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Analysis of urinary cathepsin C for diagnosing Papillon–Lefèvre syndrome

Yveline Hamon[1,2,3,4], Monika Legowska[5,*], Patricia Fergelot[6,7,8,*], Sandrine Dallet-Choisy[1,2], Louise Newell[9], Lise Vanderlynden[1,2], Ali Kord Valeshabad[10], Karina Acrich[11], Hadi Kord[12], Charalampos Tsamakis [9], Fanny Morice-Picard[13], Ian Surplice[9], Jerome Zoidakis[14], Karen David[11], Antonia Vlahou[14], Shivanna Ragunatha[15], Nikoletta Nagy[16,17,18], Katalin Farkas[16,17,18], Márta Széll[16,17,18], Cyril Goizet[6,8], Beate Schacher[19], Maurizio Battino[20,21], Abdullah Al Farraj Aldosari[22], Xinwen Wang[23], Yang Liu[24], Sylvain Marchand-Adam[1,2], Adam Lesner[5], Elodie Kara[25], Sevil Korkmaz-Icöz[26], Celia Moss[9,27], Peter Eickholz[19], Alain Taieb[13], Salih Kavukcu[28], Dieter E. Jenne[3,4], Francis Gauthier[1,2] and Brice Korkmaz[1,2]

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1 INSERM U-1100 "Centre d'Etude des Pathologies Respiratoires", Tours, France
2 Université François Rabelais, Tours, France
3 Comprehensive Pneumology Center, Institute of Lung Biology and Disease (iLBD), German Center for Lung Research (DZL), Munich, Germany
4 Max Planck Institute of Neurobiology, Planegg-Martinsried, Germany
5 Faculty of Chemistry, University of Gdansk, Gdansk, Poland
6 Service de Génétique Médicale, Hôpital Pellegrin, CHU Bordeaux, France
7 Centre de Référence des Anomalies du Développement Embryonnaire, Bordeaux, France
8 INSERM U-1211, Laboratoire Maladies Rares: Génétique et Métabolisme, Université de Bordeaux, France
9 Birmingham Children's Hospital, UK
10 University of Colorado Denver, Aurora, CO, USA
11 Metropolitan Hospital Center, Medical Genetics Clinic, New York, NY, USA
12 Bone Joint and Connective Tissue Disease Research Center (BJCRC), Department of Rheumatology, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran
13 Centre de Référence pour les Maladies Rares de la Peau, Service de Dermatologie Adulte et Pédiatrique, CHU de Bordeaux and INSERM U-1035, University of Bordeaux, France
14 Biotechnology Division, Biomedical Research Foundation, Academy of Athens, Greece
15 Department of Dermatology, Venereology, and Leprosy, Sri Siddhartha Medical College, Tumkur, India
16 Department of Medical Genetics, University of Szeged, Hungary
17 MTA SZTE Dermatological Research Group, University of Szeged, Hungary
18 Department of Dermatology and Allergology, University of Szeged, Hungary
19 Department of Periodontology, Johann Wolfgang Goethe-University Frankfurt, Germany
20 Department of Clinical Sciences, Università Politecnica delle Marche, Ancona, Italy
21 Centre for Nutrition & Health, Universidad Europea del Atlantico, Santander, Spain
22 Department of Prosthetic, College of Dentistry, King Saud University, Riyadh, Kingdom of Saudi Arabia
23 Department of Oral Medicine and Periodontology, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi, China
24 Department of Periodontology, PLA 309 Hospital, Beijing, China
25 ReproPharm, Centre INRA-Val de Loire, Domaine de l'Orfrasière, Nouzilly, France
26 Department of Cardiac Surgery, Heidelberg University Hospital, Germany
27 University of Birmingham, Edgbaston, UK
28 Division of Pediatric Nephrology, Department of Pediatrics, School of Medicine, Dokuz Eylül University, İzmir, Turkey

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Correspondence
B. Korkmaz, "Centre d'Etude des

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* that completely abrogate the proteolytic activity of this cysteine proteinase. Most often, a genetic analysis to enable early and rapid diagnosis of PLS is unaffordable or unavailable. In this study, we tested the hypothesis that

Pathologies Respiratoires", INSERM U-1100, Université François Rabelais, Faculté de Médecine, 37032 Tours, France
Fax: 0033 2 47 36 60 46
Tel: 0033 2 47 36 62 53
E-mail: brice.korkmaz@inserm.fr

*These authors contributed equally to this work.

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active CatC is constitutively excreted and can be easily traced in the urine of normal subjects. If this is true, determining 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. Of 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 for 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 early and severe periodontitis, causing loss of both the primary and permanent teeth [1–3]. The prevalence of PLS is one to four cases per million people, and the carrier rate is 2–4 per 1000 [4]. There is no gender predilection, but parental consanguinity has been reported in > 50% of cases [4,5]. Palmoplantar keratoderma may be visible at birth or 1–2 months thereafter, but, generally, keratoderma and periodontitis develop in parallel between the sixth month and the fourth 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 per cent of homozygous mutations were missense mutations, 25% were nonsense mutations, 23% were frameshift mutations, and 2% were other types of mutation [12]. In addition to the classic form of PLS, six cases with late-onset periodontal disease and/or late-onset palmoplantar lesions have been reported [13]. Haim–Monk syndrome, which is 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 (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 noncovalent bonds, with a total molecular mass of ~ 200 kDa [18,19]. Each subunit is composed of three polypeptide chains: an 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, and 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

Abbreviations
AMC, aminomethylcoumarin; APN, aminopeptidase N; CatC, cathepsin C; PLS, Papillon–Lefèvre syndrome.

both mature CatC and proCatC (unpublished). We hypothesized that urinary tract and/or renal epithelial cells also produce CatC constitutively in healthy individuals; if this is true, CatC should be absent in the urine of PLS patients, and monitoring of CatC in the urine could be utilized as an early, simple, reliable and low-cost diagnostic technique for PLS.

Results

ProCatC 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, and Ab3) against 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 apparent molecular masses 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 (Fig. 1). The presence of proCatC and mature CatC did not vary with the timepoint of urine sampling, or with the age or sex of the donor (Fig. 2A,B). All of the 75 urine samples hydrolyzed the CatC substrate Gly-Phe-aminomethylcoumarin (AMC), and the observed proteolytic activities were fully inhibited by the CatC nitrile inhibitor Thi-Phe-CN (Fig. 2C). On the basis of 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 proCatC or mature CatC in plasma by immunoblotting. We found, however, that human bladder cells (T24/T24M) and Martin–Darby canine kidney epithelial cells produced and secreted proCatC but not the mature proteinase. To determine 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 24 h of incubation at 37 °C. No change in this ratio was observed, strongly suggesting that proCatC-processing active proteinases do not occur in urine (data not shown).

