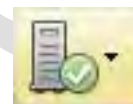


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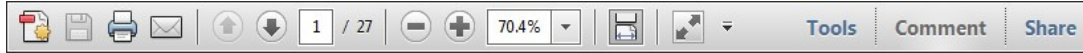


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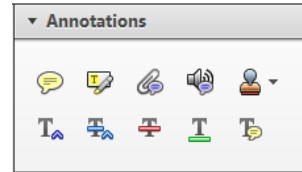
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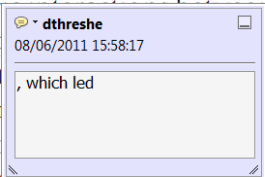


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standard framework for the analysis of microeconomic activity. Nevertheless, it also led to the development of a number of strategic approaches. The number of competitors in an industry is that the structure of the industry is a main component. At the industry level, are externalities important? (Mankiw henceforth) we open the 'black b



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there is no room for extra profits as mark-ups are zero and the number of firms (net) values are not determined by market clearing. Blanchard ~~and Kiyotaki~~ (1987), perfect competition in general equilibrium. The effects of aggregate demand and supply shocks in a classical framework assuming monopolistic competition and an exogenous number of firms

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dynamic responses of mark-ups consistent with the VAR evidence

sation by Markov processes. The number of competitors and the impact on the structure of the sector is that the structure of the sector



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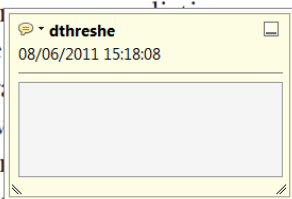


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and supply shocks. Most of the time, the number of competitors and the impact on the structure of the sector is that the structure of the sector



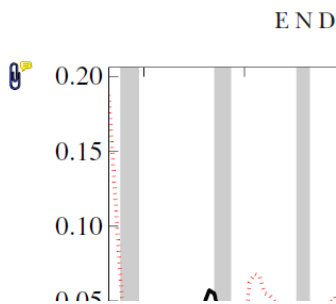
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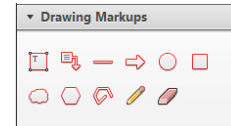
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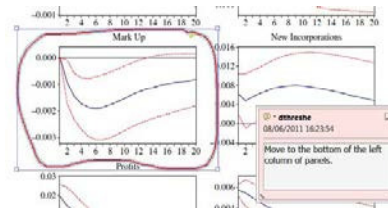


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# Analysis of urinary cathepsin C for diagnosing Papillon–Lefèvre syndrome

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## Keywords

cathepsin C; diagnostic method; Papillon–Lefèvre syndrome; protease; urine analysis

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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 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

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technique. All 75 urine samples from healthy control subjects (aged 3 months to 80 years) contained proteolytically active CatC and its pro-form, 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 non-covalent 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 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

## Abbreviations

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

urine could be utilized as an early, simple, reliable and low-cost diagnostic technique for PLS.

