The impact of peptidase activity on clear cell renal cell carcinoma survival

Gorka Larrinaga,1,2,5 Lorena Blanco,2,5 Begoña Sanz,2,5 Itxaro Perez,1,2,5 Javier Gil,2,5 Miguel Unda,3 Leire Andrés,4,5 Luis Casis,2 and José I. López4,5

1Department of Nursing I, School of Nursing, University of the Basque Country (UPV/EHU), Bizkaia, Spain; 2Department of Physiology, Faculty of Medicine and Odontology, University of the Basque Country (UPV/EHU), Bizkaia, Spain; 3Department of Urology, Basurto University Hospital, Bizkaia, Spain; 4Department of Pathology, Cruces University Hospital, Bizkaia, Spain; and 5BioCruces Research Institute, Bizkaia, Spain

Submitted 21 August 2012; accepted in final form 25 September 2012

RENAL CANCER ACCOUNTS ROUGHLY for 4% of adult malignancies, with over 64,000 new cases and 13,500 deaths expected in 2012 in the United States (52). Epidemiological data reveal that its incidence has been increasing steadily in Europe and in the United States during the last few years (16, 52). By contrast, the lowest rates of renal cancer incidence are reported in Africa and Asia (52). The 2004 WHO classification of renal carcinomas in adults recognizes a wide spectrum of histological subtypes, harboring some of their distinct molecular signatures (31). Among them, clear cell renal cell carcinoma (CCRCC), a distinct neoplasm related to chromosome 3 disorders, is by far the commonest histological subtype of renal cell carcinomas, accounting for >80% of cases (11).

Renal cell carcinomas still continue to be a health problem of major concern, not only owing to their relative frequency in Western countries but also because of their well-known traditional resistance to current chemo- and radiotherapy protocols. Albeit promising results have been obtained very recently with immunotherapy and other targeted therapies, surgery still remains the only treatment with a significant impact on survival (36). Survival is, in this context, an issue related to early diagnosis, and huge resources are being invested to discover renal cell carcinomas confined to the organ (30) because prognosis in these cases is significantly better.

Apart from the clinical approach, many other strategies are underway to know more about the biology of renal cell carcinomas and find useful clues to improving life expectancy of patients with this neoplasm. Among them, angiogenesis, local invasiveness, and metastatic potential significantly impact the prognosis of disease (19). Peptidases and matrix metalloproteinases (MMPs) play a key role in these processes, regulating peptides involved in cell signaling, angiogenesis, and extracellular matrix degradation (6, 9). These enzymes are present in most human tissues including renal parenchyma, where they play distinct physiological roles (18, 38, 54).

The expression and activity patterns of different proteases vary in malignant tumors and are correlated with tumor aggressiveness and poor outcome (6, 9). Changes in the expression of neutral endopeptidase (NEP, also known as CD13), aminopeptidase N (APN, also known as CD13), and dipeptidyl peptidase IV (DPP-IV, also known as CD26) in tumor tissue and serum of lung, colorectal, and pancreatic carcinomas have been associated with worse survival rates and poor prognoses (12, 14, 20, 22, 37, 55). With regard to kidney neoplasms, MMP 2, 7, 9, and 10 influence the survival of patients with renal cell carcinoma and have been proposed as prognostic markers and therapeutic targets in these tumors (35, 43, 48). The expression of other proteases such as kallikreins is inversely correlated with renal cancer survival (41). Peptidases have not been correlated so far to renal cell carcinoma survival.

NEP, APN, and DPP-IV have been proposed as molecular markers of kidney neoplasms (18, 25, 56). Our previous studies (4, 5, 27–29, 58, 59) have demonstrated that the activity, protein expression, and mRNA levels of these three and other peptidases are selectively altered in several subtypes of renal cell neoplasms (CCRCC, papillary renal cell carcinoma, chromophobe renal cell carcinoma, and renal oncocytoma). What is more, we have found that several peptidase activities are significantly increased in high-stage and high-grade CCRCC, suggesting the involvement of these enzymes in the biological aggressiveness of these neoplasms (4, 27, 29, 59).

