monomer of the molecule. The structure was visualized with the Rasmol program. The functional annotations of the protein, including the amino acids corresponding to the active site were obtained from the well-curated database UniProt.

Enzymatic activity of CatC

CatC activity was determined by measuring the amount of 7-amino-4-methyl coumarin (NHMec) released by hydrolysis of a specific substrate (glycyl-L-arginine-7-amido-4-methylcoumarin, Bachem, UK, www.bachem.com) on incubation with sonicated peripheral blood leukocytes, as described previously [9].

Antioxidant enzyme activity

Catalase activity was determined in cellular lysate by monitoring H_2O_2 decomposition at 240 nm [10]. SOD activity was determined on the basis of the inhibition of the formation of NADH−phenazine methosulfate-nitroblue tetrazolium formazan [11].

Western Blotting for mitochondrial protein

Whole cellular lysate from fibroblasts was prepared by gentle shaking with a buffer containing 0.9% NaCl, 20 mM Tris-ClH, pH 7.6, 0.1% triton X-100, 1 mM phenylmethylsulfonylfluoride and 0.01% leupeptine. The protein content was determined by the Bradford method. Electrophoresis was carried out in a 10–15%

acrylamide SDS/PAGE and proteins were transferred to Immobilon membranes (Amersham Pharmacia, Piscataway, NJ). Next, membranes were washed with PBS, blocked over night at 4°C and incubated with the respective primary antibody solution (1:1000). Membranes were then probed with their respective secondary antibody (1:2500). Immunolabeled proteins were detected using a chemiluminescence method (Immun Star HRP substrate kit, Bio-Rad Laboratories Inc., Hercules, CA). Western blot image was quantified using ImageJ software (see: http://rsb.info.nih.gov/ij/download.html).

Mitochondrial ROS production

Mitochondrial ROS generation in BMCs and fibroblasts was assessed by MitoSOX™ red, a red mitochondrial superoxide indicator. MitoSOX Red is a novel fluorogenic dye recently developed and validated for highly selective detection of superoxide in the mitochondria of live cells. MitoSOX™ Red reagent is live-cell permeant and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide and exhibits red fluorescence.

Flow cytometry. Approximately 1 x 10^6 cells were incubated with 1µM MitoSOX™ red for 30 min at 37°C, washed twice with PBS, resuspended in 500 µl of PBS and analyzed by flow cytometry in an Epics XL cytometer, Beckman Coulter, Brea, California, USA (excitation at 510 nm and fluorescence detection at 580 nm).

Intracellular ROS production

The intracellular ROS levels were determined using the probe CellROX® Orange reagent (Invitrogen™, Life Techonoliges, Milan, Italy) according to the manufacturer's instructions. Briefly, on the first day of the assay cells were seeded in a 6-well plate at a density of 1.5 x 10^5 cell/well and allowed to adhere for 16–18 h. The cells were treated

with CellROX® Orange Reagent which was added to 1 mL of complete medium at a 1:500 (v/v) dilution. Samples were incubated for 30 min at 37 °C, centrifuged at 320 x g once to remove medium and excess dye, and then resuspended in PBS. After labeling with CellROX® Orange Reagent, cells were analysed with the Tali® Image-Based cytometer (Invitrogen™, Life Techonoliges, Milan, Italy) collecting 20-fields per sample. Control cells were used to determine baseline levels of intracellular ROS and to set the fluorescence threshold for the Tali® instrument. Each treatment was carried out in three replicates and the final results were expressed as fold increase compared to control.

Oxygen consumption rate (OCR)

Oxygen consumption rate (OCR) was assessed in real-time using the Extracellular Flux Analyzer XF-24 (Seahorse Bioscience, North Billerica, MA, USA) according to the manufacturer's protocol, which allows to measure OCR changes after injecting up to four different compounds (stimulators, inhibitors or substrates) affecting bioenergetics. Cells were seeded at a density of 5 x 10^4 cell/well into the XF-24 cell culture microplate with the complete growth media and then incubated for 24 h. Before starting measurements, cells were placed in a running DMEM medium (supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium Pyruvate, and without serum) and pre-incubated for 20 min at 37°C in the absence of CO_2 in the XF Prep Station incubator (Seahorse Bioscience, Billerica MA, USA). Cells were transferred to the XF-24 Extracellular Flux Analyzer and after an OCR baseline measurement a profiling of mitochondrial function was performed by sequential injection of the following compounds : oligomycin (final concentration 2.5 µg/mL) at injection in port A, 2,4-dinitrophenol (2,4-DNP) (final concentration 1 mM) at injection in port B, and antimycin/rotenone (final concentration 10 µM/1µM) at injection in port C. A minimum

of five wells were utilized per condition in any given experiment. Data are expressed as pMol of O_2 consumed per minute normalized to 1000 cells (pMol O_2/1000 cells/min).

Electron microscopy

Fibroblasts were fixed for 15 min in the culture plates with 1.5% glutaraldehyde in culture medium, then for 30 min in 1.5% glutaraldehyde-0.1 M NaCacodylate/HCl, pH 7.4. They were then washed three times in 0.1 M NaCacodylate/HCl, pH 7.4 for 10 min and post-fixed with 1% OsO_4-H_2O, pH 7.4 for 30 min. After dehydration in increasing concentrations of ethanol, 5 min for each step: 50,70, 90 and three times 100%, impregnation steps and inclusion were performed in Epon and finally polymerized at 60°C for 48 h. 60–80 nm sections were obtained using an ultramicrotome Leica ultracut S (Leitz Microsystems, Wetzlar, Germany) and contrasted with uranyl acetate and lead citrate. Observations were performed on a Zeiss LEO 906 E (Oberkochen, Germany) transmission electron microscope.

Proliferation rate

Two hundred thousand fibroblasts were cultured in the absence or presence of rCatC (0.25µg) for 24, 48, 72, and 120h. After discharging supernatant with dead cells, cell counting was performed from 3 high power fields using an inverted microscope and a 40X objective.

Cathepsin B release

CatB redistribution from lysosomes/autophagolysosomes to the cytosol was assessed by immunofluorescence techniques using antibodies against CatB and LAMP-I as a marker of lysosomal/autophagolysosomal compartment. In control fibroblasts, CatB-specific immunostainings reveal cytoplasmic puncta structures that are surrounded by

lysosomal/autophagolysosomal membrane proteins such as LAMP-I. After lysosomal permeabilization, the immunofluorescence detection of CatB reveals a diffuse staining throughout the entire cell.

Galectin puncta

Lysosomal permeabilization was also detected by the galectin puncta assay as previously described [12].

Recombinant Cathepsin C production

Sequence was designed from the literature and an external service (GeneArt Invitrogen) was used for its synthesis inside a bacterial vector with ampicillin resistance gen. Construct codifying for recombinant CatC was synthesized and cloned into a pBAC4x baculovirus transfer vector under the control of the polyhedrin promoter. Sf21 cell line was co-transfected using a linearized modified Autographa californica nuclear polyhedrosis virus DNA and the created transfer plasmid was transferred by a lipofectamin-mediated method as specified by the manufacturer (Invitrogen™). The Sf21 cells were sown in 6 wells-plates (1×106 cells/plate). After attachment, the cells were infected with the recombinant baculovirus at MOI of 5 for 72 h at 27 °C in darkness. Based on titration of virus obtained by quantitative PCR and the amount of virus required, the volumes were calculated to infect cells. The infected cells and culture supernatant were harvested and separated by centrifugation at 800×g for 5min at 4ºC. Cell pellet was homogenized in PBS 1X and protease inhibitor, 1 mM PMSF, then sonicated with an ultrasonic processor Sonopuls HD2070 (Bandelin) for 3×10 s at 30% potency. Once the clarified fractions were obtained by centrifugation with recombinant proteins, these were used in cell cultures assays.

Statistical Analysis

Data in the figures is given as mean ± SD. Data between different groups were analyzed statistically by using ANOVA on Ranks with Sigma Plot and Sigma Stat statistical software (SPSS for Windows, 19, 2010, SPSS Inc. Chicago, IL, USA). For cell-culture studies, Student's t test was used for data analyses. A value of $p<0.05$ was considered significant.

Results

Genetic characterization shows homozygous and heterozygous mutations in CatC with clinical phenotype

Three patients were selected for this study. Patient one was a 31-year-old man with hyperkeratosis in the palmoplantar region and on his hands and moderate periodontitis (**Figure 1A**). After genetic characterization, the patient was a compound heterozygote for two nonsense CTSC mutations (c.96T>G and c401G>A, numbered according to reference cDNA sequence NM_001814.4). The first mutation has been previously described [13] and the second is a novel mutation. Both genetic changes were observed in the mother and father respectively without phenotypic manifestation, while the brother did not show any mutation. Patient 2 was a 21-year-old woman with hyperkeratosis in the palmoplantar region and on her joints and moderate periodontitis (**Figure 1B**). After genetic characterization, the patient was a compound homozygote for one nonsense CTSC mutation, c.1286G>A, previously described [14]. This genetic change was observed in the mother and father in heterozigosis without phenotypic manifestation, while the brother did not show any mutation. Patient 3 was a XX-year-old woman previously described (patient L in Table 2, reference 2) with hyperkeratosis in the palmoplantar region elbows and hands, and suffering from severe periodontitis that led to edentulism.