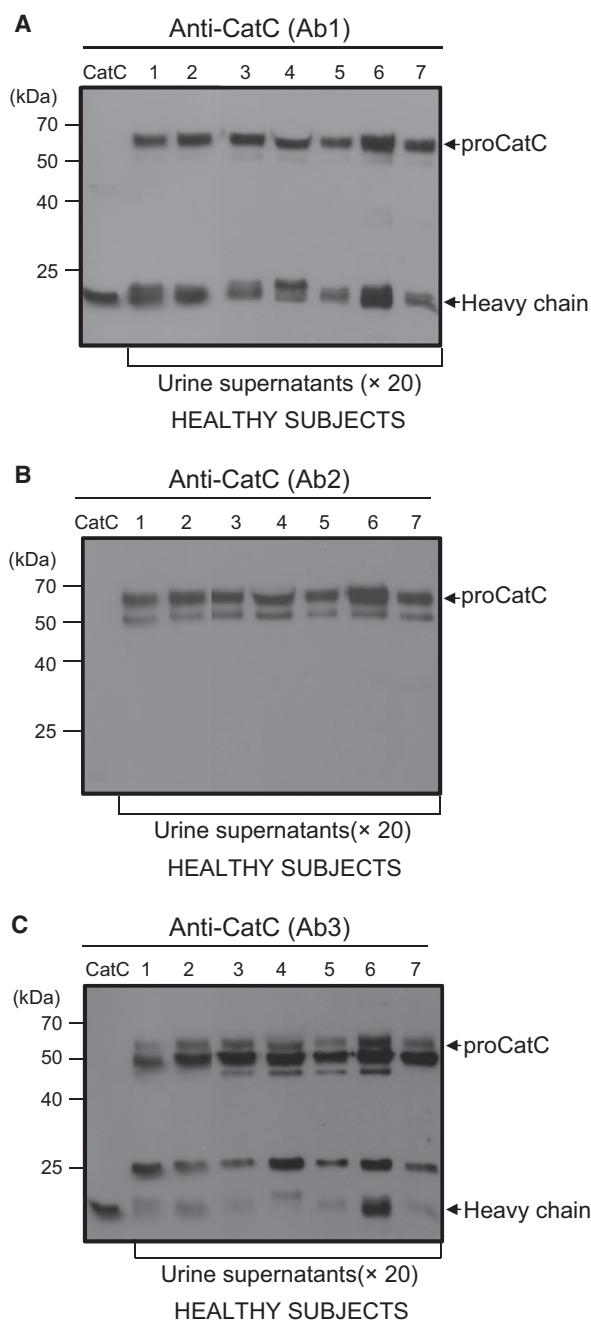
## Results

### 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 all peptidic 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 (not shown).

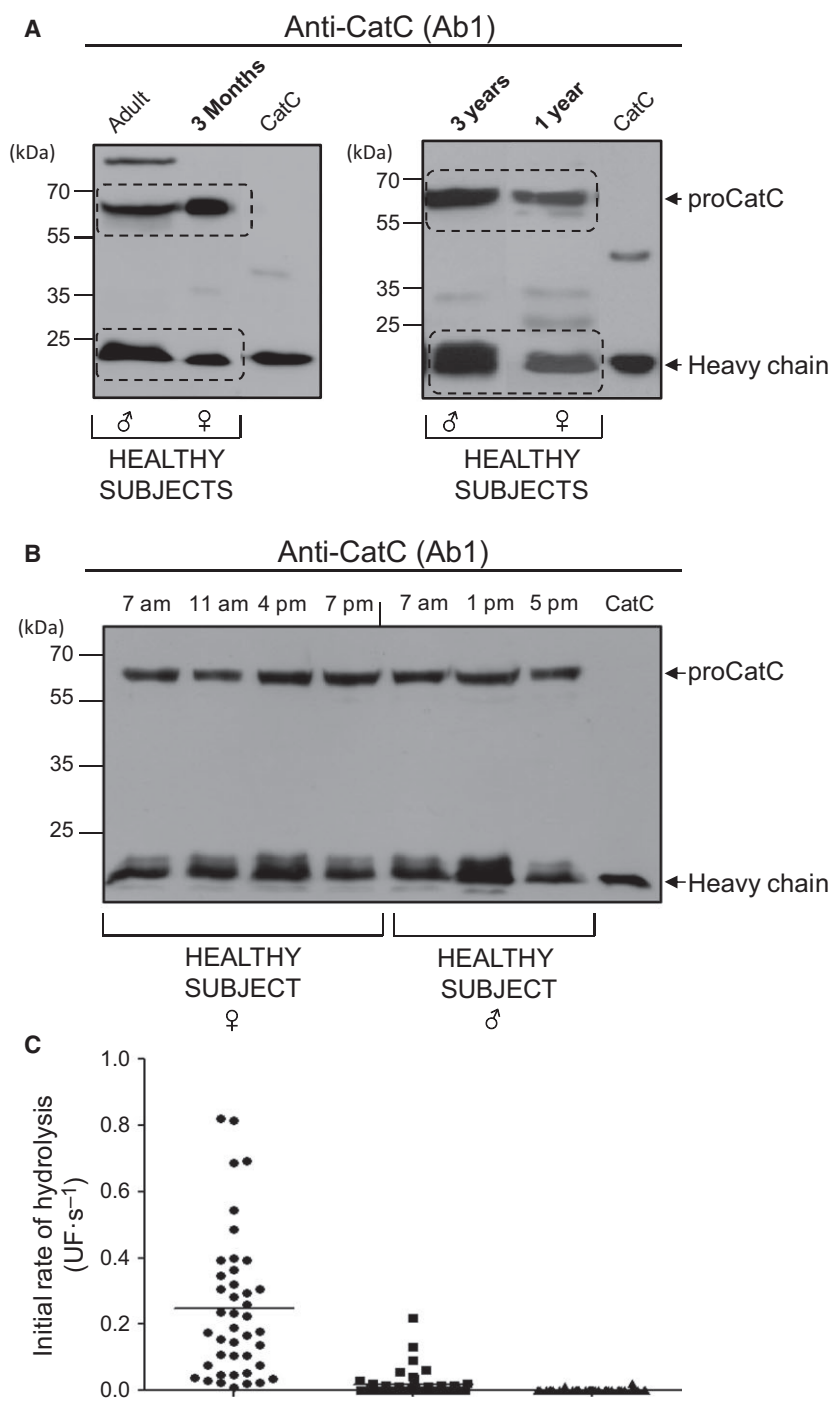
### CatC in urine of patients with a PLS phenotype

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 ( $\times 120$ ), 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 either nonsense, frameshift or missense



**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.

mutations in *CTSC* (Table 1). In spite of the higher concentration of PLS urine samples ( $\times 120$  versus  $\times 20$  in controls), no proCatC or active CatC was



**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  $\mu$ M), and its inhibition by E64c (100  $\mu$ M) and Thi-Phe-CN (5  $\mu$ M). UF.

4

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

1 genetic analysis was therefore performed, but no  
2 mutation of *CTSC* was found, indicating that he did  
3 not suffer from classic PLS. The second CatC-positive  
4 patient was a 16-year-old French teenager (P31) who  
5 had developed palmoplantar psoriasiform skin  
6 changes with tapered distal phalanges as observed in  
7 Haim–Munk syndrome (Fig. 4A,B). Again, the genetic  
8 analysis did not reveal any loss-of-function mutation  
9 in *CTSC*. Urinary CatC activity was fully inhibited by  
10 the specific CatC inhibitor Thi-Phe-CN. The urine of  
11 the mother with periodontitis and anomalies of  
12 deciduous teeth (Fig. 4C) also contained proCatC and  
13 mature CatC (Fig. 4D). Mature CatC was present in  
14 a lysate of white blood cells from the son and his  
15 mother, at levels similar to those in a healthy subject.  
16 Furthermore, proteolytically active elastase-like pro-  
17 teinases were present in white blood cell lysates, indi-  
18 cating that functional CatC was present in cells and  
19 tissues (data not shown).

## 21 Discussion

23 Clinical signs of classic PLS become apparent by the  
24 age of 1–5 years, when dry, scaly patches appear on  
25 the skin of the palms and soles, and severe inflamma-  
26 tion starts to affect the surrounding gingiva and bone  
27 of primary teeth, leading to their rapid loss. Bacterial  
28 skin infections such as abscesses may also occur [26].  
29 The genetic etiology of PLS has been clearly estab-  
30 lished by sequencing of *CTSC* [1,3,12] and whole  
31 exome sequencing [27]. However, it still remains  
32 unclear why a lack of CatC activity leads to specific  
33 dermatological lesions and severe destructive periodon-  
34 titis. A plausible explanation for the latter is deficiency  
35 of the antimicrobial peptide LL-37 in the gingiva,  
36 allowing for infection with *Aggregatibacter actino-*  
37 *mycetemcomitans* and the development of severe peri-  
38 odontal disease [28]. Proteinase 3 releases this peptide  
39 from a precursor molecule after neutrophil activation  
40 [29]. The lack of CatC activity in PLS patients almost  
41 completely blocks the activity of neutrophil serine pro-  
42 teases and reduces the levels of their proforms in neu-  
43 trophils [21,30,31]. On the other hand, mutations in  
44 completely different genes might induce a PLS-like  
45 syndrome with similar dermatological lesions and  
46 destructive periodontitis, but very different with regard  
47 to comorbidities and optimal treatment regimens [32].  
48 Several sporadic cases with a PLS-like clinical pheno-  
49 type have been reported in the literature [13,33,34],  
50 and two examples of a PLS-like syndrome have been  
51 included in this study. Because of the variable manifes-  
52 tations of the classic PLS syndrome, elucidation of its  
53 phenotypic variability requires functional and

biochemical assays as well as *CTSC* analysis. Other-  
wise, 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 surpris-  
ing 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 intracellu-  
lar 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 imple-  
mentation of a strict oral hygiene regimen can mini-  
mize 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]. Demon-  
strating 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 popu-  
lations 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 phe-  
notype–genotype correlation in PLS and overlapping  
syndromes. Such a simple, rapid and low-cost screen-  
ing test based on CatC excretion in the urine of chil-  
dren and newborns can now be developed for early  
diagnosis and timely therapy to prevent aggressive  
periodontitis. When available, the test should take a



**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.

Patients	Ethnicity	Gender	Age (years)	Clinical manifestations	Mutation	Urinary CatC		
						ProCatC	Mature CatC	CatC activity
P1	French	F	55	Palmoplantar hyperkeratosis, severe periodontitis	c.96T>G <sup>a</sup> (p.Y32X) Nonsense	–	–	–
P2	Indian	M	15	Transgradient palmoplantar keratoderma, periodontitis	c.912C>A <sup>b</sup> (p.Y304X) Nonsense	–	–	–
P3	Hungarian	F	4	Palmoplantar hyperkeratosis, periodontitis	c.681delCATA <sup>c</sup> (p.T188fsX199) Frameshift	–	–	–
P4	Hungarian	F	13	Palmoplantar hyperkeratosis, severe periodontitis	c.681delCATA <sup>c</sup> (p.T188fsX199) Frameshift	–	–	–
P5	Pakistani	F	14	Palmoplantar hyperkeratosis, periodontitis	(p.W433L) <sup>d</sup> Missense	–	–	–
P6	Pakistani	M	9	Palmoplantar hyperkeratosis, periodontitis, cerebral abscess	c.815G>C <sup>e</sup> (p.R272P) Missense	–	–	–
P7	Pakistani	F	13	Palmoplantar hyperkeratosis, periodontitis	c.815G>C <sup>e</sup> (p.R272P) Missense	–	–	–
P8	Pakistani	M	15	Palmoplantar hyperkeratosis, periodontitis	c.815G>C <sup>f</sup> (p.R272P) Missense	–	–	–
P9	Italian	F	42	Palmoplantar hyperkeratosis, severe periodontitis	c.1141delC <sup>g</sup> (p.L381fsX393) Frameshift	–	–	–
P10	Hungarian	F	12	Palmoplantar hyperkeratosis, severe periodontitis	c.901G>A <sup>a</sup> (p.G301S) Missense	–	–	–
P11	Hungarian	F	4	Palmoplantar hyperkeratosis, severe periodontitis	c.901G>A <sup>a</sup> (p.G301S) Missense	–	–	–
P12	Erythrean	M	12	Palmoplantar hyperkeratosis, severe periodontitis, tinea capitis	c.755A>T <sup>h</sup> (p.Q252L) Missense	–	–	–
P13	Erythrean	M	15	Palmoplantar hyperkeratosis, severe periodontitis	c.755A>T <sup>h</sup> (p.Q252L) Missense	–	–	–
P14	Moroccan	M	19	Mild palmoplantar hyperkeratosis, severe periodontitis	c.854C>T <sup>i</sup> (p.P285L) Missense	–	–	–
P15	Moroccan	M	35	Palmoplantar hyperkeratosis, severe periodontitis, edentulous by now	c.854C>T <sup>i</sup> (p.P285L) Missense	–	–	–
P16	German	F	24	Palmoplantar hyperkeratosis, severe periodontitis, edentulous by now	c.566–572Del <sup>i</sup> (T189FS199X) Frameshift	–	–	–
P17	German	M	48	Severe palmoplantar hyperkeratosis, late onset of severe periodontitis (22 years of age), liver abscess	Completely heterozygous <sup>i</sup> c.322A>T/c.436delT (p.K108X/ p.S146fs153X) Nonsense/frameshift	–	–	–
P18	German	M	27	Palmoplantar hyperkeratosis, severe periodontitis	Completely heterozygous <sup>i</sup> c.947T>G (p.L316R) c.1268G>C (p.W423S) Missense	–	–	–

5

**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 <sup>l</sup> (p.R132G) Missense	–	–	–
P20	Chinese	M	22	Palmoplantar psoriasiform appearance, severe periodontitis	c.394 C>G <sup>l</sup> (p.R132G) Missense	–	–	–
P21	Mauritian	M	5	Palmoplantar hyperkeratosis, periodontitis	n.i	–	–	–
P22	Mauritian	F	15	Palmoplantar hyperkeratosis, periodontitis	n.i	–	–	–
P23	Puerto Rican	F	31	Palmoplantar hyperkeratosis, severe periodontitis	c.116G>C <sup>a</sup> (p.W39S) Missense	–	–	–
P24	Persian	F	33	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C <sup>a</sup> (p.R272P) Missense	–	–	–
P25	Persian	M	9	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C <sup>a</sup> (p.R272P) Missense	–	–	–
P26	Persian	M	4	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C <sup>a</sup> (p.R272P) Missense	–	–	–
P27	Saudi arabian	M	17	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C <sup>a</sup> (p.R272P) Missense	–	–	–
P28	Saudi Arabian	M	24	Palmoplantar hyperkeratosis, severe periodontitis	c.815G>C <sup>a</sup> (p.R272P) Missense	–	–	–
P29	Pakistani	M	3	Palmoplantar hyperkeratosis, periodontitis	c.815G>C <sup>f</sup> (p.R272P) Missense	–	–	–
P30	Turkish	M	20	Palmoplantar hyperkeratosis, severe periodontitis, corneal leukoma	No mutation <sup>a</sup>	+	+	+
P31	French	M	16	Palmoplantar psoriasiform appearance, anomalies of deciduous teeth with no net periodontitis	No mutation <sup>a</sup>	+	+	+

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

few minutes and will be manageable by any technician at the hospital laboratory.

## Experimental procedures

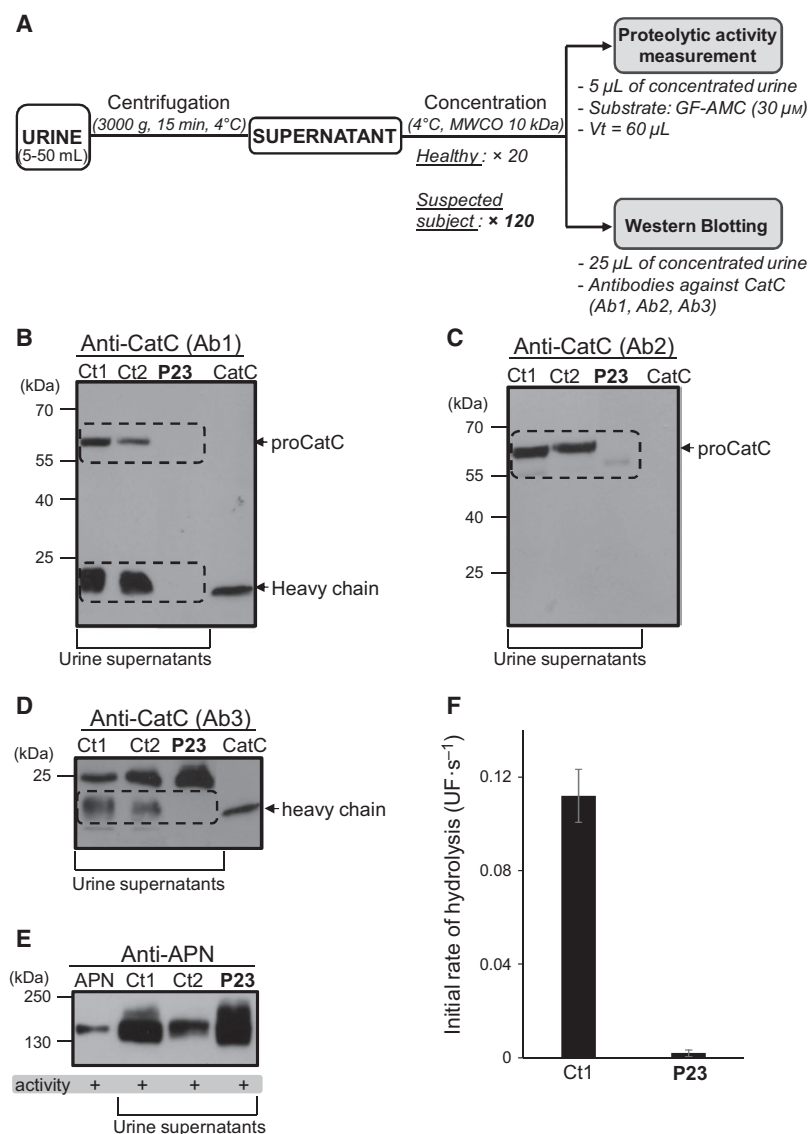
### Reagents

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, Oxfordshire, UK). The mouse mAb against APN was from Santa Cruz Biotechnology. Gly-Phe-AMC was from

Enzyme Systems Products (Illkirch, France) and H-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 Lesner (University of Gdansk, Poland). EDTA was from Merk (Darmstadt, Germany), and bestatin, the specific inhibitor of APN, was from Santa Cruz (Heidelberg, Germany).

### Urine collection

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



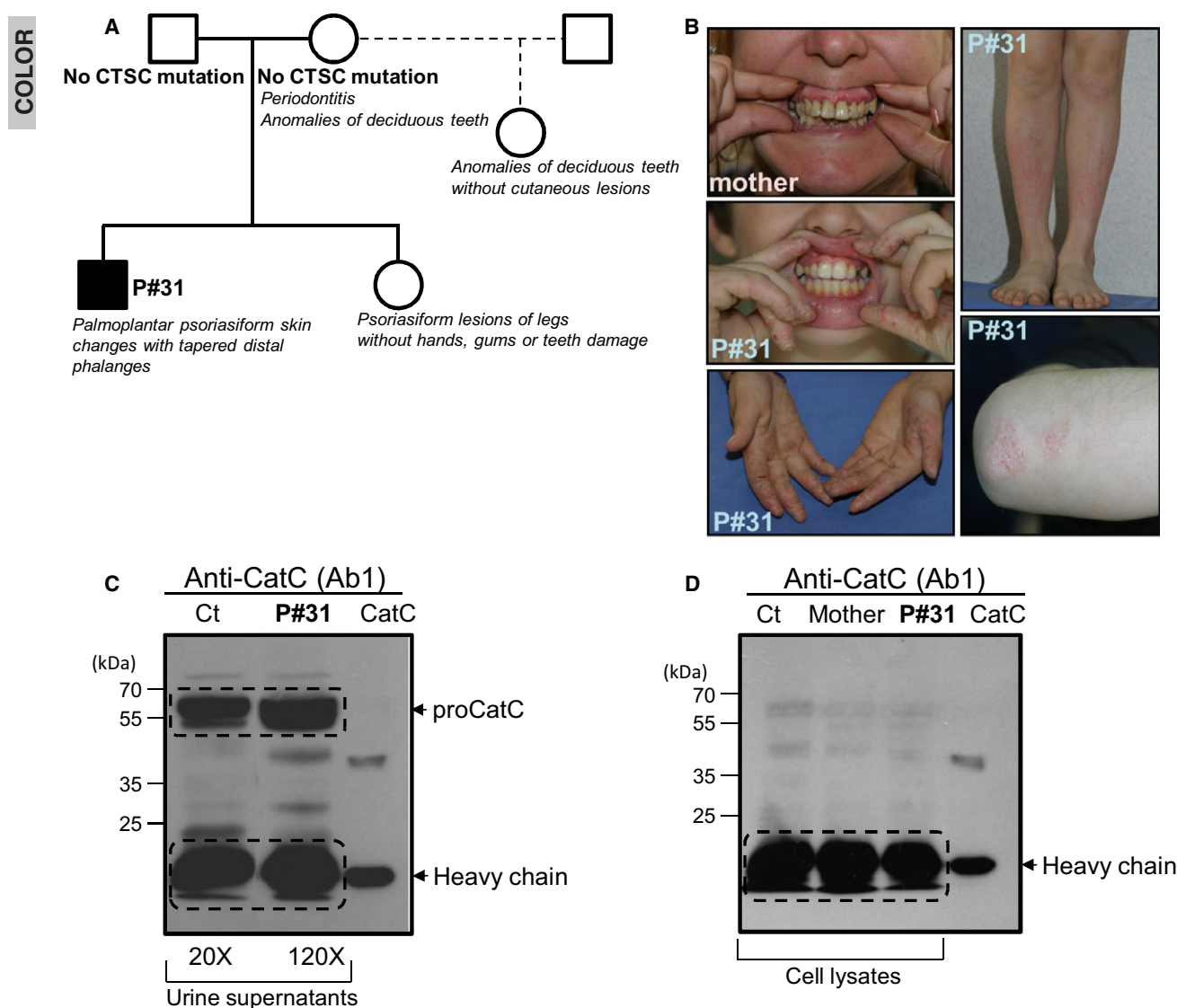
**Fig. 3.** Absence of CatC in urine from a PLS patient. (A) Protocol for analysis of urinary CatC. After receipt of PLS samples, urine samples were centrifuged to eliminate cells and debris, and then concentrated 120 times, i.e. six times more than the urine of healthy subjects, to analyze the presence 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  $\mu$ M). Recombinant CatC and APN were used as controls. MWCO, molecular weight cutoff; UF.

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, UK), 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 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 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 **11** EVO100 ReliaPrep (Tecan, Promega).

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 **12** (Agilent Technologies, CA, USA), 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 **13** then one probe every 350 bp in the 300-kb regions apart. 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 CZ performed **14** 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 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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