The aim of this study was to define the impact of nine peptidases measured by fluorometric assay on the survival of patients with CCRCC and give additional information for the prognostic assessment of these patients.

METHODS

Patients. All the experiments carried out in this study comply with current Spanish and European Union legal regulations.
Table 1. Peptidase activities of membrane-bound and soluble fractions and CCRCC patient survival (Log-rank test)

<table>
<thead>
<tr>
<th>Peptidase Activities of Membrane-Bound Fraction with Cutoff Values</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>APN, 5,500 UP/mg protein</td>
<td>0.029</td>
</tr>
<tr>
<td>NEP, 2 UP/mg protein</td>
<td>0.496</td>
</tr>
<tr>
<td>DPPIV, 8,000 UP/mg protein</td>
<td>0.867</td>
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<tr>
<td>CAP, 1,300 UP/mg protein</td>
<td>0.088</td>
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<tr>
<td>mAPB, 2,500 UP/mg protein</td>
<td>0.315</td>
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<tr>
<td>mASP, 850 UP/mg protein</td>
<td>0.323</td>
</tr>
<tr>
<td>mPGI, 100 UP/mg protein</td>
<td>0.2</td>
</tr>
<tr>
<td>mPEP, 500 UP/mg protein</td>
<td>0.549</td>
</tr>
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</table>

Peptidase Activities of Soluble Fraction with Cutoff Values | P  |
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>PSA, 21,000 UP/mg protein</td>
<td>0.059</td>
</tr>
<tr>
<td>APB, 7,000 UP/mg protein</td>
<td>0.046</td>
</tr>
<tr>
<td>ASP, 175 UP/mg protein</td>
<td>0.102</td>
</tr>
<tr>
<td>PGI, 100 UP/mg protein</td>
<td>0.494</td>
</tr>
<tr>
<td>PEP, 3,500 UP/mg protein</td>
<td>0.096</td>
</tr>
<tr>
<td>sAPN, 3,400 UP/mg protein</td>
<td>0.015</td>
</tr>
<tr>
<td>sCAP, 1,000 UP/mg protein</td>
<td>0.027</td>
</tr>
<tr>
<td>sDPPIV, 1,300 UP/mg protein</td>
<td>0.012</td>
</tr>
</tbody>
</table>

CCRCC, clear cell renal cell carcinoma. See the text for peptidase definitions. Statistically significant results appear in bold (Log-rank test; P < 0.05).

Tumor and non-tumor renal tissue were analyzed in a prospective series of 79 consecutive nephrectomies in CCRCC. Males largely predominated in the series (63 M/16 F), the average age being 62 yr (range, 26–80 yr). AJCC (15) and Furhman’s (17) method were predominated in the series (63 M/16 F), the average age being 62 yr (range, 26–80 yr). AJCC (15) and Furhman’s (17) method were applied to assign stage and grade, respectively. A total of 51 cases (64.5%) were low-stage (organ confined, pT1/2) tumors, and 28 (35.5%) high-stage (non-organ confined, pT3/4). Grade assignment was balanced in the series, with 40 cases being low grade (G1/2) and 39 high grade (G3/4). Clinical follow-up was closed on December 31, 2011, and a total of 22 patients (27.8%) had died of disease by that time. Mean follow-up was 63.3 months (range, 4–131 months).

**Tissue specimens.** Current protocols for handling tumor-containing kidney specimens (10) were followed in all cases. Cases were received fresh in the pathology laboratory and represented tumor tissue samples of surgical specimens. Selected tissue was frozen with isopentane and stored at −80°C within the first 30 min of removal until the enzyme assays were performed. Nephrectomies were sliced and fixed in formalin for 24 h. Tissue sampling for paraffin embedding was performed as previously reported (10).