The human CatC sequence is processed into a mature form and it produces a trimer consisting of the exclusion domain, heavy chain and light chain, which are bound by disulfide bonds. The active enzyme is composed by a tetramer of these heterotrimers (**Figure 1D, WT**). Evaluation of the effect of the amino acid changes on the protein structure of CatC showed the region colored in grey corresponding to the lost sequence of the one allele. In patient 1, one of the cysteine of the disulfide bond is lost, and it could affect the 3D structure of the exclusion domain with important consequences on the function (**Figure 1D, P1**). In patient 2, the amino acids in the active site are all conserved, not affecting the active site of the enzyme, but probably influencing the structure, by the folding adopted by the light and heavy chains inducing a possible effect in the function (**Figure 1D, P2**). In patient 3, only one amino acid in the active site is conserved with a possible more affected function (**Figure 1D, P3**). Accordingly, enzymatic activity of CatC was observed reduced in all patients (**Figure 1E**).

Fibroblasts from PLS patients show metabolic alterations

Because dermatological symptoms are an important clinical manifestation of PLS, we evaluated the metabolic status of skin fibroblasts from PLS patients. Fibroblasts from patients presented a reduced growth ratio and abnormal morphology (**Figure 2A and B**). We also investigated the mitochondrial functionality on fibroblasts from PLS patients by measuring the OCR values in control and PLS fibroblasts, exposed sequentially to four modulators of oxidative phosphorylation (OXPHOS): oligomycin (an inhibitor of F_1F_o-ATPase or complex V), 2,4-DNP (uncoupling of the OXPHOS electron transport chain) and antimycin/rotenone (complex I and III inhibitors respectively) (**Figure 2C**). The basal OCR was markedly affected in fibroblasts from PLS patients compared to controls (**Figure 2C**). The spare respiratory capacity (SRC) of cells -an indicator of how close a cell is operating to its bioenergetics limit- was

obtained by calculating the mean of OCR values after injection of 2,4-DNP minus the basal respiration. Fibroblasts from PLS patients showed a significant decrease of SRC compared to control cells (**Figure 2D**). These metabolic alterations were accompanied by increased oxidative stress characterized by high levels of reactive oxygen species (ROS) and mitochondrial superoxide production and reduced activity of the antioxidant enzymes, superoxide dismutase and catalase (**Figure S1A-D**).

Autophagic flux is impaired in PLS

Degradative substrates are cleared intracellularly by a complex macro-autophagic response [15]. By Western blot and mRNA expression, levels of ATG12 and the autophagy marker LC3-II were markedly increased in fibroblasts from PLS (**Figure 3A-B**). Furthermore, PLS showed an increment in p62/SQSTM1 accumulation, an established marker of autophagic clearance (**Figure 3A**). Next, we performed autophagic flux analysis using the Bafilomycin A1 (BafA1), a specific inhibitor of vacuolar H^+-ATPases and a blocker of autophagosome-lysosome fusion, to check for autophagosome/autophagolysosomal formation. As expected, BafA1 treatment in control cells led to a significant increase in the amount of LC3-II, suggesting that autophagic flux is normal (**Figure 3C**). However BafA1 treatment in PLS fibroblasts had a reduced effect on LC3-II expression levels (**Figure 3C**), indicating that autophagic flux was impaired (**Figure 3C**). Electron microscopy analysis of fibroblasts from the patients revealed abundant multilamellar bodies and increased autophagosome number as indicated by the accumulation of double-membrane vesicle structures (**Figure 4**).

Lysosomal permeability is associated to PLS

Degradation of autophagic substrates/autophagosome takes place in the lysosomal compartment by acidic proteases, such as cathepsins. Reduced autophagic flux with the consequent autophagosome accumulation can be a consequence of reduced autophagosome–lysosome fusion or inefficient lysosomal degradation [16]. According to this, we determined whether the impaired autophagic flux is associated to lysosomal dysfunction. Because the cysteine Cats family includes CatB, C, H, K, and L [17], we analyzed the level of other important Cat, CatB. Surprisingly, CatB protein levels were increased in fibroblasts from patients with PLS (**Figure 5A**). Thus, we hypothesized that lysosomal protease CatB may play an important role in the pathophysiology of PLS through its release from the lysosomal/autophagolysosomal compartment to the cytosol. In healthy fibroblasts, CatB signal colocalized with the lysosomal/autophagolysosomal marker LAMP-1 indicating that it was located within lysosomes/autophagolysosomes (**Figure 5B**). However, in PLS fibroblasts, the CatB signal was diffuse through the cytosol and it did not colocalize completely with the LAMP-I marker suggesting lysosome/autophagolysosome membrane permeabilization (LMP) (**Figure 5B**). This LMP was also confirmed by the detection of galectin puncta at leaky autophagolysosomes (**Figure 6**) as it has been described that galectin translocation to phagosomal and lysosomal membranes is a marker of vacuole lysis and/or permeabilization [18]. Interestingly, this CatB release and lysosomal alteration were accompanied by NLRP3-inflammasome activation (**Figure 5C**).

Recombinant CatC improves growth and induces partial restoration of autophagic flux in fibroblasts

Because mutation in patients induces an important reduction of the enzymatic activity of CatC, which is associated to an impairment of autophagic flux and lysosomal dysfunction, we tested the effect of a recombinant CatC (rCatC) in fibroblasts from

patients with PLS. First, the rCatC was produced by baculovirus system in insect cell cultures according to the methods described in Material and methods section [19]. Three extracts were isolated and tested, supernatant and cellular extracts: insoluble and soluble extracts. Soluble extract showed more enzyme concentration (tested by Dot Blot) of rCatC (Figure S2). Immunoblot analysis of the purified rCatC revealed three polypeptides with molecular masses of approximate 55 kDa, 25 kDa, and 7.8 kDa (Figure S3). The amount of 55 kDa polypeptides was much larger than that of the other two. Flatworm CatC expressed in insect cells also exhibited similar three bands of 55 kDa, 25 kDa, and 7.8 kDa polypeptides after purification. Enzymatic activity assay showed increased enzymatic activity in the soluble extract (Figure S4).

After comparing three different doses, rCatC treatment in fibroblasts from a representative patient showed increased ratio of cell growth in low doses of 0.25µg. However, control fibroblasts showed and important growth decrement in a dose-dependent manner (**Figure 7A-C**). Enzymatic activity of CatC was observed to be partially recovered in the fibroblasts from the patient after 120h of rCatC treatment (**Figure 7D**). By Western blot analysis, levels of the autophagy marker LC3-II were markedly increased in fibroblasts from control fibroblasts after rCatC treatment. No significant changes concerning LC3-II were found in fibroblasts from PLS, taking into account the increased basal levels of the patient (**Figure 7E**). Furthermore, the increased level of p62/SQSTM1 accumulation in PLS were recovered by the rCatC treatment after 120h showing an autophagic clearance (**Figure 7E**).

rCatC improves lysosomal permeability associated to PLS

Because we previously showed lysosomal permeability associated to the pathophysiology of the fibroblasts from PLS, the potential effect of the rCatC on this

aspect was also explored. The increased levels of CatB protein previously observed were decreased in fibroblasts from the patients with PLS after rCatC treatment (**Figure 7E**). Thus, partial co-colocalization of CatB signal with the LAMP-1 marker suggested a reduction of LMP (**Figure S5**). This reduction of the LMP, was also confirmed after no detection of galectin puncta at leaky autophagolysosomes (**Figure 8**).

Discussion

The dearth of knowledge about the molecular bases of PLS leads to a loss of therapeutic tools. Previous works have been oriented to the description of new mutations and several pathophysiological characteristics such as oxidative stress, neutrophilic function impairment without deepening in molecular mechanisms. The present report provides experimental evidence about metabolic and autophagic impairment in CatC-deficient fibroblasts from PLS patients. First, we assessed metabolic changes in cultured fibroblasts. As expected, a reduction of mitochondrial metabolic rate was observed. This entailed a significant reduction in cell growth. Then, we provided experimental evidence for the activation of autophagy in CatC-deficient fibroblasts. From an ultrastructural and functional point of view, autophagy is characterized by the formation of double-membraned vesicles called autophagosomes. Autophagic process is initiated by the formation of a phagophore or nucleation membrane to which cytoplasmic content is targeted. This phagophore is elongated and results in a new autophagosome which engulfs material marked for degradation. Finally, mature autophagosomes fuses with lysosomes, resulting in autophagolysosomes, and the content is degraded via catalytic enzymes [15]. Enhanced autophagy may be due to either increased autophagosome formation or impaired clearance of the autophagosomes. Because the autophagosome is an intermediate structure, the number of autophagosomes observed at any specific time point is a result of the balance between the rate of their generation and the rate of their

conversion into autophagolysosomes. As LC3-II overexpression can evoke either an increment of autophagy or an impairment of the autophagic flux, these LC3-II changes must be interpreted together with the p62/SQSTM1 levels [20]. We observed an impaired autophagic flux determined by expression of LC3-II and increased expression of proteins involved in clearance pathways like p62/SQSTM1 and confirmed by BafA1 assay and TEM. Interestingly, this blockade of autophagosome fusion with lysosomes or autophagic flux occurs in multiple lysosomal diseases like Niemann pick, Gaucher and Pompe diseases [21-23].

Oxidative stress has been associated to the pathophysiology of PLS [2] and we observed increased ROS production accompanied by reduced antioxidant defenses. Oxidative stress is associated to the induction of lysosomal instability and membrane permeabilization [24]. Previously, reduction in some cysteine Cats family members like CatL have been related to upregulate the CatB and with autophagy dysfunction [25] in a compensatory transcriptional upregulation with increased enzymatic activation of CatB. Furthermore, in a lysosomal disease such as Niemann pick disease, the expression of mature protein forms for both CatB and CatD are upregulated with accumulation of autophagosomes [26]. Our findings show reduced enzymatic activity of CatC with a compensatory upregulation of mature CatB proteins leading to a lysosomal permeabilization. All these disturbances on the machinery of autophagy and lysosome metabolism could explain several of the clinical phenotype characteristics of PLS. Autophagy is a process not only involved in the degradation and recycling of macromolecules including proteins, lipids and carbohydrates, for the synthesis of essential components and as an energy supply, but also in the innate and adaptive immunity against microbial invasion [7]. Accordingly, it is not surprising that defects in the autophagy process have been linked to a wide range of diseases increasing

susceptibility to infections [7]. Thus, many patients with PLS show increased susceptibility to infections and periodontal disease [2], which probably could be associated with autophagy dysfunction. Autophagy is also involved in the homeostasis of the skin, which is the first line of defense against many different insults. So, autophagy might have a critical role in the development and progression of skin diseases. However, the exact mechanism by which autophagy dysfunction could be involved in the development of skin diseases is still unknown. Nevertheless, several skin diseases like vitiligo, psoriasis, hyperpigmentation or the skin alteration from systemic lupus erythematosus, have been related autophagy defects [27] which might lead to inflammatory cytokine production mediated by p62/SQSTM1, as reported for psoriatic skin [28]. Accordingly, in the present study a similar autophagy dysfunction with p62/SQSTM1 in PLS was found, which has been associated with increased production of inflammatory cytokines [29]. Furthermore, the compensatory upregulation of mature CatB found here was accompanied by a NLRP3-inflammasome activation. NLRP3-inflammasome complex is a molecular platform which activates innate immune defenses through the maturation of proinflammatory cytokines (interleukins IL-1β and IL-18). This complex is activated by a wide variety of danger signals such as potassium efflux out of the cell, mitochondrial ROS, translocation of NLRP3 to the mitochondria, cardiolipin, or the release of cathepsins, like CatB, into the cytosol after lysosomal destabilization [30]. So, inflammatory characteristics of PLS could be explained by NLRP3-inflammasome activation.

Finally, the treatment and management of PLS is poor and is limited to oral approach and dermatological damage treatment with emollients, salicylic acid and topical steroids, with toxic effects in many cases [31]. Therefore, it is important to have effective pharmacological treatments for PLS based on the molecular etiology of the disease.

Accordingly, we designed a strategy to correct several pathophysiological markers shown in this study. Enzyme replacement therapies have been proposed as an interesting approach in diseases based on lysosomal alterations [32]. Thus, we treated fibroblasts from PLS patients with a rCatC isolated from insect cells. Autophagy dysfunction was corrected with a reduction of lysosomal permeabilization and partial reduction of CatB. All these changes led to an improvement in cellular growth.

To the best of our knowledge, this study shows for the first time a new molecular basis of the PLS and new pharmacological targets. Furthermore, the use of recombinant CatC presents an interesting therapeutic approach which will need new studies before being considered as a treatment in humans. New research lines will be needed to establish putative therapeutic uses of recombinant CatC by oral or topic administration.

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Author Disclosure Statement

All the Authors declare that no conflict of interest exists for any of them.

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Figure legends

Figure 1. A-C. Pedigree analysis and clinical characteristic of the patients. Black or gray symbols denote people with different mutations. Symbols with two colors denote two heteroplasmic mutations. Squares: man, circles: woman. **D.** 3D-structure of a cathepsin C monomer. The protein is processed into a proteolytically mature active enzyme consisting of 3 chains: an exclusion domain (flesh color), a heavy chain (green color) and a light chain (violet color). WT: Canonical structure highlighting a disulfid bond keeping the folding of the exclusion domain, and the three amino acids of the active site (pink color). P1: Patient 1. The sort sequence colored in orange corresponds to one allele, and the region colored in grey corresponds to the lost sequence of the other allele. Note that one of the cysteines of the disulfide bond is lost, and it could affect the 3D structure of the exclusion domain. P2: Patient 2. The region colored in grey corresponds to the lost sequence in the mutant. Note that the amino acids in the active site are all conserved. P3: Patient 3. The region colored in grey corresponds to the lost sequence in the mutant. Note that only one amino acid in the active site is conserved. **E.** Enzymatic activity of CatC in homogenate from skin fibroblasts. Data represent the mean – SD of three separate experiments. ***$p < 0.001$ between controls and PLS patients.

Figure 2.- Abnormalities in various aspects of bioenergetic function. **A.** Morphological changes of fibroblasts from patients compared with control. **B.** Cell growth determined in healthy and PLS fibroblasts. **C.** Oxygen consumption rate (OCR) in cells from control and PLS patients. OCR was monitored using the Seahorse XF-24 Extracellular Flux Analyzer with the sequential injection of oligomycin (2.5μg/mL), 2,4-DNP (1mM), antimycin (10μM)/rotenone (1μM) at the indicated time point. **D.** The spare respiratory capacity (SRC) of FM fibroblasts showed a significant decrease compared to

control fibroblasts. Data represent the mean±SD of three separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{**}P < 0.001$ between control and PLS patients.

Figure 3.- A. Autophagic protein expression levels of ATG12, LC3-I (top panels, top band), LC3-II (top panels, bottom band), and p62 were determined in control and PLS fibroblast cultures by Western blot analysis, as described in Materials and Methods. ATG12 band represents the Atg12-Atg5 conjugated form. GAPDH was used as a loading control. **B.** Impaired autophagic flux in PLS fibroblasts. Determination of LC3-II in the presence and absence of bafilomycin A1 in control (CTL) and PLS fibroblasts (patients). Control and PLS fibroblasts were incubated with bafilomycin A1 (100 nM for 12 h). Total cellular extracts were analyzed by immunoblotting with antibodies against LC3. GAPDH was used as a loading control. For control cells, data are for experiments on 2 different control cell lines.

Figure 4.- Ultrastructure of PLS fibroblasts. A. Control fibroblasts showing mitochondria with typical ultrastructure. **B.** Laminar bodies (black arrows) and autophagosome (white arrows) present in PLS fibroblasts. Scale bar 10 µm (low magnification) and 2 µm (high magnification).

Figure 5.- Cathepsin B relases in PLS fibroblasts. A. Protein expression levels of CatB B were determined in control and PLS fibroblast cultures by Western blotting, as described in Materials and Methods. **B.** Immunofluorescence of CatB in control and pathological cells. Note that in PLS fibroblasts CatB diffuses throughout the cytosol. **C.** NLRP3-inflammasome protein expression levels of NLRP3 (top band) and Caspase 1 (intermediate bands), were determined in control and PLS fibroblast cultures by Western blot analysis, as described in Materials and Methods. GAPDH was used as a loading control.

Figure 6.- Representative fluorescence images of fibroblasts from control and PLS. Cells were fixed and stained with anti-Galectin-3 antibodies (green) and anti-LAMP-I (red). Nuclei were stained with Hoechst 33342 (blue). Increased Galectin-3- puncta and colocalization of Galectin-3 and LAMP-I puncta are shown in patients.

Figure 7.- A-C. Cell growth with rCatC determined in healthy and representative PLS fibroblasts. **D.** Enzymatic activity of CatC in homogenate from skin fibroblasts after 120h of rCatC treatment. Data represent the mean – SD of three separate experiments. *$p < 0.01$ between controls and control treated with rCatC; $^{a}p < 0.01$ between PLS and PLS treated with rCatC. **E.** Autophagic protein expression levels of LC3 and p62, CatB were determined in control and representative PLS fibroblast cultures after rCatC treatment by Western blot analysis, as described in Materials and Methods. GAPDH was used as a loading control. For control cells, data are for experiments on 2 different control cell lines.

Figure 8.- Representative fluorescence images of fibroblasts from control and PLS to evaluate the effect of the rCatC in lysosomal permeabilization. Cells were fixed and stained with anti-Galectin-3 antibodies (green) and anti-LAMP-I (red). Nuclei were stained with Hoechst 33342 (blue). Increased Galectin-3- puncta and colocalization of Galectin-3 and LAMP-I puncta are shown in patients.

SUPPLEMENTARY DATA

Autophagic dysfunction in Papillon Lefèvre is restored by recombinant Cathepsin C treatment

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Running Title: Autophagic dysfunction in Papillon Lefèvre

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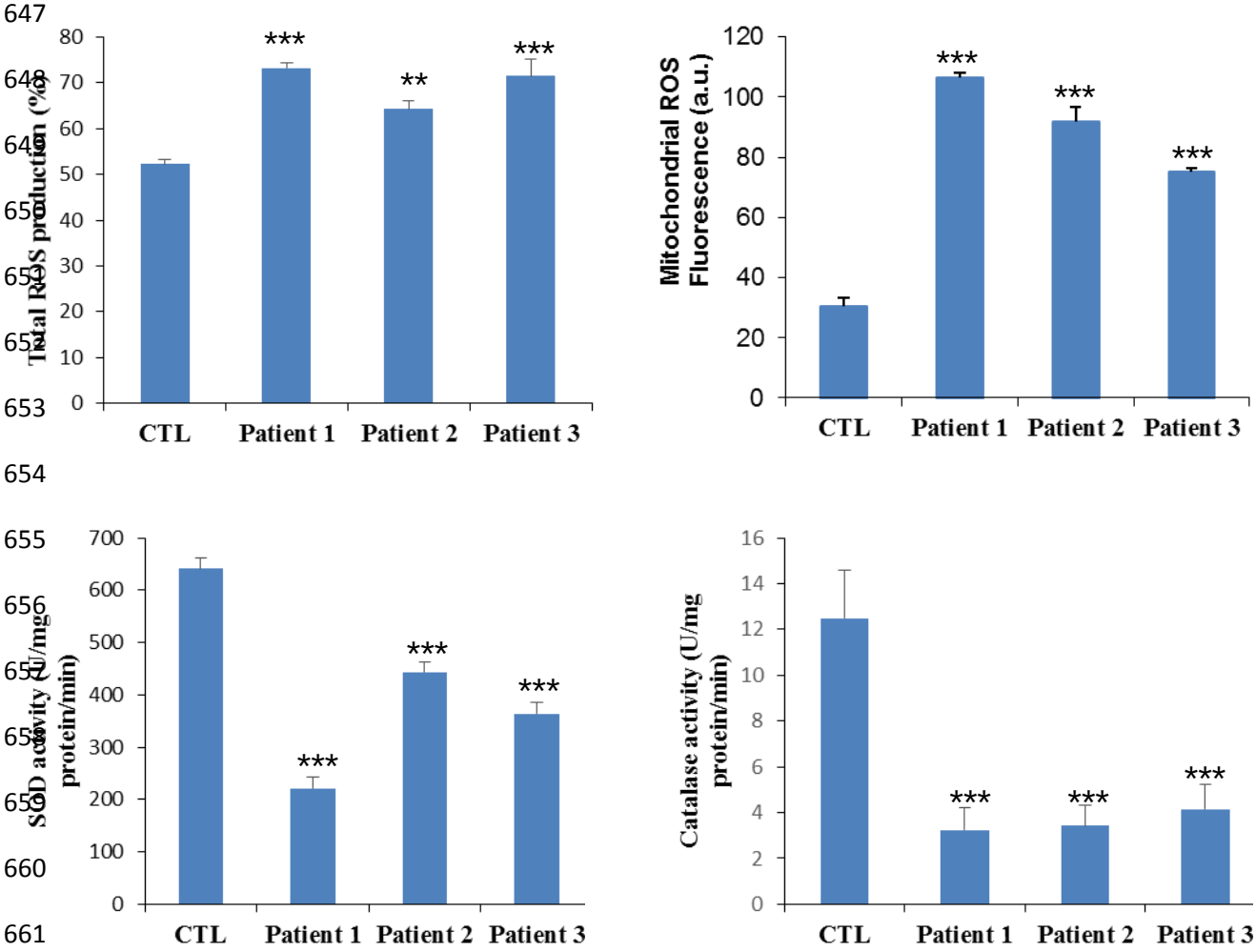
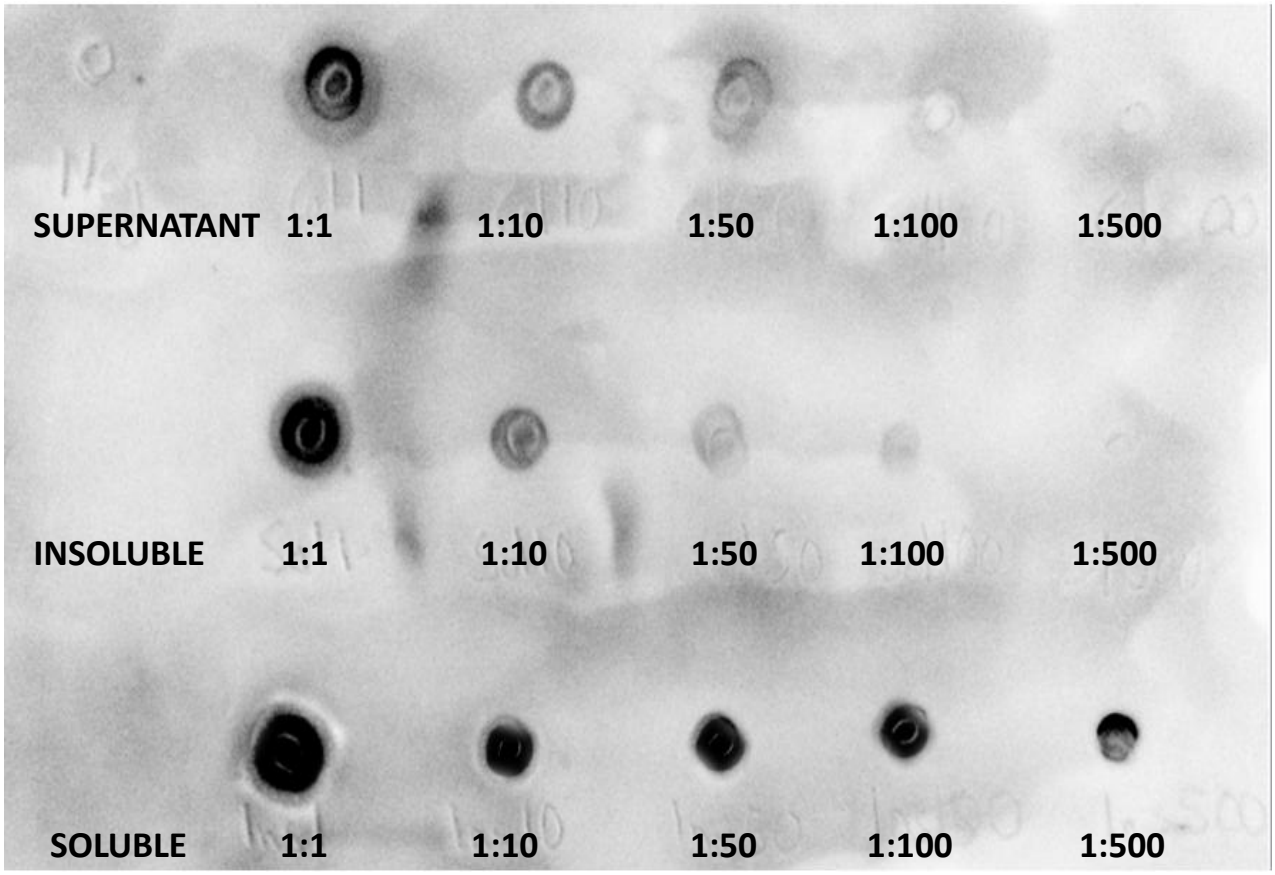
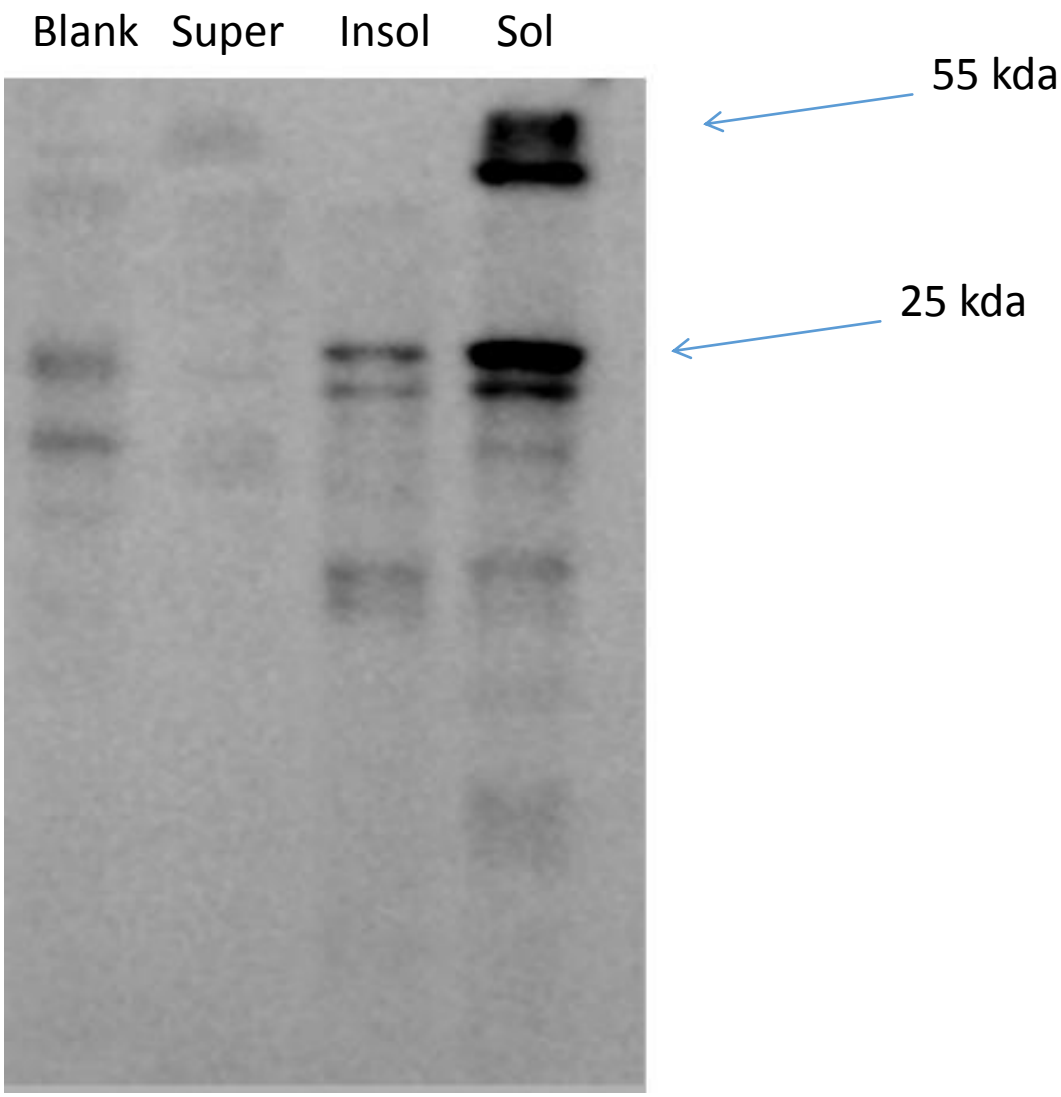


Supplementary figure 1. Oxidative stress and oxidative damage levels in fibroblasts from PLS patients. (A and B) Total ROS and mitochondrial ROS production were analyzed in fibroblasts from control and PLS patients by flow cytometry as described in Material and Methods. (C and D) Antioxidant enzymes SOD and catalase (CAT) activities were also analyzed in fibroblasts from control and PLS patients as described in Material and Methods. Data represent the mean±SD of three separate experiments.*P < 0.05, **P < 0.01 and ***P < 0.001 between control and PLS patients.

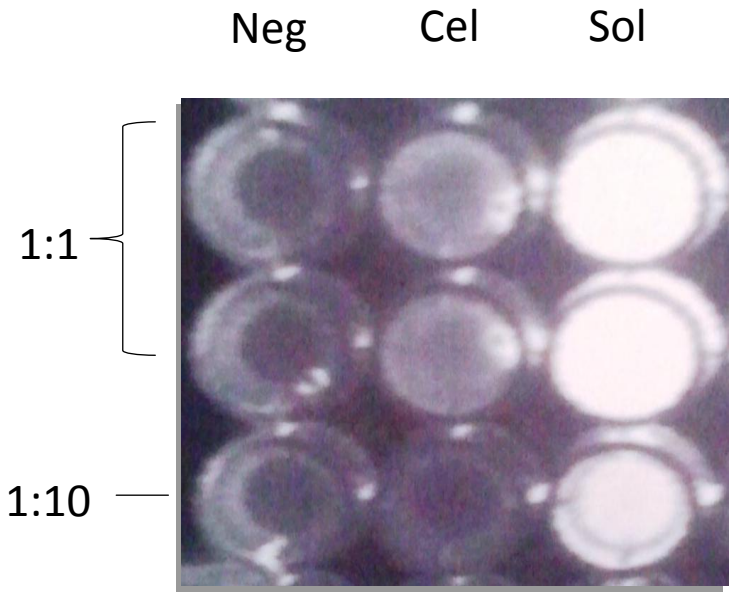


Supplementary figure 2. Detection of CatC protein in theof three different extracts (cell extract and the soluble and insoluble extract) determined by Dot Blot.

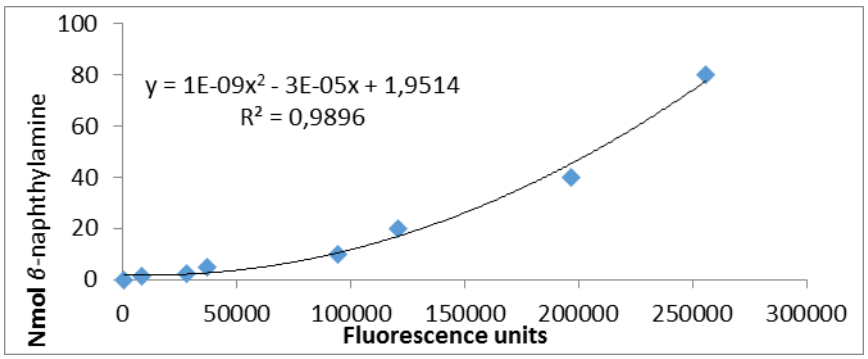


Supplementary figure 3. Immunoblot analysis of the insoluble extract revealed three polypeptides with molecular masses of approximate 55 kDa, 25 kDa, and 7.8 kDa. Super: supernatant, Insol: insoluble, Sol: soluble.

A

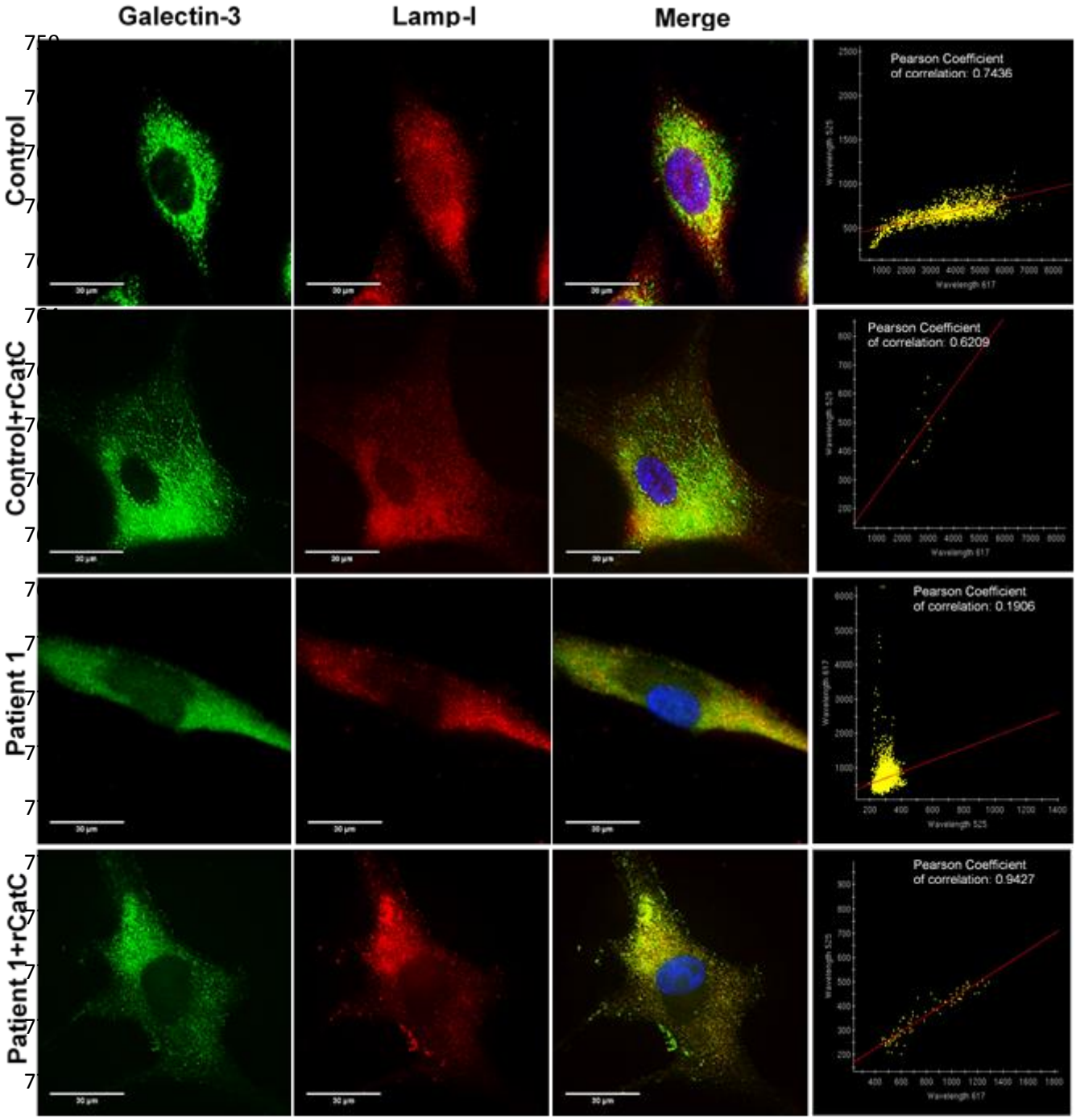


B



	Negative	Cell	Soluble
Mean	17810	50023,5	186078,5
SD	6940	9337	82154

Supplementary figure 4. Enzymatic activity of celular and soluble extracts.



Supplementary figure 5. Representative fluorescence images of fibroblasts from control and PLS with and without rCatC. Cells were fixed and stained with anti-Galectin-3 antibodies (green) and anti-LAMP-I (red). Nuclei were stained with Hoechst 33342 (blue). Increased Galectin-3- puncta and colocalization of Galectin-3 and LAMP-I puncta are shown in patients.

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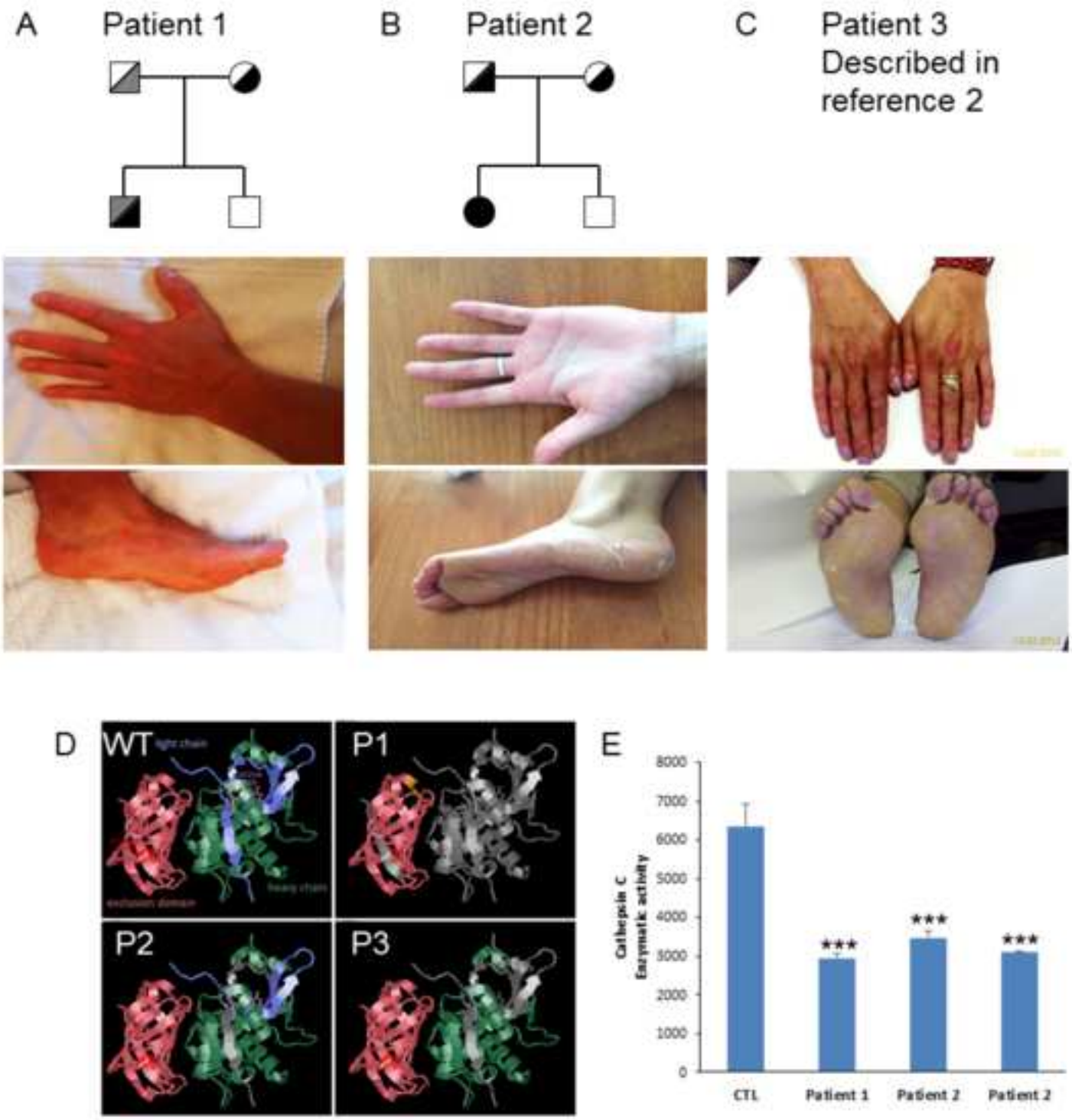

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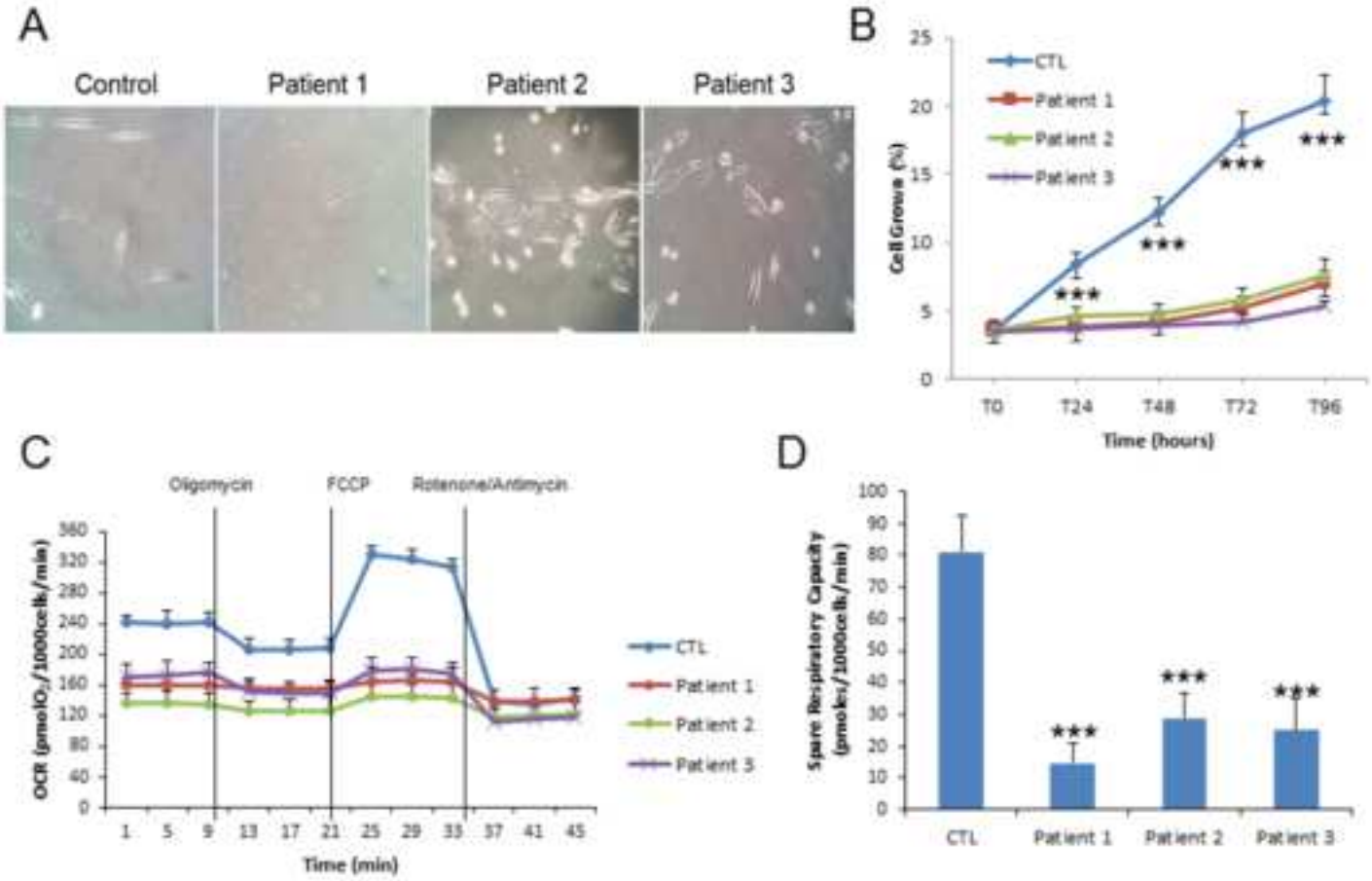

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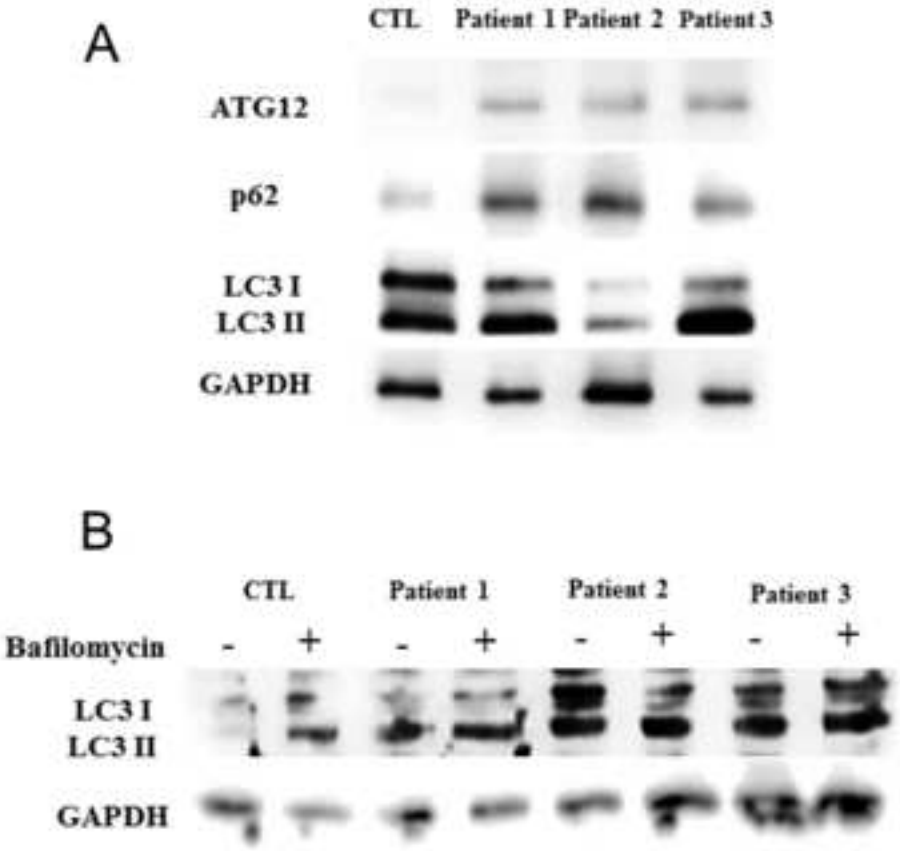

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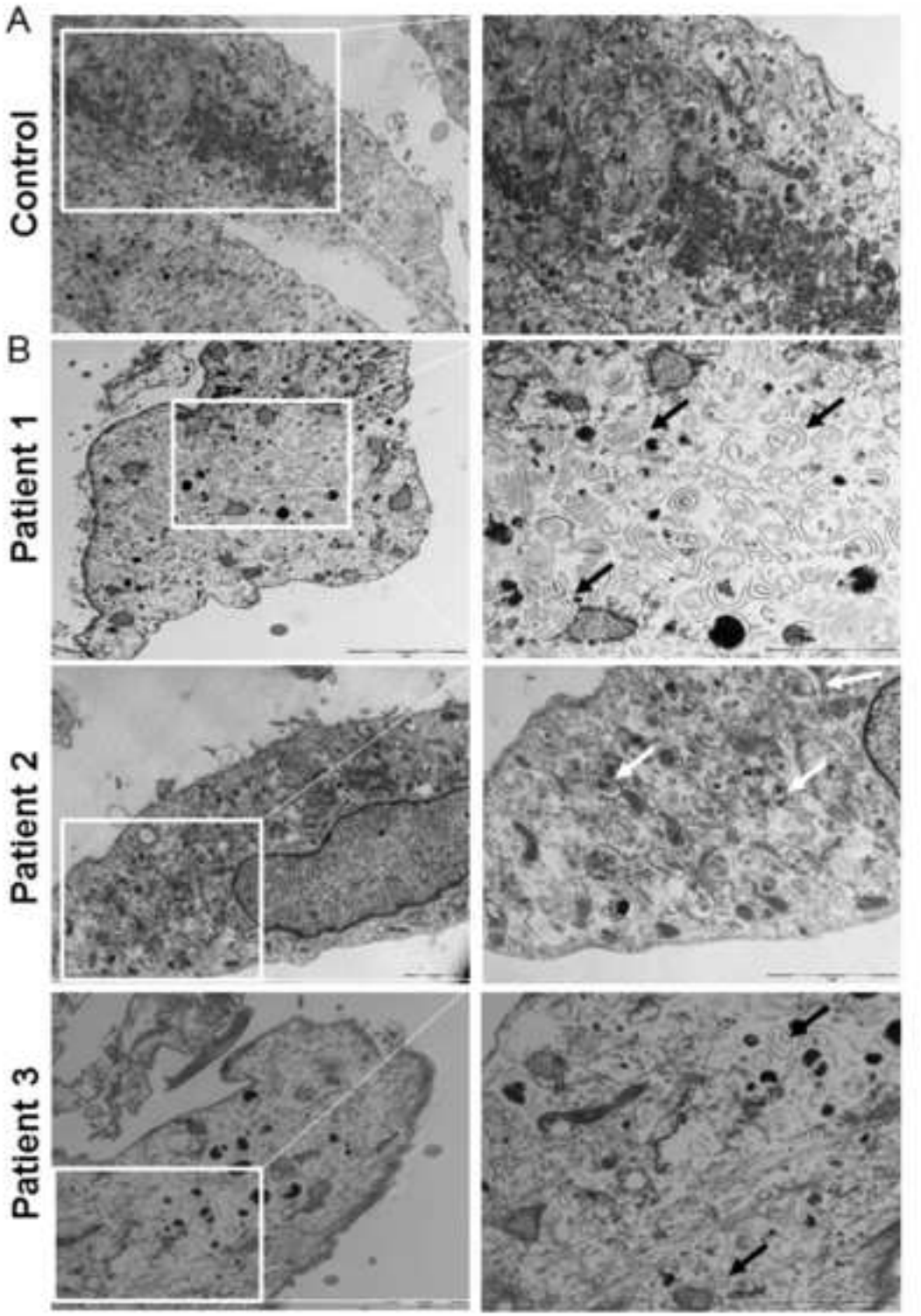

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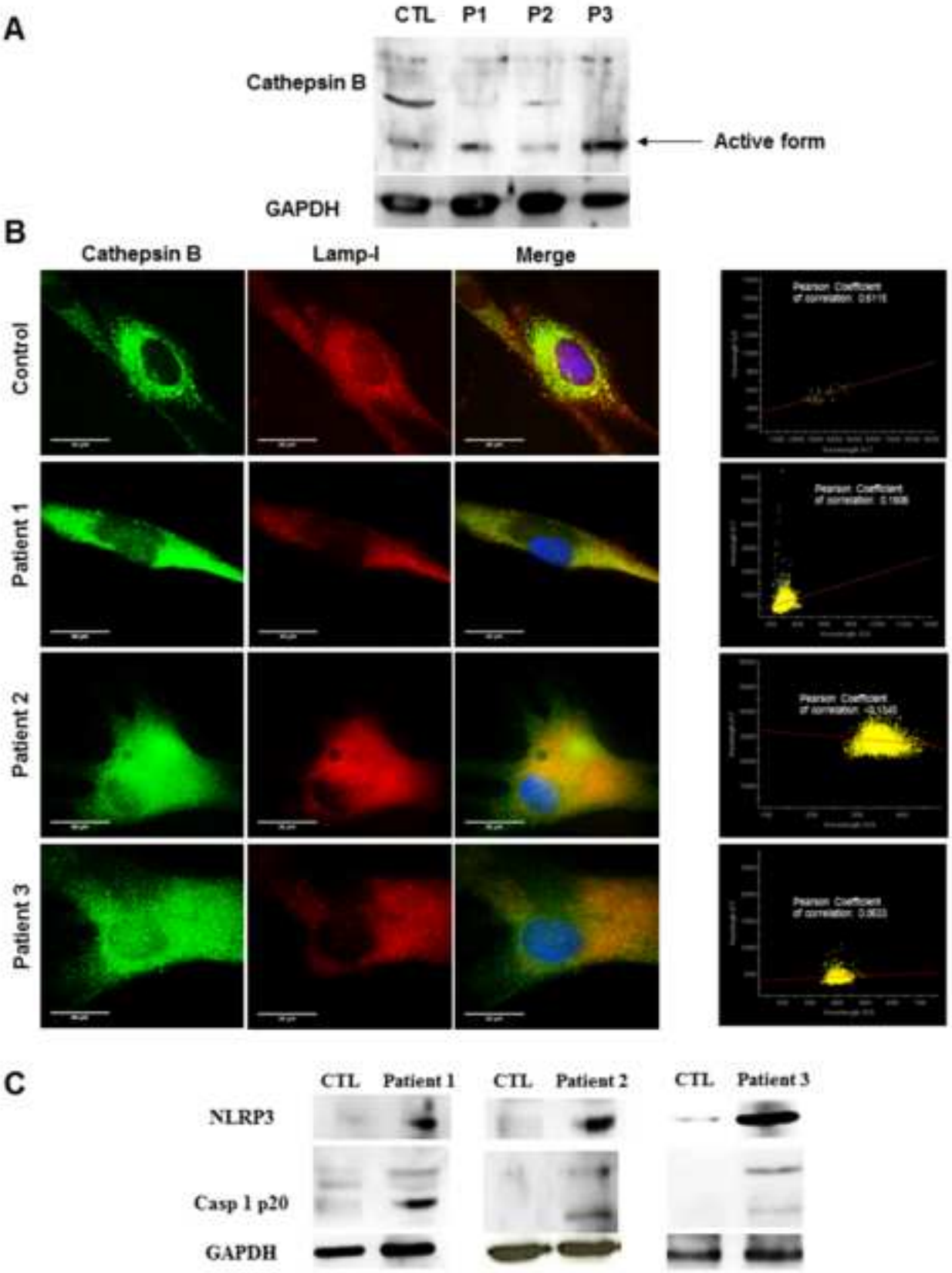

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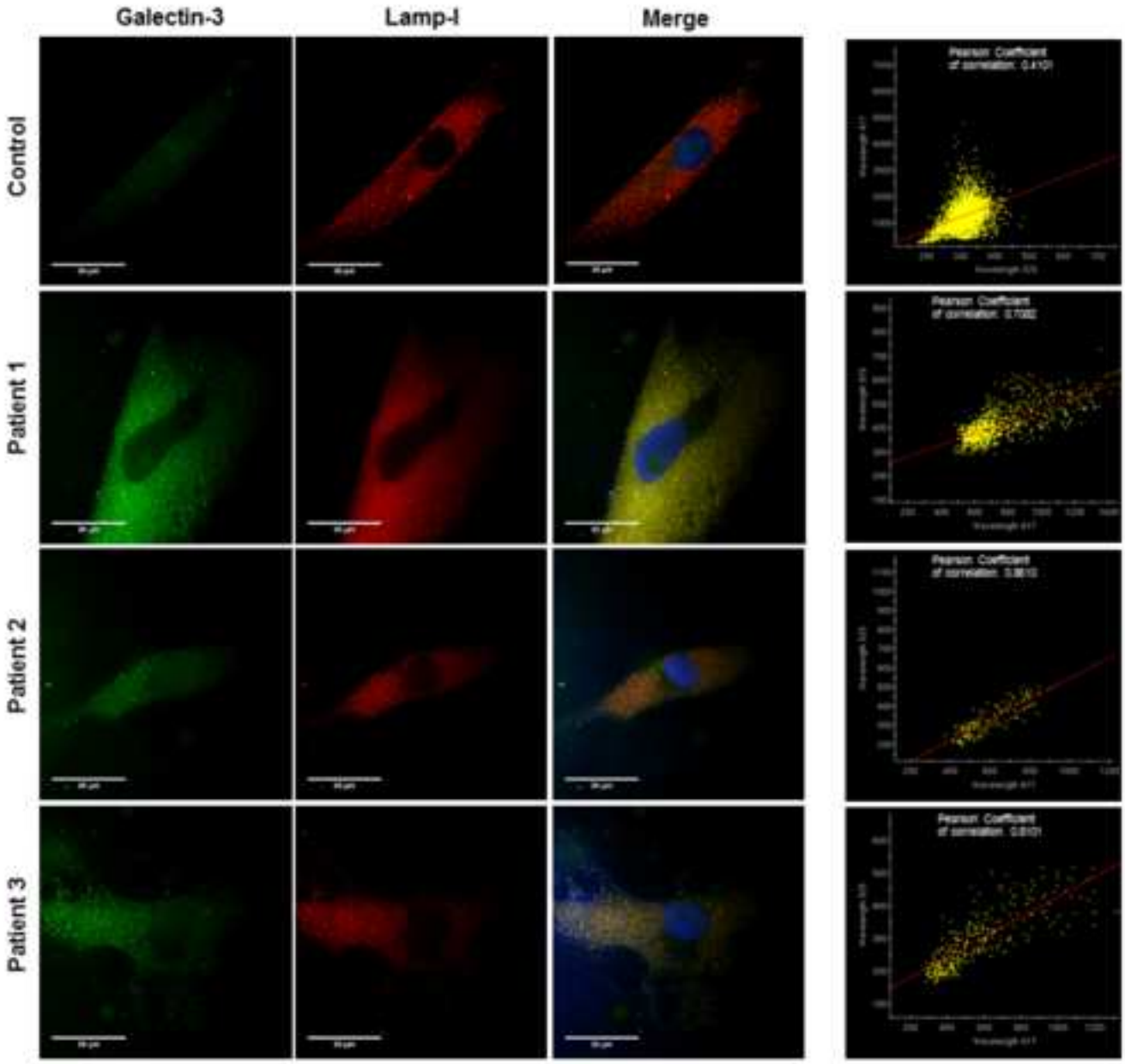

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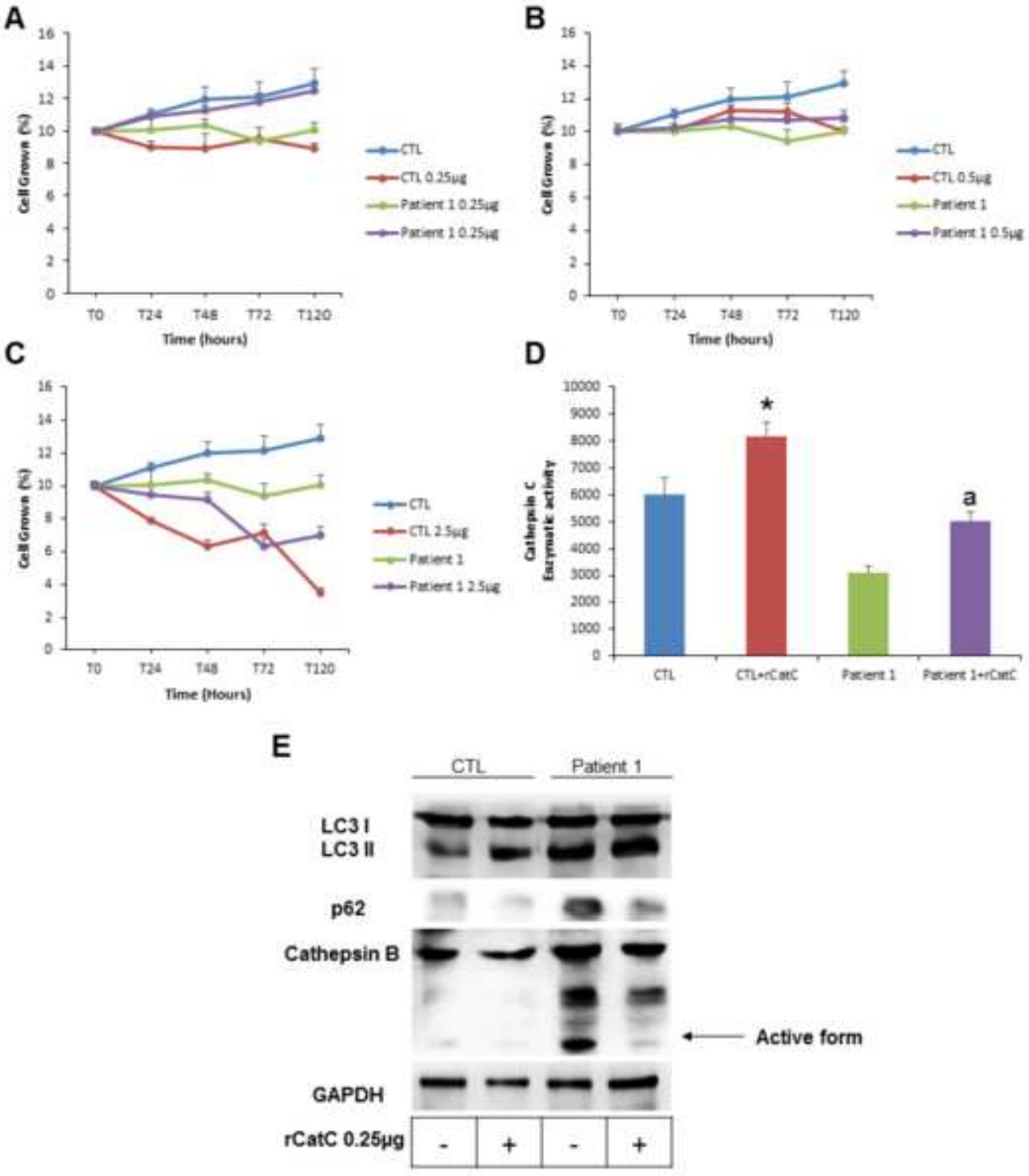

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