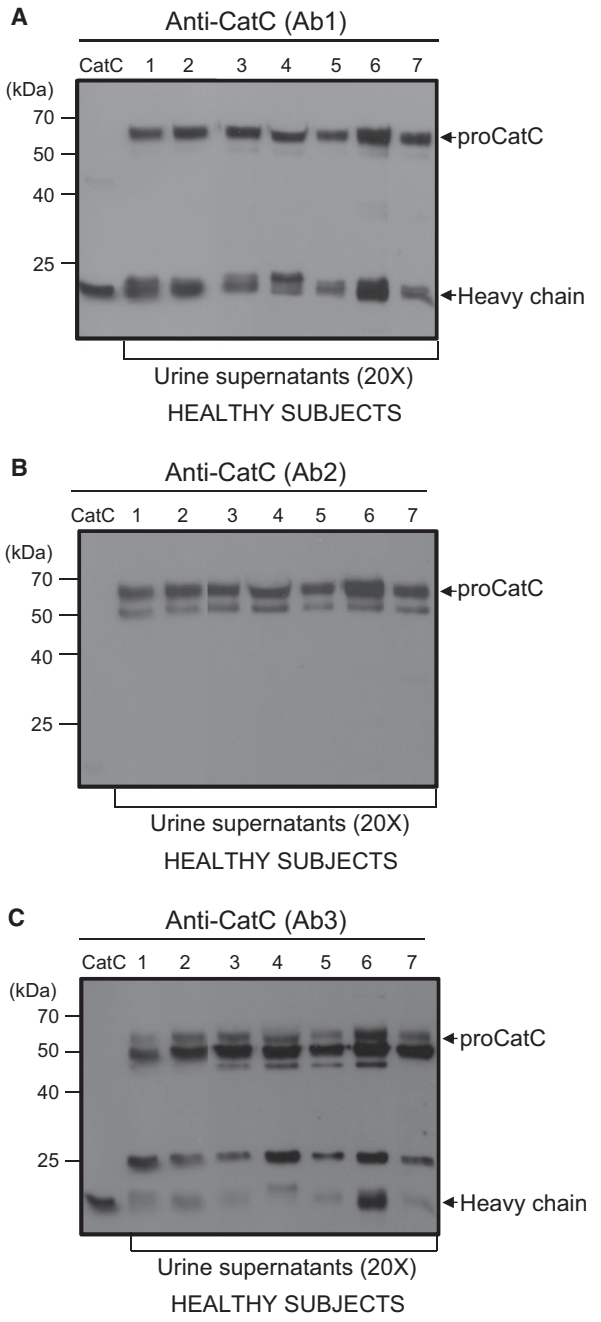

Fig. 1. CatC in urine from healthy subjects. Immunoblots of 20-fold-concentrated urine samples collected from healthy control subjects, with different antibodies against CatC. (A) Ab1 binds to an epitope on the heavy chain. (B) Ab2 binds to the propeptide region. (C) Ab3 binds to another epitope on the heavy chain of CatC. Recombinant CatC was used as a control (left lane). The extra bands observed with Ab2 and Ab3 correspond to nonspecific interactions.

CatC in urine of patients with a PLS phenotype

Thirty-one 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 (×120), and analyzed for the presence and activity of CatC as described for control subjects (Fig. 3A). A sequencing-based PLS diagnosis was previously established for 21 patients, allowing the identification of

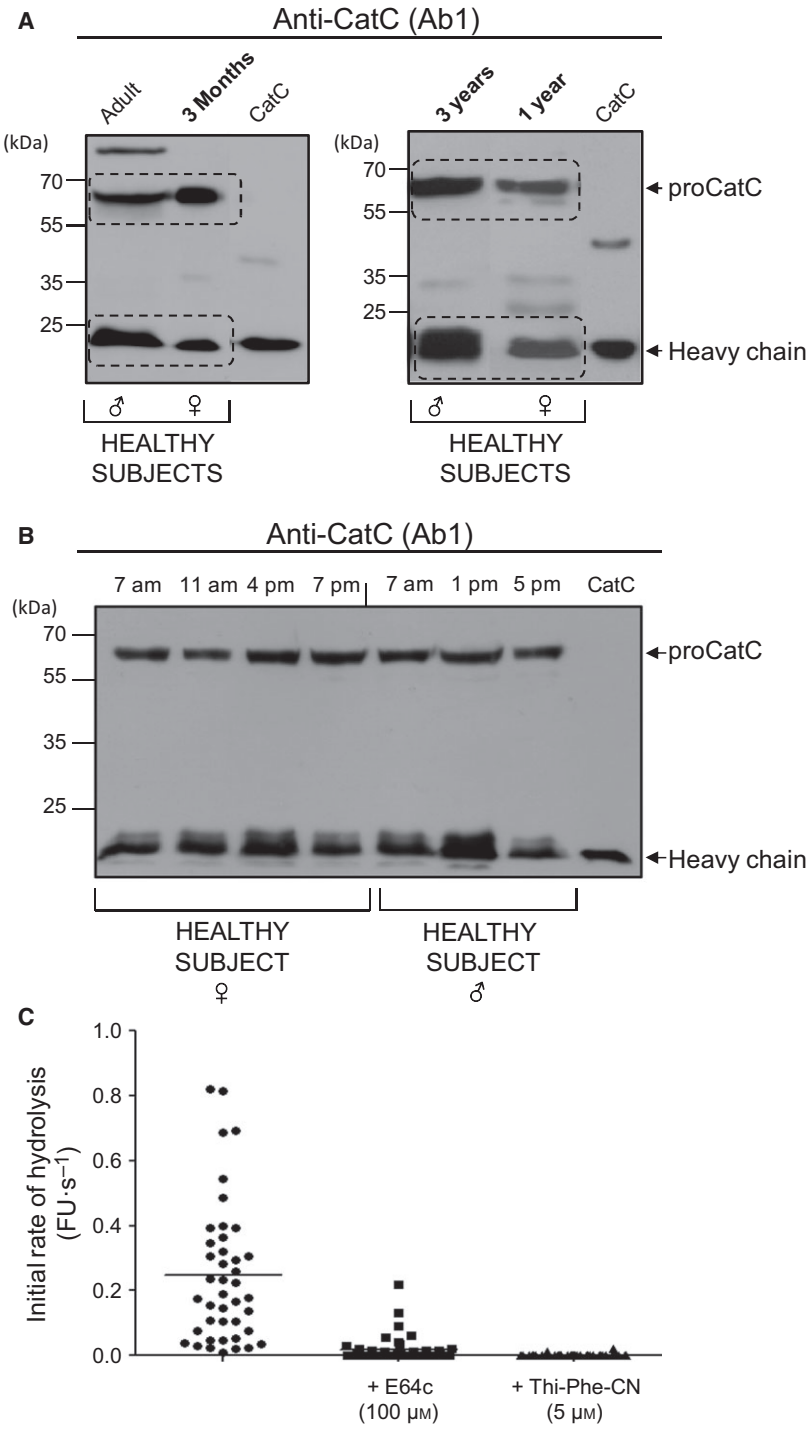

Fig. 2. Continuous presence of CatC in urine from healthy children and adults. (A) Immunoblots of 20-fold-concentrated urine samples collected from healthy children at the ages of 3 months, 1 year and 3 years as compared with those for a healthy adult, with Ab1. (B) Immunoblots of 20-fold-concentrated urine samples collected at different times of the day from one healthy woman and one healthy man, with Ab1. Recombinant CatC was used as a control. (C) CatC activity in 20-fold-concentrated urine samples from 50 healthy control subjects determined with the fluorogenic substrate Gly-Phe-AMC (30 μM), and its inhibition by E64c (100 μM) and Thi-Phe-CN (5 μM). FU, fluorescence units.

either nonsense, frameshift or missense mutations in *CTSC* (Table 1). In spite of the higher concentration of PLS urine samples (×120 versus × 20 in controls), no proCatC 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 based only on clinical features (Table 1), eight had no proCatC or mature CatC in their urine, and we confirmed the PLS diagnosis by genetic analysis allowing detection of the mutation (Table 1; Fig. 3). The other two patients, however, had proCatC and mature CatC in their urine. The first patient was a

20-year-old Turkish boy (P30) who presented with the classic dental and dermatological 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. A detailed genetic analysis was therefore carried out, but no mutation of *CTSC* was found, indicating that he did not suffer from classic PLS. The second CatC-positive patient was a 16-year-old French teenager (P31) who had developed palmoplantar psoriasiform skin changes with tapered distal phalanges as observed in Haim–Munk syndrome (Fig. 4A,B). Again, the genetic analysis did not reveal any loss-of-function mutation in *CTSC*. Urinary CatC activity was fully inhibited by the specific CatC inhibitor Thi-Phe-CN. The urine of the mother with periodontitis and anomalies of deciduous teeth (Fig. 4A,B) also contained proCatC and mature CatC as observed in the urine of her son (Fig. 4C). Mature CatC was present in a lysate of white blood cells from the son and his mother, at levels similar to those in a healthy subject (Fig. 4D). Furthermore, 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 classic PLS become apparent by the age of 1–5 years, when dry, scaly patches appear on the skin of the 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 *CTSC* [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 *Aggregatibacter 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 almost completely blocks the activity of neutrophil serine proteases and reduces the levels of their proforms 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 comorbidities 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 have been included in this study. Because of the variable manifestations of the classic PLS, 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 proproteinase by epithelial and myeloid cells and their precursors, and is activated by a multistep mechanism, possibly involving several proteinases [1,36,37]. Thus proCatC 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 proCatC or active CatC that can be easily characterized and analyzed with 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 was of great value in the early diagnosis of PLS. In 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. Whereas nonsense and frameshift mutations are expected to affect the expression of mutated proteins, it is surprising for missense mutations to do so. 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 gives it 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, and oral retinoid therapy can clear the skin lesions and improve the quality of life of patients with 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 intrafamilial 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

Table 1. Demographic and clinical data of patients with a PLS phenotype. P12 and P13 are brothers. P14 and P15 are brothers. P3 and P4 are sisters. P6 and P7 are siblings. P21 and P22 are siblings. P8 and P29 are from the same family. P24, P25 and P26 are from the same family. F, female; M, male.

						Urinary CatC		
Patients	Ethnicity	Gender	Age (years)	Clinical manifestations	Mutation	ProCatC	Mature CatC	CatC activity
P1	French	F	55	Palmoplantar hyperkeratosis, severe periodontitis	c.96T>G[a] (p.Y32X) Nonsense	–	–	–
P2	Indian	M	15	Transgradient palmoplantar keratoderma, periodontitis	c.912C>A[b] (p.Y304X) Nonsense	–	–	–
P3	Hungarian	F	4	Palmoplantar hyperkeratosis, periodontitis	c.681delCATACAT[c] (p.T188fsX199) Frameshift	–	–	–
P4	Hungarian	F	13	Palmoplantar hyperkeratosis, severe periodontitis	c.681delCATACAT[c] (p.T188fsX199) Frameshift	–	–	–
P5	Pakistani	F	14	Palmoplantar hyperkeratosis, periodontitis	(p.W433L)[d] Missense	–	–	–
P6	Pakistani	M	9	Palmoplantar hyperkeratosis, periodontitis, cerebral abscess	c.815G>C[e] (p.R272P) Missense	–	–	–
P7	Pakistani	F	13	Palmoplantar hyperkeratosis, periodontitis	c.815G>C[e] (p.R272P) Missense	–	–	–
P8	Pakistani	M	15	Palmoplantar hyperkeratosis, periodontitis	c.815G>C[f] (p.R272P) Missense	–	–	–
P9	Italian	F	42	Palmoplantar hyperkeratosis, severe periodontitis	c.1141delC[g] (p.L381fsX393) Frameshift	–	–	–
P10	Hungarian	F	12	Palmoplantar hyperkeratosis, severe periodontitis	c.901G>A[a] (p.G301S) Missense	–	–	–
P11	Hungarian	F	4	Palmoplantar hyperkeratosis, severe periodontitis	c.901G>A[a] (p.G301S) Missense	–	–	–
P12	Erythrean	M	12	Palmoplantar hyperkeratosis, severe periodontitis, tinea capitis	c.755A>T[h] (p.Q252L) Missense	–	–	–
P13	Erythrean	M	15	Palmoplantar hyperkeratosis, severe periodontitis	c.755A>T[h] (p.Q252L) Missense	–	–	–
P14	Moroccan	M	19	Mild palmoplantar hyperkeratosis, severe periodontitis	c.854C>T[i] (p.P285L) Missense	–	–	–
P15	Moroccan	M	35	Palmoplantar hyperkeratosis, severe periodontitis, edentulous by now	c.854C>T[i] (p.P285L) Missense	–	–	–
P16	German	F	24	Palmoplantar hyperkeratosis, severe periodontitis, edentulous by now	c.566–572Del[i] (T189FS199X) Frameshift	–	–	–
P17	German	M	48	Severe palmoplantar hyperkeratosis, late onset of severe periodontitis (22 years of age), liver abscess	Compound heterozygous[i] c.322A>T/c.436delT (p.K108X/ p.S146fs153X) Nonsense/frameshift	–	–	–
P18	German	M	27	Palmoplantar hyperkeratosis, severe periodontitis	Compound heterozygous[i] c.947T>G (pL316R) c.1268G>C (pW423S) Missense	–	–	–

Table 1. (Continued).

Patients	Ethnicity	Gender	Age (years)	Clinical manifestations	Mutation	Urinary CatC		
						ProCatC	Mature CatC	CatC activity
P19	Chinese	M	22	Palmoplantar psoriasiform appearance, severe periodontitis	c.394 C>G[j] (p.R132G) Missense	–	–	–
P20	Chinese	M	22	Palmoplantar psoriasiform appearance, severe periodontitis	c.394 C>G[j] (p.R132G) Missense	–	–	–
P21	Mauritian	M	5	Palmoplantar hyperkeratosis, periodontitis	Not identified	–	–	–
P22	Mauritian	F	15	Palmoplantar hyperkeratosis, periodontitis	Not identified	–	–	–
P23	Puerto Rican	F	31	Palmoplantar hyperkeratosis, severe periodontitis	c.116G>C[a] (p.W39S) Missense	–	–	–
P24	Persian	F	33	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C[a] (p.R272P) Missense	–	–	–
P25	Persian	M	9	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C[a] (p.R272P) Missense	–	–	–
P26	Persian	M	4	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C[a] (p.R272P) Missense	–	–	–
P27	Saudi Arabian	M	17	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C[a] (p.R272P) Missense	–	–	–
P28	Saudi Arabian	M	24	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C[a] (p.R272P) Missense	–	–	–
P29	Pakistani	M	3	Palmoplantar hyperkeratosis, periodontitis	c.815G>C[f] (p.R272P) Missense	–	–	–
P30	Turkish	M	20	Palmoplantar hyperkeratosis, severe periodontitis, corneal leukoma	No CatC mutation[a]	+	+	+
P31	French	M	16	Palmoplantar psoriasiform appearance, anomalies of deciduous teeth with no net periodontitis	No CatC mutation[a]	+	+	+

[a] Cathepsin C mutations identified in this work. [b] Ragunatha *et al.*, 2015 [39]. [c] Farkas *et al.*, 2013 [43]. [d] Identified by Professor N. Thakker, Academic Unit of Medical Genetics, St Mary's Hospital, Manchester UK. [e] Kanthimathinathan *et al.*, 2013 [8]. [f] Taibjee *et al.*, 2005 [26]. [g] Bullon *et al.*, 2014 [44]. [h] Schacher *et al.*, 2006 [45]. [i] Noack *et al.*, 2008 [46]. [j] Xinwen *et al.* 2015 [47].

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.

Experimental procedures

Reagents

Recombinant CatC and APN were from Unizyme Laboratories (Hørsholm, Denmark) and R&D Systems (Lille, France), respectively. Antibodies against CatC included: mouse mAb (Ab1) (Santa Cruz Biotechnology, Heidelberg, Germany), goat polyclonal antibody (Ab2) (R&D Systems), and goat polyclonal antibody (Ab3) (Everest Biotech, Oxforshire, UK). The mouse mAb against APN was from Santa Cruz Biotechnology. Gly-Phe-AMC was from Enzyme Systems Products (Illkirch, France) and Ala-AMC was from Bachem (Weil am Rhein, Germany). The cysteine proteinase inhibitor E64c [(2S,3S)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane)] was from Sigma-Aldrich (St Louis, MO, USA), the specific inhibitor of CatC, Thi-Phe-CN [(β-2-thienyl)-L-alanyl-L-phenylalanine nitrile)], was provided by Dr Adam Lesner (University of Gdansk, Poland). EDTA was from Merk (Darmstadt, Germany), and bestatin, the specific inhibitor of APN, was from Santa Cruz Biotechnology (Heidelberg, Germany).

Urine collection

Urine samples were collected from 31 PLS patients from European countries (France, Germany, the UK, Hungary,

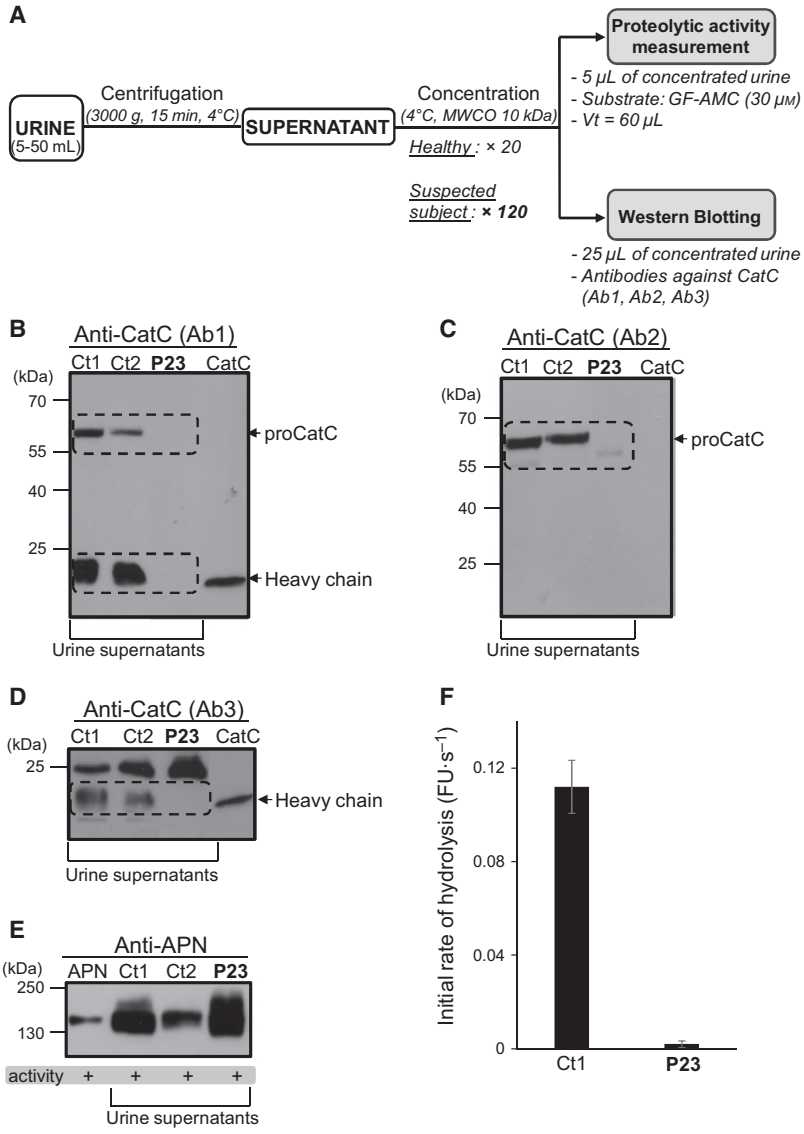

Fig. 3. Absence of CatC in urine from a PLS patient. (A) Protocol for analysis of urinary CatC. After receipt of PLS samples, urine samples were centrifuged to eliminate cells and debris, and then concentrated 120 times, i.e. six times more than the urine of healthy subjects, to analyze the presence and the proteolytic activity of CatC as described in Experimental procedures. (B) Immunoblots of 20-fold-concentrated urine samples collected from two healthy control subjects (controls Ct1 and Ct2) and of 120-fold-concentrated urine samples collected from one representative PLS patient (P23), with Ab1, (C) Ab2, (D) Ab3, and (E) antibody against APN. (F) CatC activity in 20-fold-concentrated urine samples from one healthy control subject (Ct1) and of 120-fold-concentrated urine samples collected from one representative PLS patient (P23), determined with the fluorogenic substrate Gly-Phe-AMC (30 µM). Recombinant CatC and APN were used as controls. MWCO, molecular weight cutoff; FU, fluorescence units.

Italy, and Turkey), from Asian countries (China, India, Iran, and 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 the local Ethics Review Boards for the 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 with Vivaspin 15R concentrators (molecular mass cutoff, 10 kDa; Sartorius, Goettingen, Germany) and stored at 4 °C.

Western blotting

Briefly, urine samples were electrophoretically separated on a 10% SDS/PAGE gel under reducing conditions for CatC analysis and under nonreducing conditions for APN analysis, and then transferred to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). After saturation, membranes were incubated with antibodies against CatC (Ab1 and Ab3, diluted 1 : 500; Ab2, diluted 1 : 1000) or with mAb against APN (diluted 1 : 500). After washing, membranes were incubated with peroxidase-conjugated anti-mouse IgG (diluted 1 : 10 000) or anti-goat IgG (diluted 1 : 20000) (Sigma-Aldrich), as appropriate. Bound antibodies were detected by chemiluminescence (ECL Plus Western Blotting Kit Detection Reagents; GE Healthcare, Illkirch, France), according to the manufacturer's instruction.

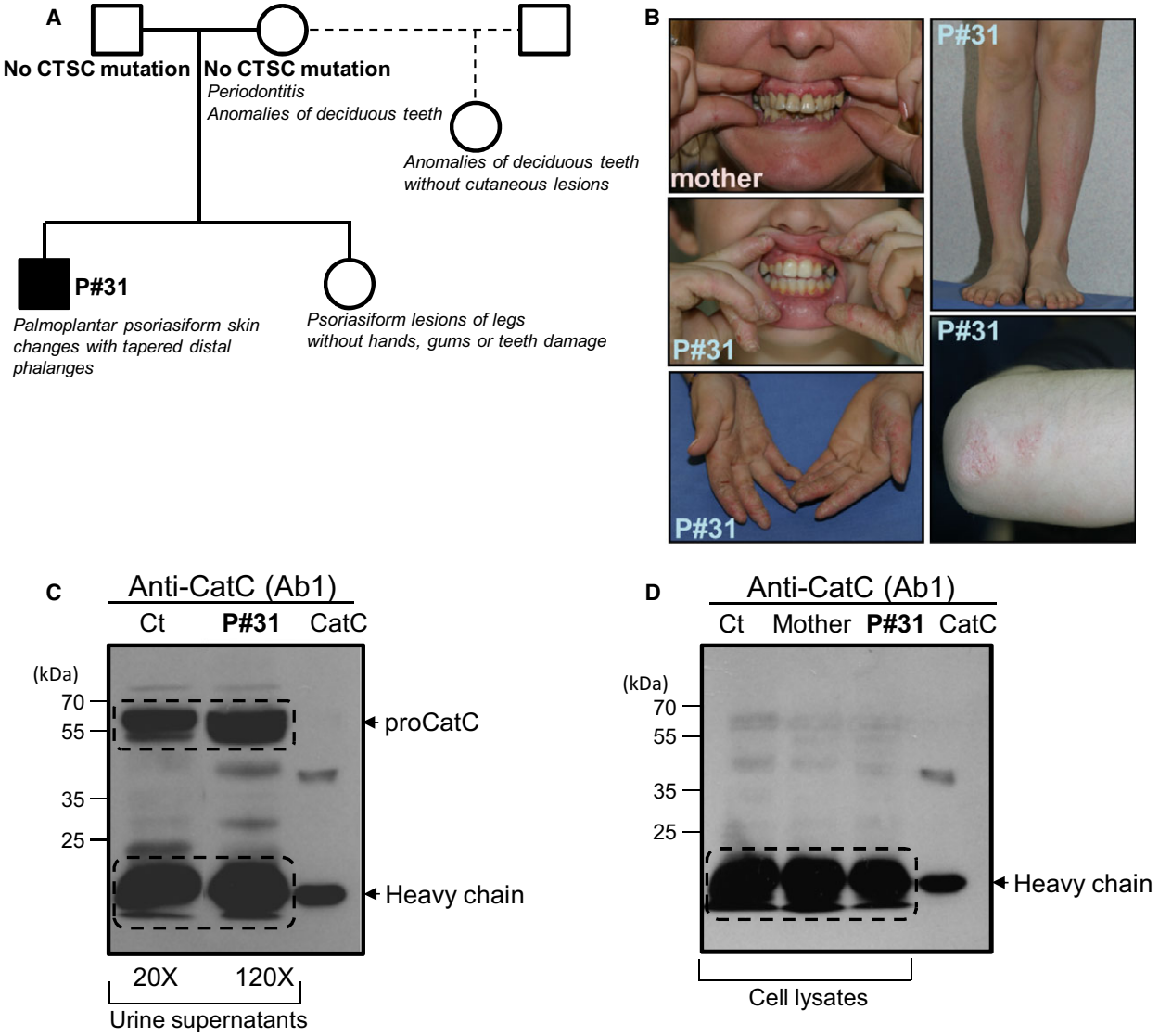

Fig. 4. Presence of CatC in urine from an atypical French patient without CTSC mutation. (A) Pedigree of a 16-year-old individual (P31) with suspected PLS. (B) The dermatological and dental features of the patient (P31) and his mother. (C) Immunoblotting analysis with Ab1 of 120-fold-concentrated urine samples from the patient (P31) as compared with 20-fold-concentrated urine samples from a healthy control subject (Ct). (D) Western blotting of white blood cell lysates from the patient (P31), the patient's mother and a healthy control subject (Ct) with Ab1. Recombinant CatC was used as a control.

Enzyme assays

Assays were carried out at 37 °C in 50 mM sodium acetate, 30 mM NaCl, 1 mM EDTA and 2 mM DTT (pH 5.5) for CatC, and in 50 mM Tris (pH 7) for APN. Proteolytic activity was measured in 5 μL of urine supernatants with 30 μM Gly-Phe-AMC in a total volume of 60 μL for CatC, or with 50 μM 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 by incubating urine samples with the selective CatC inhibitor Thi-Phe-CN [41] prior to substrate addition. Recombinant CatC and APN were used as controls. 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 to 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 or not supplemented with 10% FBS, at 37 °C. Martin–Darby

canine kidney epithelial cells were cultured in Eagle's minimum essential medium, supplemented or not supplemented with 10% FBS, at 37 °C. Cells were lysed in PBS containing 0.5% Nonidet P-40 (IGEPAL 630), and, after centrifugation (10 000 ***g***, 15 min, 4 °C), the lysate was stored at −20 °C. Cell supernatants were concentrated 30 times with Vivaspin 15R concentrators (molecular mass cutoff, 10 kDa; Sartorius). Proteins in lysates and in supernatants were quantified with a bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA).

Genetic investigations

Patient's genomic DNA and, when available, parent's DNA were extracted from EDTA blood samples with EVO100 ReliaPrep (Tecan, Promega, Lyon, France).

For PCR and sequencing reactions, *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 with 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 Human Genome Variation Society recommendations.

A deletion search was performed with custom array comparative genomic hybridization: custom microarrays (8000 × 60 000) were designed with e-array web software (Agilent Technologies, Waghauesel-Wiesental, Germany), with the similarity score filter in order to select highly specific probes. A total of 3141 probes were distributed: approximately one probe every 100 bp in *CTSC* and 50 kb around the gene, and then one probe every 350 bp in the 300 kb region either side. DNA was labeled (cyanine 3 or cyanine 5) with 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 PLS. Scanning of the microarrays was performed with a G2565CA scanner (Agilent Technologies). Data analysis was carried out with software 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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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

B. Korkmaz, Y. Hamon and M. Legowska planned experiments; Y. Hamon, M. Legowska, P. Fergelot, S. Dallet-Choisy, L. Vanderlynden and C. Goizet performed experiments; B. Korkmaz, F. Gauthier, D. E. Jenne, Y. Hamon, M. Legowska, P. Fergelot and S. Dallet-Choisy analyzed data; B. Korkmaz, F. Gauthier, D. E. Jenne, Y. Hamon, and C. Moss wrote the paper; B. Korkmaz supervised the project. All other authors contributed samples or other essential material (urine/blood).

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