**Sample preparation.** Explanted tissue samples were homogenized in 10 mM Tris-HCl buffer at pH 7.4 for 30 s at 800 rpm using a Heidolph PZR 50 Selecta homogenizer and ultracentrifuged in a Centrikon-T-2070 Kontron Instruments apparatus at 100,000 g for 35 min. The resulting supernatants were used to measure soluble enzyme activities: aminopeptidase B (APB), aspartyl aminopeptidase (ASP), pyroglutamyl peptidase I (PGI), prolinal endopeptidase (PEP), puromycin-sensitive aminopeptidase (PSA), sAPN, cystinyl aminopeptidase (sCAP), and sDPPIV. To avoid contamination with soluble enzymes, the resulting pellets were washed three times by suspension in 10 mM Tris-HCl buffer at pH 7.4. The pellets were then homogenized in 10 mM Tris-HCl buffer at pH 7.4 and centrifuged at low speed (800 g) for 3 min to purify the samples. The supernatants thus obtained were used to determine membrane-bound enzyme activities: APN, cystinyl aminopeptidase (CAP), DPPIV, neutral endopeptidase (NEP), mAPB, mASP, mPGI, and mPEP. All steps were carried out at 4°C.

**Enzyme assays.** Peptidase activities were measured by incubating samples with a saturating (0.125 mM) concentration of fluorogen-derivatized substrates following the method described by Yoshimoto et al. (61), Mantle et al. (32), and Silveira et al. (53). Alanine aminopeptidase activities (PSA and APN) were measured in triplicate using Ala-naphthylamide as a substrate. Incubations with the specific

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**A**

**B**

Multivariate analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fuhrman’s grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (G1-G2)</td>
<td>1.056&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.911&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>High (G3-G4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
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<td></td>
</tr>
<tr>
<td>Low (T1-T2)</td>
<td>1.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.333&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>High (T3-T4)</td>
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<td></td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 7 cm</td>
<td>1.522&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.365&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>&gt; 7 cm</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>APN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 5500 UP/mg prot</td>
<td>2.155&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.123&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>&gt; 5500 UP/mg prot</td>
<td>2.658&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.035&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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Fig. 1: A: patient survival curves according to membrane-bound aminopeptidase N (APN/CD13) activity levels (Kaplan-Meier method). B: variables associated with survival in multivariate analysis (Cox regression model) with APN. a: Results in the first step of the backward conditional method. None of the 4 variables reached statistical significance. b: Result of the final step, where only the APN/CD13 appears as an explanatory variable in the regression model.
PSA inhibitor puromycin (40 μM) were performed in parallel to discriminate between the PSA and APN forms of total alanine aminopeptidase activity. DPPIV and PEP activities were assayed using H-Gly-Pro-β-naphthylamide and Z-Gly-Pro-β-naphthylamide, respectively. A NEP assay was performed by incubating samples with a saturating concentration of N-dansyl-t-Ala-Gly-pNO2-Phe-Gly (DAGNP; a dansyl derivative). APB (basic) and ASP activity was quantified with Arg-/H9252/H9252 (DAGNPG; a dansyl derivative). APB (basic) and ASP activity was quantified with Arg-β-naphthylamide and Asp-β-naphthylamide. Omega peptidase (PGI) activity was measured fluorometrically using pGlu-β-naphthylamide as a substrate. CAP activity was analyzed using t-cystine-di-β-naphthylamide. These assays are based on the fluorescence of products generated from substrate hydrolysis by the enzyme. Reactions were started by adding 30–50 μl of the sample to 1 ml of the appropriate incubation mixture depending on the enzyme and substrate analyzed as follows: PSA, APN, APB, PEP, and PGI activities (50 mM phosphate buffer at pH 7.4 for PSA, APN, and PGI activities, pH 6.5 for APB, and 0.125 mM aminoacyl-β-naphthylamide); and ASP, DPPIV, CAP, and NEP activities (50 mM Tris·HCl buffer at pH 7.4 for ASP and DPPIV, pH 5.9 for CAP, and 0.125 mM aminoacyl-β-naphthylamide or (D)AG(pN)PG). After 30-min incubation at 37°C, 1 ml of 0.1 M sodium acetate buffer (pH 4.2) was added to the mixture to terminate the reaction. The released product was determined by measuring the fluorescent intensity at 412 nm with excitation at 345 nm for β-naphthylamidine, and at 342 nm for (D)AG(pN)PG with a Shimadzu RF-540 spectrofluorometer. Blanks were used to determine background fluorescence. Relative fluorescence was converted into picomoles of product using a standard curve constructed with increasing concentrations of β-naphthylamine or (D)AG.

