Analysis of urinary cathepsin C for diagnosing Papillon-Lefèvre syndrome

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Abbreviations: CatC, cathepsin C; PLS, Papillon-Lefèvre syndrome, APN, aminopeptidase N; AMC, aminomethylcoumarin

Keywords: cathepsin C, Papillon-Lefèvre syndrome, protease, urine analysis, diagnostic method

Abstract

Papillon-Lefèvre syndrome (PLS; OMIM: 245000) is a rare disease characterized by severe periodontitis and palmoplantar keratoderma. It is caused by mutations in both alleles of the cathepsin C (CatC) gene *CTSC* which completely abrogate the proteolytic activity of this cysteine proteinase. A genetic analysis most often is unaffordable or unavailable to establish an early rapid diagnosis of PLS. In this study, we tested the hypothesis that active CatC is constitutively excreted and can be easily traced in the urine of normal subjects. If true, its absence in the urine of patients would be an early, simple, reliable, low cost and easy diagnostic technique. All 75 urine samples from healthy control subjects (aged 3 months to 80 years) contained proteolytically active CatC and its proform as revealed by kinetic analysis and immunochemical detection. From the urine samples of 31 patients with a PLS phenotype, 29 contained neither proteolytically active CatC nor the CatC antigen so that the PLS diagnosis was confirmed. CatC was detected in the urine of the other two patients and genetic analysis revealed no loss-of-function mutation in *CTSC* indicating that they suffer from a PLS-like condition but not from PLS. Screening the absence of urinary CatC activity soon after birth and early treatment before the onset of PLS manifestations will help to prevent aggressive periodontitis and loss of many teeth and should considerably improve the quality of life of PLS patients.

INTRODUCTION

Papillon-Lefèvre syndrome (PLS, OMIM: 245000) is a rare inherited autosomal recessive disorder characterized by palmoplantar hyperkeratosis and an early and severe periodontitis, causing loss of both the primary and permanent teeth [1-3]. The prevalence of PLS is 1 to 4 cases per million people and the carrier rate is 2 to 4 per 1000 [4]. There is no gender predilection but parental consanguinity has been reported in more than 50% of cases [4, 5]. Palmoplantar keratoderma may be visible at birth or 1 to 2 months thereafter, but generally, keratoderma and periodontitis develop in parallel between the 6th month and the 4th year of life of the patient often beginning with the eruption of the first teeth [6]. Other features include intellectual disability, intracranial calcifications, recurrent skin infections, hyperhidrosis and liver or cerebral abscesses [5, 7, 8].

The disease-causing gene *CTSC,* encoding cathepsin C (CatC) [1, 3], is located on chromosome 11q14-21[9-11]. To date, 75 mutations have been reported in PLS patients, of which 68% were homozygous. Fifty percent of homozygous mutations were missense, 25% nonsense, 23% frameshift, and 2% were other types of mutations [12]. In addition to the classic form of PLS, 6 cases with late-onset periodontal disease and/or late-onset palmoplantar-lesions were reported [13]. Haim-Monk syndrome, allelic to PLS has been described in a Jewish community in India, with arachnodactyly, acroosteolysis and onychogryphosis as additional features [14]. The diagnosis of PLS is based on clinical signs and is generally confirmed by *CTSC* sequencing. This latter procedure, however, has several drawbacks: high costs in contrast to the low socio-economic status of patients from countries with frequent intrafamilial marriages; the uncertain interpretation of rare benign mutations and/or the unavailability of an appropriate platform for DNA preparation and sequence analysis [15, 16].

CatC, also known as dipeptidyl peptidase I (DPPI, EC 3.4.14.1) is a lysosomal cysteine exopeptidase belonging to the papain superfamily of cysteine peptidases [17]. Functional CatC is a tetrameric enzyme consisting of four identical subunits linked together by non covalent bonds with a total molecular mass of approximately 200 kDa [18, 19]. Each subunit is composed of three polypeptide chains: a N-terminal fragment or exclusion domain (~13 kDa), a heavy chain (~23 kDa) and a light chain (~7 kDa) [19]. It has an important role in the activation of various granular serine proteinases from neutrophils (proteinase 3, elastase, cathepsin G, NSP-4) [20, 21], mast cells [22], cytotoxic T-lymphocytes and natural killer cells [23].

We have recently shown that a proteolytically active CatC is secreted by activated neutrophils in lung fluids from patients with chronic inflammatory lung diseases, which makes it a marker of neutrophilic lung inflammation (Hamon et al., *submitted*). We also observed that proCatC, but not the mature proteinase, was secreted by bronchial epithelial cells (Hamon et al., *submitted*) and that MCF-7 epithelial cells secrete both mature and proCatC (*unpublished*). In the present study, we hypothesize that urinary tract and/or renal epithelial cells also produce CatC constitutively in healthy individuals; if true, CatC should be absent in the urine of PLS patients, and monitoring CatC in the urine could be utilized as an early, simple, reliable, low cost diagnostic technique for PLS.