Protein determination. The protein concentration of soluble and membrane-bound fractions of kidney tissues was measured in triplicates of protein by the Bradford method (7), using BSA (1 mg/ml) as a calibrator. Results were recorded as units of peptidase (UP) per milligram of protein (UP/mg prot). One unit of peptidase activity is the amount of enzyme required to release one picomole of β-naphthylamine per minute. Fluorogenic assays were linear with respect to hydrolysis time and protein content.

Immunohistochemistry. Immunohistochemical studies for APN (NeoMarkers, clone 38C12, working dilution 1:80) were performed in an automated immunostainer (Dako Autostainer Link48) using formalin-fixed and paraffin-embedded material from a randomly selected group of 20 cases following routine protocols.

Statistical analysis. Kaplan-Meier curves and a log-rank test were performed for univariate analysis to compare survivals. A Cox regression multivariate analysis was used to test the independent effects of clinical and pathological variables and peptidase activity on survival. In the multivariate analysis, a stepwise selection procedure (backward conditional method) was used to select the final optimal model. Cutoff points for enzyme values are reflected in Table 1. Only classic histopathological parameters (grade, tumor diameter, and stage) were considered in the analysis. For such a purpose, Furhman grades were grouped as low (G1/2) and high (G3/4) grades, tumor diameter as ≤7 cm, >7 cm, and stage as low (pT1) and high (pT2–4) stages. A value of P < 0.05 was considered statistically significant. SPSS 19.0 software was used for the statistical analysis.

RESULTS

Table 1 shows the cutoff points for the analyzed peptidases and their corresponding log-rank test results.

APN. Five-year survival of patients with CCRCC was better (P = 0.029) when the membrane-bound activity of this enzyme was equal to the Bradford method (7), using BSA (1 mg/ml) as a calibrator. Results were recorded as units of peptidase (UP) per milligram of protein (UP/mg prot). One unit of peptidase activity is the amount of enzyme required to release one picomole of β-naphthylamine per minute. Fluorogenic assays were linear with respect to hydrolysis time and protein content.

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or lower than 5,500 UP/mg of protein (Fig. 1A). In the multivariate analysis (Fig. 1B), survival correlated with the activity of this fraction of this enzyme in the final step of the backward conditional method \((P = 0.035)\). In the same sense, 5-yr survival was better \((P = 0.015)\) when the soluble fraction was equal to or lower than 3,400 UP/mg of protein (Fig. 2A). Cox regression also showed that the soluble fraction of this peptidase was an independent factor of survival \((P = 0.02)\) (Fig. 2B).

By immunohistochemistry, APN was found in the membrane and cytoplasm of CCRCC and in luminal cells in the proximal convoluted tubule (Fig. 3).

**APB.** The analysis of this enzyme yielded results opposite those obtained with the rest of peptidases analyzed; that is, when its activity was equal or lower than 7,000 UP/mg protein the accumulated survival of CCRCC patients was significantly worse \((P = 0.046)\) with the Kaplan-Meier curves (Fig. 4A). Results did not reach a significant level in the Cox analysis (Fig. 4B). However, this enzyme remained as the sole explanatory parameter in the final step of the backward conditional method (Fig. 5B).

**DISCUSSION**

Several studies have proposed that protease expression and activity may have a predictive value for the survival of patients with renal cell carcinoma and, therefore, that these enzymes could be useful as prognostic tumor markers in this disease. Special attention has been focused on the analysis of MMPs (35, 43, 48), and little is known about the role of other proteases, such as peptidases, in the prognosis of renal cell carcinoma.

We started a prospective study on the activity and expression of a pool of peptidases in several histological subtypes of renal cancer (4, 5, 27–29, 58, 59). We observed that the activity of some peptidases like APN, CAP, and DPPIV was increased in high-grade and advanced stage CCRCC (4, 27, 29, 59). In this work, we show that 5-yr survival of patients with CCRCC was shorter if the activity of these three peptidases was high. By contrast, high activity levels of APB correlates with significantly longer 5-yr survival. These data suggest the involvement of peptidases in the biological aggressiveness of CCRCC and support the usefulness of these proteases as prognostic markers of this disease.

APN is a metalloprotease that catalyzes NH2-terminal amino acids removal from peptides, with a preference for neutral residues. APN is widely distributed in human tissues (42) and is highly expressed in the tubular renal system (18). We have found this enzyme in the proximal tubules of the nephron and in CCRCC, with strong immunoreaction in the membrane and mild in the cytoplasm. Owing that this enzyme is not expressed in distal nephron-derived tumors such as chromophobe renal cell carcinoma and renal oncocytoma (18), we suggest that APN could be a useful immunohistochemical marker in the differential diagnosis between these renal cell neoplasms, same as happens with NEP (65).

APN has been traditionally described as a membrane-bound peptidase, but some authors have also detected soluble forms in the cytosol (33), intratumoral fluids (57), and serum of patients with cancer (37). High activity levels of both membrane-bound and soluble fractions of APN appear in the present study as independent factors of poor prognosis. This finding agrees with those of other researchers who found an association of high activity levels of this enzyme with angiogenesis, tumor invasiveness, and worse survival (20, 22, 37, 55, 60). This enzyme is being investigated as a target for anticancer therapy (60).
Different approaches have been proposed: the direct inhibition of the enzyme with molecules like bestatin and the use of APN as a peptide-homing receptor in a malignant environment or as an enzymatic activator of prodrugs (21, 55, 60). Our data support the relevance of APN in the aggressiveness of CCRCC and point to this enzyme as a molecular target for new therapies in this disease.

DPPIV is a cell surface glycoprotein which is highly expressed in several epithelial cells. However, a soluble fraction has also been described (12, 13, 39). This peptidase has multifunctional properties dependent or independent of its catalytic activity, and it is quite well established that it may play pro- and antitumor effects depending on the tumor type (9, 13, 26). Thus it has been reported that high tissue expression and high serum levels are associated with prolonged survival in mesothelioma and in colorectal cancer patients (13, 46, 48). By contrast, the overall survival of ovarian carcinoma was worse in patients with high DPPIV expression (64). We have previously found that soluble DPPIV activity was increased in high-grade and advanced stage CCRCC (59), and here we confirm that high soluble DPPIV activity is associated with poor survival rates. These data suggest a role of this enzyme in the aggressiveness of CCRCC and point to DPPIV as a potential therapeutic target. In this sense, Inamoto et al. (23) have demonstrated that the anti-DPPIV/CD26 treatment in human renal cell carcinoma mouse xenograft models drastically inhibited tumor growth and enhanced survival, suggesting a potential clinical use of this treatment in DPPIV/CD26-positive renal cell carcinomas.