RESULTS

Pro and mature CatC in urine of healthy subjects

We first screened 20-fold concentrated urine samples from 75 healthy individuals of various ages ranging from 3 months to 80 years for the presence of CatC. Using three different commercial antibodies (Ab1, Ab2, Ab3) to CatC we found that Ab1 and Ab3 recognized two different epitopes of the heavy chain whereas Ab2 recognized one epitope in the propeptide region. We observed two immunoreactive bands with an apparent molecular

mass of ~23 kDa and ~60 kDa in 100% of the urine samples. These bands corresponded to the heavy chain of mature CatC and to proCatC, respectively (Figure 1). The presence of pro and mature CatC did not vary with the timepoint of urine sampling, or with the age or sex of the donor (Figures 2A&B). All of the 75 urine samples hydrolyzed the CatC substrate Gly-Phe-AMC and all peptidic activities were fully inhibited by the CatC nitrile inhibitor Thi-Phe-CN (Figure 2C). Based on the rate of hydrolysis of Gly-Phe-AMC by recombinant CatC, we estimated the concentration of active CatC to be in the 1-10 nanomolar range in normal urine. We did not observe any pro or mature CatC in plasma by immunoblotting. We found however that human bladder cells (T24/T24M) and Martin-Darby canine kidney (MDCK) epithelial cells produced and secreted proCatC but not the mature proteinase. To learn whether proCatC is converted into active CatC in urine, we compared the ratio between proCatC and fully processed and active CatC in a urine sample before and after a 24h incubation time at 37°C. No change in this ratio was observed strongly suggesting that proCatC processing active proteinases do not occur in urine (*not shown*).

CatC in urine of patients with a PLS phenotype

31 urine samples were collected from clinically diagnosed or suspected PLS patients from different European, American and Asian countries (Table 1). Urine samples were centrifuged upon receipt, concentrated (x120) and analyzed for the presence and activity of CatC as described for control subjects (Figure 3A). A sequencing-based PLS diagnosis was previously established for 21 patients allowing the identification of either nonsense, frameshift or missense mutations in the *CTSC* (Table 1). In spite of the greater concentration of PLS urine samples (x120 *vs* x20 in controls) no pro or active CatC was detected in any of these patients whatever the type of mutation (Table 1). We used the presence and the activity of aminopeptidase N (APN) [24] as a positive control to check the quality of the urine sample. Of the 10 remaining patients whose PLS diagnosis was only based on clinical features (Table

1) eight had no pro or mature CatC in their urine and we confirmed the PLS diagnosis by a genetic analysis allowing detection of the mutation (Table 1, Figure 3). The other two patients, however, had pro and mature CatC in their urine. The first patient was a 20-year-old Turkish boy (P#30) who presented the classical dental and dermatologic characteristics of PLS [25]. He also had a bilateral, hypertrophic-looking corneal leukoma and was suspected to suffer from PLS but no genetic analysis had been performed so far. A detailed genetic analysis was therefore performed, but no mutation of *CTSC* was found indicating that he did not suffer from classical PLS. The second CatC positive patient was a 16-year-old French teenager (P#31) who had developed palmoplantar psoriasiform skin changes with tapered distal phalanges as observed in Haim-Munk syndrome (Figure 4A&B). Again, the genetic analysis did not reveal any loss-of-function mutation in the *CTSC* gene. Urinary CatC activity was fully inhibited by the specific CatC inhibitor Thi-Phe-CN. The urine of the mother with periondontitis and anomalies of deciduous teeth (Figure 4C), also contained pro and mature CatC (Figure 4D). Mature CatC was present in a lysate of white blood cells from the son and his mother, at levels comparable to a healthy subject. Further, proteolytically active elastase-like proteinases were present in white blood cell lysates indicating that functional CatC was present in cells and tissues (*data not shown*).

DISCUSSION

Clinical signs of classical PLS become apparent by the age of one to five years when dry, scaly patches appear on the skin of palms and soles, and severe inflammation starts to affect the surrounding gingiva and bone of primary teeth, leading to their rapid loss. Bacterial skin infections such as abscesses may also occur [26]. The genetic etiology of PLS has been clearly established by sequencing of the *CTSC* gene [1, 3, 12] and whole-exome sequencing [27]. However, it still remains unclear why a lack of CatC activity leads to specific dermatological lesions and severe destructive periodontitis. A plausible explanation for the

latter is deficiency of the antimicrobial peptide LL-37 in the gingiva, allowing for infection with *A. actinomycetemcomitans* and the development of severe periodontal disease [28]. Proteinase 3 releases this peptide from a precursor molecule after neutrophil activation [29]. The lack of CatC activity in PLS patients blocks almost completely the activity of neutrophil serine proteases and reduces the level of their proform in neutrophils [21, 30, 31]. On the other hand, mutations in completely different genes might induce a PLS-like syndrome with similar dermatological lesions and destructive periodontitis, but very different with regard to co-morbidities and optimal treatment regimens [32]. Several sporadic cases with a PLS-like clinical phenotype have been reported in the literature [13, 33, 34] and two examples of a PLS-like syndrome have been included in this study. Because of the variable manifestations of the classical PLS syndrome, elucidation of its phenotypic variability requires functional and biochemical assays as well as *CTSC* analysis. Otherwise PLS could be underdiagnosed [35].

CatC is expressed as a pro-proteinase by epithelial and myeloid cells and their precursors and is activated by a multistep mechanism possibly involving several proteinases [1, 36, 37]. Thus pro- and/or mature CatC may be constitutively present in a variety of cells and tissues including renal and/or bladder epithelial cells. Here, we evaluated the hypothesis that urine from healthy and diseased persons is in fact a reliable source of pro- or active CatC that can be easily characterized and analyzed by enzymatic and/or immunochemical methods. In this study, we found that the absence of active CatC and its proform in the urine was a strong and reliable indicator for PLS and of great value for the early diagnosis of PLS. By contrast 100% of urine samples from control subjects of any age and gender contained measurable amounts of active CatC. Nonsense, frameshift and missense mutations all resulted in a total absence of CatC or CatC fragments in the urine of PLS patients. While predictable for nonsense and frameshift mutations, it is surprising for missense mutations to

affect the expression of mutated proteins. It could be that missense mutations in *CTSC* alter the constitutive secretion of the mutated CatC or induce its intracellular degradation. Intracellular degradation would also explain the systematic loss of CatC activity observed with a missense mutation even if not predicted by the structure-based analysis [19]. This was corroborated by the recent observation that a missense mutation in the propeptide of CatC resulted in the absence of CatC in mature neutrophils [31].

The ready availability of large volumes of urine has many advantages over saliva and gingival crevicular fluids which contain only small amounts of CatC activity in healthy subjects [28]. The early implementation of a strict oral hygiene regimen can minimize the progression of periodontitis, while oral retinoid therapy can clear the skin lesions and improve quality of life in PLS [38-40]. Demonstrating the absence of urinary CatC activity soon after birth and before the onset of clinical symptoms can be used as a screening procedure for PLS in populations with a high frequency of intra-familial marriages and in close relatives of PLS patients. A test based on the absence of urinary CatC activity will facilitate phenotype-genotype correlation in PLS and overlapping syndromes. Such a simple, rapid and low cost screening test based on CatC excretion in the urine of children and newborns can now be developed for early diagnosis and timely therapy to prevent aggressive periodontitis. When available the test should take a few minutes and will be manageable by any technician at the hospital laboratory.

MATERIALS AND METHODS

Reagents

Recombinant CatC and APN were from Unizyme Laboratories (Hørsholm, Denmark) and R&D systems (Lille, France), respectively. AntiCatC antibodies included: the mouse monoclonal anti-CatC Ab (Ab1) (Santa Cruz Biotechnology, Heidelberg, Germany), the goat polyclonal anti-CatC Ab (Ab2) (R&D Systems) and the goat polyclonal anti-CatC (Ab3)

(Everest Biotech, Oxforshire, UK). The mouse monoclonal anti-APN Ab was from Santa Cruz Biotechnology. Gly-Phe-AMC was from Enzyme Systems Products (Illkirch, France) and H-Ala-AMC was supplied by Bachem (Weil am Rhein, Germany). The cysteine proteinase inhibitor E-64c ((2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane)) was from Sigma-Aldrich (St Louis, MO, USA) and the specific inhibitor of CatC, Thi-Phe-CN ((*β*-2-thienyl)-L-alanyl-L-phenylalanine nitrile) was provided by Dr Lesner (University of Gdansk, Poland). EDTA or Ethylenediaminetetraacetic acid was from Merk (Darmstadt, Germany) and the specific inhibitor of APN, bestatin was from Santa Cruz (Heidelberg, Germany).

Urine collection

Urine samples were collected from 31 PLS patients from European countries (France, Germany, UK, Hungary, Italy and Turkey), Asian Countries (China, India, Iran, Saudi Arabia) and from the USA. The 75 healthy volunteers were from France and Iran.

The study protocol was approved by the Comité de Protection des Personnes, CPP OUEST-1 (Tours, France) and by Ethics Review Boards local to patients studied, and informed consent was obtained from each individual or parent prior to enrollment. The study methodologies conformed to the standards set by the Declaration of Helsinki. Demographics and clinical data from patients are summarized in Table 1. After centrifugation (3000 g, 15 min) at 4°C to eliminate cells and debris, the urine supernatants were concentrated 20 or 120 times using Vivaspin 15R concentrators (molecular mass cutoff, 10 kDa; Sartorius, Goettingen, Germany) and store at 4°C.

Western blotting

Briefly, urine samples were electrophoretically separated on a 10% SDS-PAGE in reducing conditions for CatC analysis and in non-reducing conditions for APN analysis, and then transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). After saturation, membranes were incubated with anti-CatC Abs (Ab1 and Ab3 diluted 1:500; Ab2 diluted 1:1000) or with anti-APN mAb (diluted 1:500). After washing, membranes were incubated with peroxidase-conjugated anti-mouse IgG (diluted 1:10000) or anti-goat IgG Abs (diluted 1:20000) (Sigma-Aldrich), when appropriate. Bound Abs were detected by chemiluminescence (ECL Plus Western Blotting Kit Detection Reagents, GE Healthcare, UK) according to the manufacturer's instruction.

Enzyme assays

Assays were carried out at 37°C in 50 mM sodium acetate, 30 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol (DTT), pH 5,5 for CatC and in 50 mM Tris, pH 7 for APN. Proteolytic activity was measured using 5 µL of urine supernatants with 30 µM Gly-Phe-AMC in a total volume of 60 µL for CatC or with 50 µM H-Ala-AMC in a total volume of 100 µL for APN (excitation wavelength = 340 nm, emission wavelength = 460 nm; Spectramax Gemini (Molecular Devices, Sunnyvale, CA, USA)). The presence of CatC in urine was controlled incubating urine samples with the selective CatC inhibitor Thi-Phe-CN [41] prior to substrate addition. Recombinant CatC and APN were used as controls, respectively. For some experiments, urine samples were preincubated with 100 µM E64c, 5 µM Thi-Phe-CN, 5 mM EDTA or 100 µM bestatin for 30 min at 37°C prior the measurement of the proteolytic activity.

Cell culture

Human urinary bladder cancer T24 and T24M cells were cultured in McCoy's 5A Medium with L-glutamine (Ozyme, Saint Quentin en Yvelines, France) supplemented with or without 10% fetal calf serum (FCS) at 37 °C. MDCK epithelial cells were cultured in Eagle's Minimum Essential Medium supplemented with or without 10% fetal calf serum (FCS) at 37 °C. Cells were lysed in PBS containing 0.5% nonidet P-40 (IGEPAL 630), and after centrifugation (10000 g, 15 min at 4°C), the lysate was stored at -20°C. Cell supernatants were concentrated 30 times using Vivaspin 15R concentrators (molecular mass cutoff, 10 kDa; Sartorius). Proteins in lysates and in supernatants were quantified with a bicinchoninc acid assay (BCA) (Thermo Fisher Scientific, Rockford, IL, USA).

Genetic investigations

Patient's genomic DNA and when available parent's DNA was extracted from EDTA blood samples with the automated method EVO®100 ReliaPrep (Tecan, Promega).

PCR and sequencing reaction: *in vitro* amplification and sequencing of all *CTSC* exons and intron–exon boundaries were performed as described in [42] with minor modifications: sequencing was carried out using Big Dye Terminator v3.1 on a 3500xL Dx Genetic Analyzer (Applied Biosystems, ThermoFisher St Aubin, France). *In silico* analysis of missense mutations and database queries were conducted through the Alamut Interface (Alamut® Visual v2.3, interactive biosoftware, Rouen, France). Mutations were described in accordance with the CTSC cDNA sequence GenBank NM_001814.2 and HGVS recommendations.

Deletion search was performed using custom array CGH: custom microarrays (8 × 60K) were designed with e-array web software (Agilent Technologies, CA) using the Similarity Score Filter in order to select highly specific probes. A total of 3141 probes were distributed: about one probe every 100 bp in the *CTSC* and 50 Kb around the gene then one probe every 350 bp

in the 300 Kb regions apart. DNA was labelled (cyanine 3 or cyanine 5) using the Sure Tag DNA labeling kit from Agilent Technologies and hybridized onto the microarrays according to the manufacturer's instructions. DNA was analyzed in comparative genomic hybridization experiments with fluorochrome swapping, in a trio along with DNA from two subjects not affected by the PLS. Scanning of the microarrays was performed using a G2565CA scanner (Agilent Technologies). Data analysis was carried out with softwares from Agilent Technologies, namely Feature Extraction 10.7.3.1 and Agilent Genomic Workbench 6.0.130.24. DNA analysis was not repeated when the mutation was already known.

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AUTHOR CONTRIBUTION STATEMENT

BK, YH, ML planned experiments; YH, ML, PF, SDC, LV, CZ performed experiments;

BK, FG, DEJ, YH, ML, PF, SDC analyzed data; BK, FG, DEJ, CM wrote the paper; BK supervised the project.

All other authors contributed samples or other essential material (Urine/Blood).

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Table 1
Demographic and clinical data of patients with a PLS phenotype

Patients	Ethnicity	Gender	Age	Clinical manifestations	Mutation	Urinary CatC		
						Pro CatC	Mature CatC	CatC activity
P#1	French	F	55	Palmoplantar hyperkeratosis, severe periodontitis	c.96T>G [(a)] (p.Y32X) Nonsense	-	-	-
P#2	Indian	M	15	Transgradient palmoplantar keratoderma, periodontitis	c.912C>A [(1)] (p.Y304X) Nonsense	-	-	-
P#3	Hungarian	F	4	Palmoplantar hyperkeratosis, periodontitis	c.681delCATACAT [(2)] (p.T188fsX199) Frameshift	-	-	-
P#4	Hungarian	F	13	Palmoplantar hyperkeratosis, severe periodontitis	c.681delCATACAT [(2)] (p.T188fsX199) Frameshift	-	-	-
P#5	Pakistanian	F	14	Palmoplantar hyperkeratosis, periodontitis	(p.W433L) [(3)] Missense	-	-	-
P#6	Pakistanian	M	9	Palmoplantar hyperkeratosis, periodontitis cerebral abscess	c.815G>C [(4)] (p.R272P) Missense	-	-	-
P#7	Pakistanian	F	13	Palmoplantar hyperkeratosis, periodontitis	c.815G>C [(4)] (p.R272P) Missense	-	-	-
P#8	Pakistanian	M	15	Palmoplantar hyperkeratosis, periodontitis	c.815G>C [(5)] (p.R272P) Missense	-	-	-
P#9	Italian	F	42	Palmoplantar hyperkeratosis, severe periodontitis	c.1141delC [(6)] (p.L381fsX393) Frameshift	-	-	-
P#10	Hungarian	F	12	Palmoplantar hyperkeratosis, severe periodontitis	c.901G>A [(a)] (p.G301S) Missense	-	-	-
P#11	Hungarian	F	4	Palmoplantar hyperkeratosis, severe periodontitis	c.901G>A [(a)] (p.G301S) Missense	-	-	-
P#12	Erythrean	M	12	Palmoplantar hyperkeratosis, severe periodontitis, Tinea capitis	c.755A>T [(7)] (p.Q252L) Missense	-	-	-
P#13	Erythrean	M	15	Palmoplantar hyperkeratosis, severe periodontitis	c.755A>T [(7)] (p.Q252L) Missense	-	-	-
P#14	Moroccan	M	19	Mild palmoplantar hyperkeratosis, severe periodontitis	c.854C>T [(8)] (p.P285L) Missense	-	-	-
P#15	Moroccan	M	35	Palmoplantar hyperkeratosis, severe periodontitis, edentulous by now	c.854C>T [(8)] (p.P285L) Missense	-	-	-
P#16	German	F	24	Palmoplantar hyperkeratosis, severe periodontitis, edentulous by now	c.566-572Del [(8)] (T189FS199X) Frameshift	-	-	-
P#17	German	M	48	Severe palmoplantar hyperkeratosis, late onset of severe periodontitis (*22 years of age*), liver abscess	comp. heterozygous [(8)] c.322A>T/c.436delT (p.K108X/p.S146fs153X) Nonsense / Frameshift	-	-	-
P#18	German	M	27	Palmoplantar hyperkeratosis, severe periodontitis	comp. heterozygous [(8)] c.947T>G (pL316R) c.1268G>C (pW423S) Missense	-	-	-

P#19	Chinese	M	22	Palmoplantar psoriasiform appearance, severe periodontitis	c.394 C>G [9] (p.R132G) Missense	-	-	-
P#20	Chinese	M	22	Palmoplantar psoriasiform appearance, severe periodontitis	c.394 C>G [9] (p.R132G) Missense	-	-	-
P#21	Mauritian	M	5	Palmoplantar hyperkeratosis, periodontitis	n.i	-	-	-
P#22	Mauritian	F	15	Palmoplantar hyperkeratosis, periodontitis	n.i	-	-	-
P#23	Puerto Rican	F	31	Palmoplantar hyperkeratosis, severe periodontitis	c.116G>C [a] (p.W39S) Missense	-	-	-
P#24	Persian	F	33	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C [a] (p.R272P) Missense	-	-	-
P#25	Persian	M	9	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C [a] (p.R272P) Missense	-	-	-
P#26	Persian	M	4	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C [a] (p.R272P) Missense	-	-	-
P#27	Saudi arabian	M	17	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C [a] (p.R272P) Missense	-	-	-
P#28	Saudi arabian	M	24	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C [a] (p.R272P) Missense	-	-	-
P#29	Pakistanian	M	3	Palmoplantar hyperkeratosis, periodontitis	c.815G>C [5] (p.R272P) Missense	-	-	-
P#30	Turkish	M	20	Palmoplantar hyperkeratosis, severe periodontitis, corneal leukoma	No mutation [a]	+	+	+
P#31	French	M	16	Palmoplantar psoriasiform appearance, anomalies of deciduous teeth with no net periodontitis	No mutation [a]	+	+	+

P#12 and P#13 are brothers
P#14 and P#15 are brothers
P#3 and P#4 are sisters
P#6 and P#7 are siblings
P#21 and P#22 are siblings
P#8 and P#29 are from the same family
P#24, P#25 and P#26 are from the same family

(a) Identified in this work; (1) Ragunatha et al., 2015 [39] ; (2) Farkas et al., 2013 [43] ; (3) Identified by Prof. Nalin Thakker, Academic Unit of Medical Genetics, St Mary's Hospital Manchester UK; (4) Kanthimathinathan et al., 2013 [8] ; (5) Taibjee et al., 2005 [26] ; (6) Bullon et al., 2014 [44] ; (7) Schacher et al., 2006 [45] ; (8) Noack et al., 2008 [46] ; (9) Xinwen et al., 2015 [47].

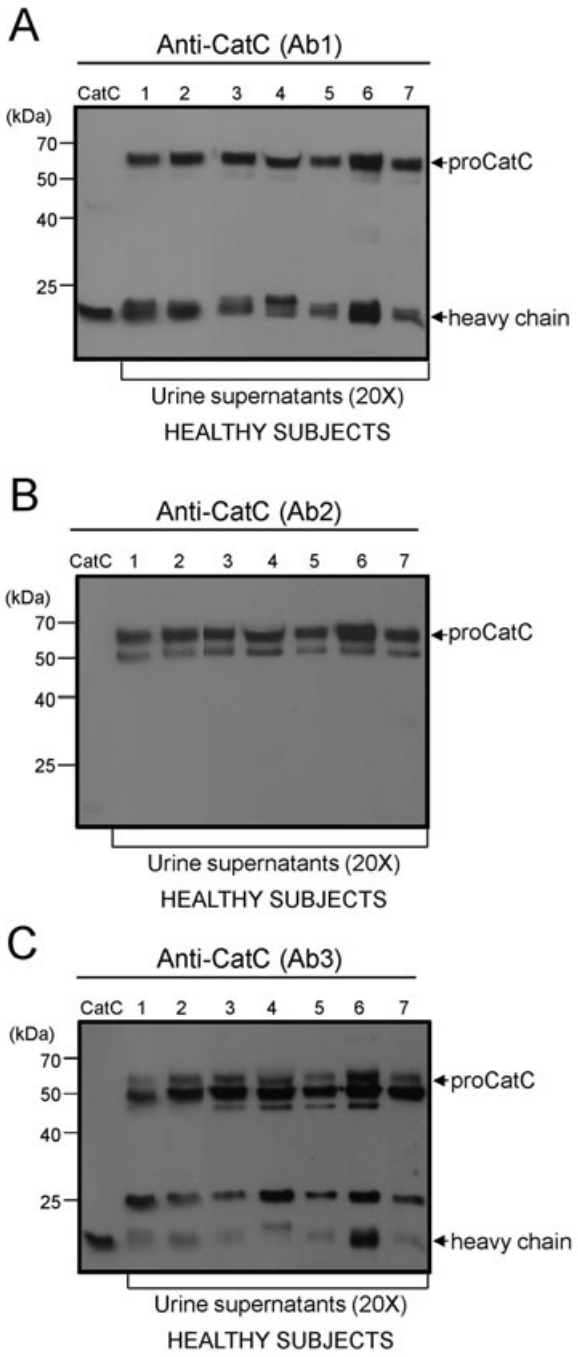

Figure 1: CatC in urine from healthy subjects. Immunoblots of 20 fold-concentrated samples of urine collected from healthy control subjects, using different anti-CatC antibodies; **(A)** anti-CatC (Ab1) recognizes an epitope on the heavy chain, **(B)** anti-CatC (Ab2) is directed to the propeptide region, **(C)** Ab3 binds to another epitope on the heavy chain of CatC. Recombinant CatC was used as a control (left lane). Extra bands observed with anti-CatC (Ab2) and anti-CatC (Ab3) correspond to non specific interactions.

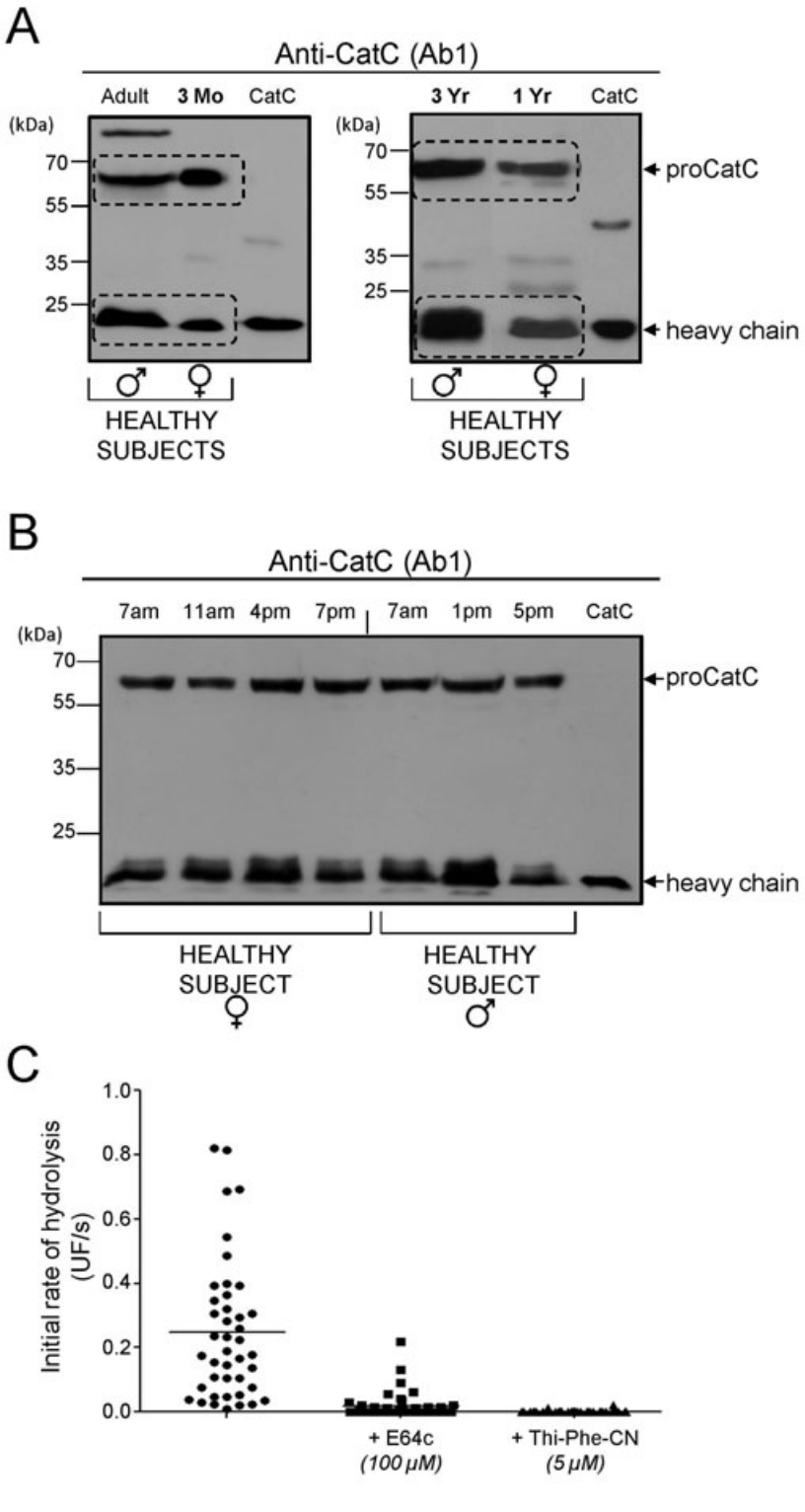

Figure 2: Continuous presence of CatC in urine from healthy children and adults. (A) Immunoblots of 20 fold-concentrated samples of urine collected from healthy children at the age of 3 months (3 mo), 1 year (1 yr) and 3 years (3 yr) compared to a healthy adult, using anti-CatC Ab1. **(B)** Immunoblots of 20 fold-concentrated samples of urine collected at different times of the day from one healthy woman and one healthy man, using anti-CatC (Ab1). Recombinant CatC was used as a control. **(C)** CatC activity in 20 fold-concentrated urines from 50 healthy control subjects using the fluorogenic substrate Gly-Phe-AMC (30 µM) and its inhibition by E64c (100 µM) and Thi-Phe-CN (5 µM).

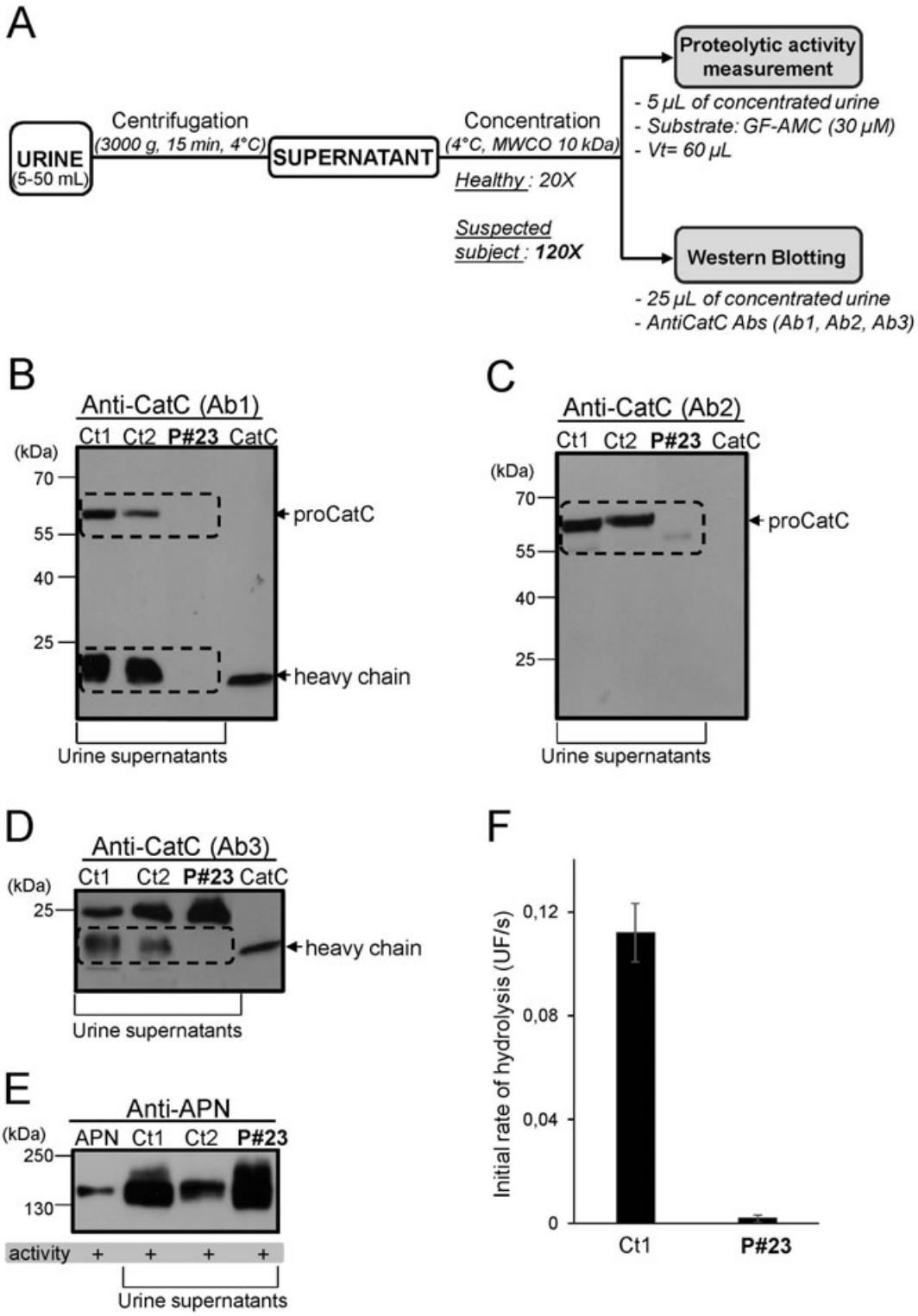

Figure 3: Absence of CatC in urine from a PLS patient. **(A)** Protocol for analysis of urinary CatC. After receipt of PLS samples, urines were centrifuged to eliminate cells and debris, and then concentrated 120 times, six times more than the urine of healthy subjects, to analyze the presence and the proteolytic activity of CatC as described in the materials and methods section. **(B)** Immunoblots of 20 fold-concentrated samples of urine collected from two healthy control subjects (controls Ct1 and Ct2) and of 120 fold-concentrated samples of urine collected from one representative PLS patient (P#23), using anti-CatC (Ab1), **(C)** anti-CatC (Ab2) **(D)** anti-CatC (Ab3) and **(E)** anti-APN. **(F)** CatC activity in 20 fold-concentrated urines from one healthy control subject (Ct1) and of 120 fold-concentrated samples of urine collected from one representative PLS patient (P#23) using the fluorogenic substrate Gly-Phe-AMC (30 μM). Recombinant CatC and APN were used as controls.

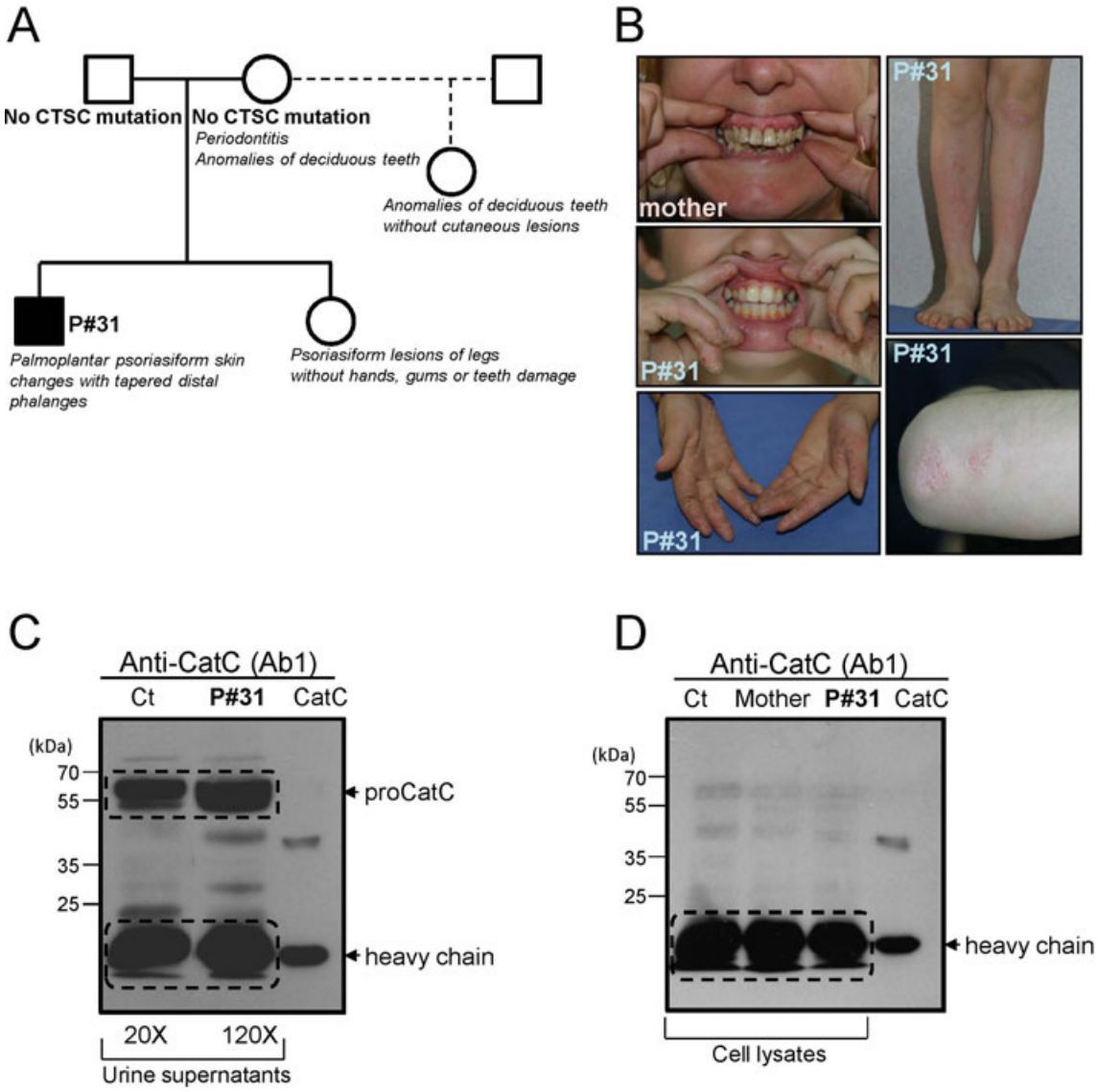

Figure 4: Presence of CatC in urine from an atypical French patient without CTSC mutation. **(A)** Pedigree of suspected 16 yr-old individual (P#31) with suspected PLS. **(B)** The dermatological and dental features of the patient (P#31) and his mother. **(C)** Immunoblotting analysis with anti-CatC (Ab1) of 120 fold-concentrated urines from the patient (P#31) compared to 20 fold-concentrated urines from a healthy control subject (Ct). **(D)** Western blotting of white blood cell lysates from the P#31 patient, his mother and a healthy control subject (Ct) using anti-CatC (Ab1). Recombinant CatC was used as a control.