Most studies on the involvement of CAP in cancer progression and prognosis have been carried out in gynecological malignancies (24). Also known as placental leucine aminopeptidase, this enzyme is highly expressed in malignant trophoblastic tumors such as choriocarcinoma, and its expression is directly related to low disease-free survival in patients with endometrial adenocarcinoma (24, 50). The kidney is one of the tissues with higher expression of CAP (34), but little is known about the role of this enzyme in renal neoplasia. We described in a previous study (27) that membrane-bound and soluble CAP activities were increased in high-grade and advanced stage CCRCC. The present study shows that high soluble CAP activity correlates with poor survival rates in the same patients, which agrees with data previously reported in gynecological cancers.

APB is a ubiquitous enzyme which has been typically described in the soluble fraction, although some authors have also reported a membrane-bound fraction in several neoplastic and nonneoplastic tissues (4, 8, 33, 40, 58). Some evidence suggests that APB may regulate immunity and cell proliferation since it is targeted or regulated by several immunomodulators and chemotherapeutic agents (8, 49, 62). Very recently, Ramirez-Exposito et al. (44) have reported increasing activity of APB and other angiotensin-converting peptidases in the serum of patients with breast cancer receiving paclitaxel and anthracycline therapies and suggested the involvement of these enzymes in the antitumor effects of these drugs. In this context, the present study shows for the first time a relationship between APB activity and cancer survival. Contrary to the observations

![Fig. 4. A: patient survival curves according to the soluble aminopeptidase B (APB) activity levels (Kaplan-Meier method). B: variables associated with survival in multivariate analysis (Cox regression model) with APB. a: Results of the first step of the backward conditional method. None of the 4 variables reached statistical significance. b: Results of the final step, where only the sAPB appears as an explanatory variable of the regression model.](http://ajprenal.physiology.org/)
in other peptidases, we have detected that higher soluble APB activity is related to longer patient survival. This finding advocates for a protective role of this enzyme in CCRCC. However, the issue needs further study because we have previously found that high membrane-bound APB activity correlated with advanced stage CCRCC (4).

Tumor cells produce peptide factors or increase their concentration to ensure tumor growth. In addition, they can increase sensitivity to these factors or promote resistance to growth-inhibitory peptides. These biological actions are intended to create loops promoting cell growth (9, 19). Several bioactive peptides and their converting peptidases play important roles in these mechanisms. Among the peptides related to the development and progression of cancer we can find angiotensins, opioid peptides, oxytocin, and several chemokines and interleukins, which are natural substrates of APN, APB, CAP, and DPPIV (1, 9, 24, 63).

The regulation of peptide growth factors in the tumor microenvironment has been classically assigned to membrane-bound peptidases (9). However, the role of soluble peptidases remains unclear. Although secretion of soluble peptidases into the extracellular space has been suggested as a possible peptide-regulatory mechanism (3, 51, 64), there is accumulated evidence in favor of intracellular trafficking and action of certain peptides and proteolytic enzymes known as intracrine action (47). In fact, it has been suggested that the peptides mentioned in the present work may also act as intracrine factors in the intracellular space, inducing cell proliferation and angiogenesis (42, 45–47). Therefore, the possibility of increas-
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...ing soluble APN, DPPIV, CAP, and APB activity as a result of intracrine peptide dysregulation in clinically aggressive forms of CCRCC should be considered in future studies.

In conclusion, the present study shows for the first time that the level of several peptidases in neoplastic tissue may be of help not only in the diagnosis of CCRCC but also in the delineation of survival of this neoplasm. With CCRCC, by far the most frequent carcinoma in the kidney, and the fact that this disease still remains a major health problem in developed countries taken into account, the arrival of peptidases as new diagnostic tools with prognostic impact may be particularly profitable in hospital practice.

ACKNOWLEDGMENTS

We thank Aranza Pérez (University of the Basque Country) for technical contributions to this study. The authors thank Prof. Adolfo Varona for invaluable help in the study of peptidases.

GRANTS

This work was supported by a grant from the Basque Government (Saiotek).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES