

# Elevated tissue expression of RANKL and RANK is associated with poorer survival rates in pancreatic cancer patients

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**Summary.** Pancreatic cancer is a highly lethal malignancy with a growing incidence reported worldwide. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, which is often diagnosed at advanced stages, making its prognosis and medical management difficult. The identification of histopathological biomarkers has allowed a more precise stratification of pancreatic cancer patients, providing additional information about their prognosis and offering possible therapeutic targets to be explored. The prognostic value of the receptor activator of nuclear factor-kappa B (RANK) and its ligand (RANKL) has been evaluated in breast and prostate tumors, however, their usefulness has not been assessed in pancreatic cancer. In the present work, we analyzed the relationship between the protein expression of RANK and RANKL with the survival of 41 patients with pancreatic cancer followed for 60 months, by performing immunohistochemistry and Kaplan-Meier curves. Our results

demonstrate a direct association of high expression levels of RANK and RANKL with poorer survival of pancreatic cancer patients in comparison to those with low/medium and null expression levels of both markers. Further studies should be conducted to explore the carcinogenic role of both components in this type of tumor, as well as additional promising translational uses.

**Key words:** Pancreatic adenocarcinoma, Receptor activator of nuclear factor-kappa B (RANK), Receptor activator of nuclear factor-kappa B ligand (RANKL), cancer survival, Prognostic biomarkers

## Introduction

Pancreatic cancer is a highly lethal gastrointestinal cancer with a five-year survival comprised between 5-15% and an overall survival of 6% (Puckett and Garfield, 2022). According to the GLOBOCAN database registered in 2020, pancreatic cancer has an annual incidence of 495,773, being ranked in seventh place in the number of cancer-related deaths worldwide (Sung et al., 2021) However, it is estimated that in the next years, pancreatic cancer will become the second leading cause

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of cancer-related mortality in the United States and the third cause of cancer death in the European Union (Rahib et al., 2014; Ferlay et al., 2016). These data are the result of the multiple diagnostic and therapeutic challenges that pancreatic cancer presents. Despite the technological and medical advances in the last decades, many patients are still diagnosed at late stages due to the lack of effective preventive measures and screening (Pekarek et al., 2021). Indeed, only 20% of pancreatic cancer is surgically resectable at the time of diagnosis (Puckett and Garfield, 2022), giving a key role to other therapeutic alternatives like chemotherapy, radiotherapy, and immunotherapy (Pekarek et al., 2021). However, even with the breakthroughs in all these strategies, little progress has been achieved in the curative rates of these patients (Elsayed and Abdelrahim, 2021), especially because of the complex tumor environment and the need for a greater understanding of the pathology and immunology of pancreatic tumors.

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, accounting for more than 90% of all cases (Hu et al., 2021). The discovery of different tissue biomarkers by immunohistochemistry and other techniques has brought a broader comprehension of cancer pathobiology, providing multiple translational applications (Esposito et al., 2014; Luu, 2021; Szymoński et al., 2022). For instance, previous works permitted the correlation of tissue expression of certain proteins with survival rates of patients with pancreatic cancer, allowing the stratification of patients and offering potential therapeutic alternatives in patients with more advanced or severe stages of the tumor (Dell'Aquila et al., 2020; Fraile-Martinez et al., 2023; Ortega et al., 2022a-c). The receptor activator of nuclear factor-kappa B (RANK) and its ligand (RANKL) are two proteins with a demonstrated carcinogenic role in various types of tumors (Boyce and Xing, 2007a). Furthermore, previous works evidenced their potential application as translational biomarkers in certain types of malignancies like breast and prostate cancer (Chen et al., 2006; Ibrahim et al., 2011); however, little is known about the possible carcinogenic role of RANK and RANKL in PDAC, and no studies have evaluated their possible application in the stratification and prognosis of these patients.

Thus, the aim of the present research was to quantify RANK and RANKL expression in pancreatic cancer tissue obtained after pancreatectomy via immunohistochemistry and correlate their expression levels with the survival rates of these patients.

## Materials and methods

### Study design and sample collection

The current research was planned as an analytical, retrospective, observational, cohort study with long-term follow-up (60 months). We used paraffin-embedded pancreatic tissue samples from 41 patients with PDAC who underwent surgery (curative resection of pancreatoduodenectomy). The diagnosis of this entity was made in accordance with Esposito et al. (2014). Retrospective analysis was performed on the paraffin blocks, considering various details, thorough clinical information on the patients, and follow-up information.

The study was conducted in accordance with the fundamental ethical principles of autonomy, beneficence, non-maleficence, and distributive justice, and its design was in accordance with the GCP guidelines, the tenets of the most recent Helsinki Declaration (2013), and the Oviedo Convention (1997). Data and information were gathered in accordance with current data protection laws, including Regulation (EU) 2016/679 and Organic Law 3/2018 of December 5, which protects personal data.

### Immunohistochemistry and histopathological evaluation

Pancreatic tissue samples that had been encased in paraffin underwent immunohistochemical research. The protocol requirements (Table 1) included a description of the antibody recovery stage. According to known procedures, antigen/antibody responses were discovered using the avidin-biotin (ABC) complex technique with avidin-peroxidase (Ortega et al., 2021a). Samples were incubated with a 3% BSA blocker (Catalog #37525; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and PBS overnight at 4°C after being exposed to the primary antibody for 1 hour and 30 minutes. After being exposed to a biotin-conjugated secondary antibody, the samples were diluted in PBS for 90 minutes at room temperature using rabbit IgG (RG-96; Sigma-Aldrich, St. Louis, MI, USA). ExtrAvidin®-Peroxidase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), an avidin-peroxidase conjugate, was utilized for 60 min at room temperature (1:200 with PBS). The next step was to evaluate the level of protein expression using a Chromogenic Diaminobenzidine (DAB) Substrate Kit (cat. no. SK-4100; Maravai LifeSciences, San Diego, CA, USA), which was prepared just before exposure (5 mL of distilled water, 2 drops of buffer, 4 drops of DAB, and 2 drops of hydrogen peroxide). A brown stain could be

**Table 1.** Primary antibodies used, together with the dilutions and protocol specifications.

Antigen	Species	Dilution	Provider	Protocol Specifications
RANK (9A725)	Rabbit	1:500	sc-52951	10 mM Sodium citrate pH=6 before incubation with blocking solution
RANKL (12A668)	Rabbit	1:100	sc-52950	10 mM Sodium citrate pH=6 before incubation with blocking solution

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seen thanks to the signal produced by the chromogenic peroxidase substrate after 15 min at room temperature. As negative controls, portions of the same tissue were used with PBS as a blocking solution and primary antibody incubation to detect each protein. Every tissue underwent a 15-minute Carazzi hematoxylin contrast procedure at room temperature.

Using an AxioCam HRc digital camera and a Zeiss Axiophot light microscope (both from Carl Zeiss, Oberkochen, Germany), tissue sections were observed. The IRS-Score approach was used to determine the histological evaluation based on the degree of immunohistochemical staining expression (Gatto et al., 2013). As a result, pancreatic cancer histological samples from patients were categorized as having negative (0), low/moderate (1), or high expression (3). In each of the five sections, seven randomly chosen microscope fields were evaluated for each subject group. When the mean proportion of the labeled sample was greater than or equal to 5% of the total sample, positive individuals were identified. This was accomplished by averaging the research sample, as previously stated in other works (Ortega et al., 2021b; García-Montero et al., 2022), by computing the overall percentage of marked tissue in the various microscope fields. The measurement and observation of the various samples were carried out independently by two different researchers.

### Statistical and survival analysis

A test for marker normalcy was conducted (Kolmogorov-Smirnoff, all  $p < 0.001$ ). We had to use non-parametric tests to characterize the results as they did not follow a normal distribution and instead used medians and interquartile ranges with the Mann-Whitney U test. A logarithmic rank test and Kaplan-Meier curves for survival comparisons were simultaneously constructed in order to evaluate the relationship between clinicopathological and immunohistochemical factors. Finally, a univariate analysis and Cox proportional-hazards regression analysis were employed to investigate

**Table 2.** Clinical and sociodemographic characteristics of the patients diagnosed with pancreatic cancer included in the study.

Age (Median [IQR])	72.00 [45.00-88.00]
Sex (n (Ratio%))	
Men	27 (65.85)
Women	14 (34.15)
Smoking	18 (43.90)
Drinking	11 (26.83)
Obesity	2 (4.88)
Type 2 diabetes	15 (55.56)
Chronic pathologies	4 (9.76)
Prior malignant neoplasms	11 (26.83)

IQR, Interquartile range; n, number of patients. As observed, 65.85% of patients are men, 43.9% are smokers, 26.83% are important alcohol consumers, 4.88% present obesity, 55.56% have type 2 diabetes mellitus, 9.76% have chronic pathologies, and 26.83% have prior malignant neoplasms.

the relationship between the investigated immunohistochemistry parameters and the established prognosis of the variables. STATA 16.1 software (Normal, IL, USA) was used to perform all statistical analyses.  $p$ -values of 0.05 or less were regarded as significant.

## Results

### Sociodemographic and clinical features of the participants

A total of 41 patients were examined; their median age ranged from 45.00 to 88.00; of these, 27 patients (65.75%) were male and 14 (34.15%), were female. Table 2 compiles the sociodemographic and clinical characteristics. Plasma levels of the main carcinogenic markers collected routinely and the patient's tumor stage are collected in Table 3. Globally, patients with a pancreatic cancer diagnosis had an 8.00 (2.98-13.02) month median survival time.

### High tissue expression of RANKL is associated with poor survival in pancreatic cancer patients.

In our study, we observed that 21 patients with pancreatic cancer (51.22%) exhibited high expression levels of RANKL, whereas 12 (29.27%) displayed low/medium expression levels (Fig. 1A,B). Conversely, eight patients presented negative expression of RANKL (19.51%). Thus, in our study sample, 80.49% were positive for RANKL expression.

We observed that the median survival of patients with negative RANKL expression was 30 months [24.95-35.05], whereas this value was significantly lower in patients with positive RANKL expression. Specifically, in patients with low/moderate RANKL expression, the median survival was 13 months [10.45-15.54], and in those with high RANKL expression, we report a median survival of 6 months [4.255-7.745] (Fig. 1C). According to our statistical associations, a high expression of RANKL is correlated with a hazard ratio (HR) of 24.30 for mortality in comparison with low/moderate RANKL expression. The global comparisons reported a significance value of  $p=0.001$ .

### Elevated histopathological expression of RANK is related to a decreased median survival in pancreatic cancer patients.

We detected that up to 30 patients (73.17%) presented high expression levels of RANK and 7

**Table 3.** Percentages of the patient's tumor stage and plasma levels of the main carcinogenic markers collected routinely expressed as median and interquartile range.

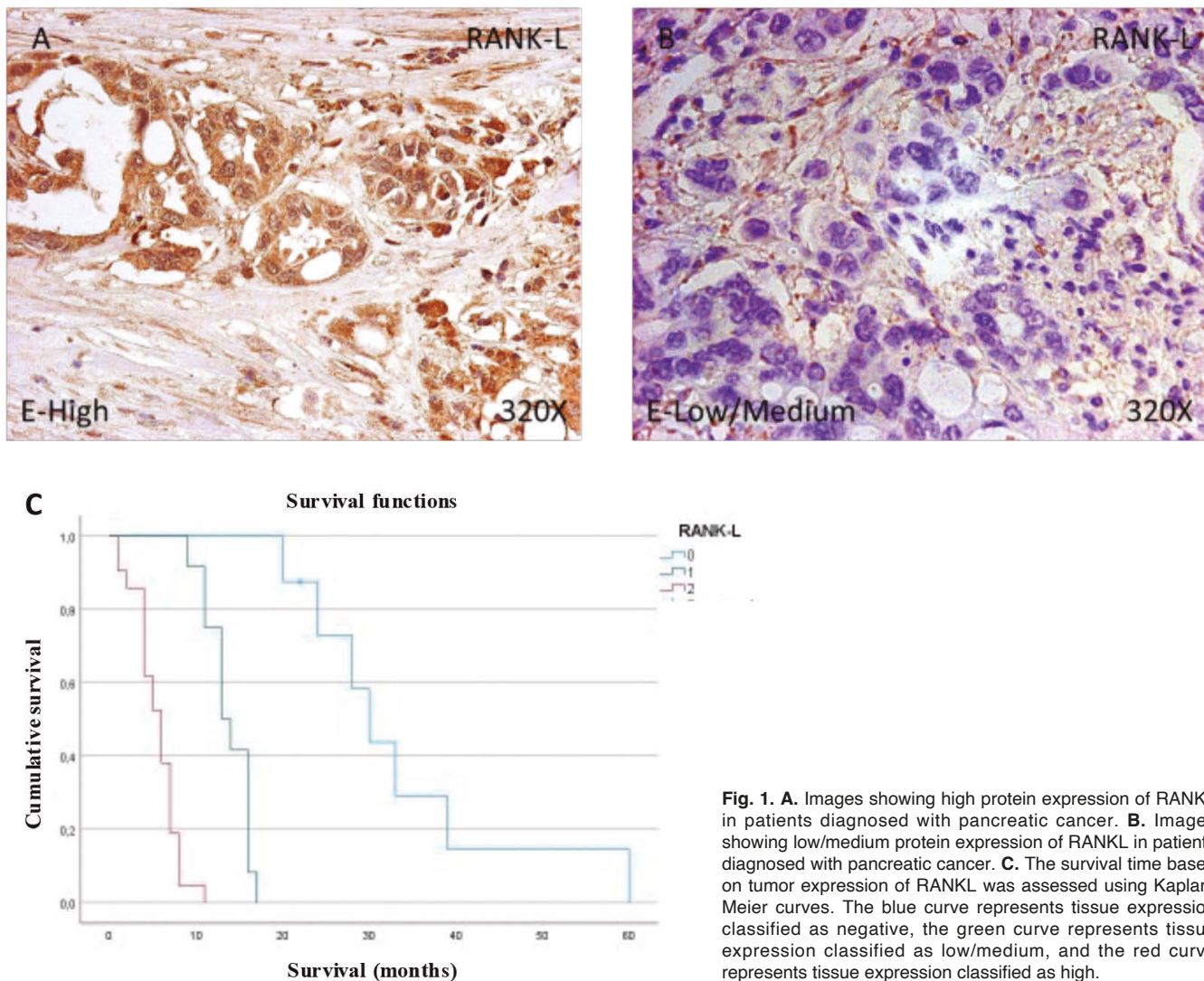
Tumor stage 4	31.71 % (13/41)
Ca 19.9 U/ml (0-37)	102.10 [44.91-805.00]
CEA ng/ml (0-5)	5.43 [2.71-11.31]
AFP ng/ml (0-13.4)	2.32 [1.46-4.39]

(17.07%) exhibited low/medium expression levels (Fig. 2A,B). Thus, 37 (90.24%) pancreatic patients were positive for RANK expression. The remaining four subjects (9.76%) displayed negative expression of this molecule.

Our results showed that the median survival of patients with negative RANK expression was 33 months [22.22-43.78], whereas this value was significantly lower in patients with positive RANK expression. Specifically, in patients with low/moderate RANK expression, the median survival was 20 months [9.735-30.265], and in those with high RANK expression, we report a median survival of 7 months [5.670-8.330] (Fig. 2C). According to our statistical associations, a high expression of RANK is correlated with an HR of 7.05 for mortality in comparison with low/moderate RANK expression. The global comparisons reported a significance value of  $p=0.001$ .

## Discussion

The identification of various tissue markers has provided insights into the fundamental pathophysiology of pancreatic adenocarcinoma and facilitated the categorization of patient risk at different disease stages, as well as the evaluation of tumor recurrence (Pekarek et al., 2022). Given the invasive nature of this type of cancer and the lack of effective screening markers, novel immunohistochemical and serological markers have the potential to serve as prognostic indicators for recurrence and aid in the exploration of potential therapeutic targets, as the survival rate in these patients is typically quite low. The present work has successfully identified two previously undiscovered histopathological markers, RANK and its ligand RANKL as potential prognostic biomarkers with promising implications for individuals diagnosed with pancreatic cancer. Specifically, our



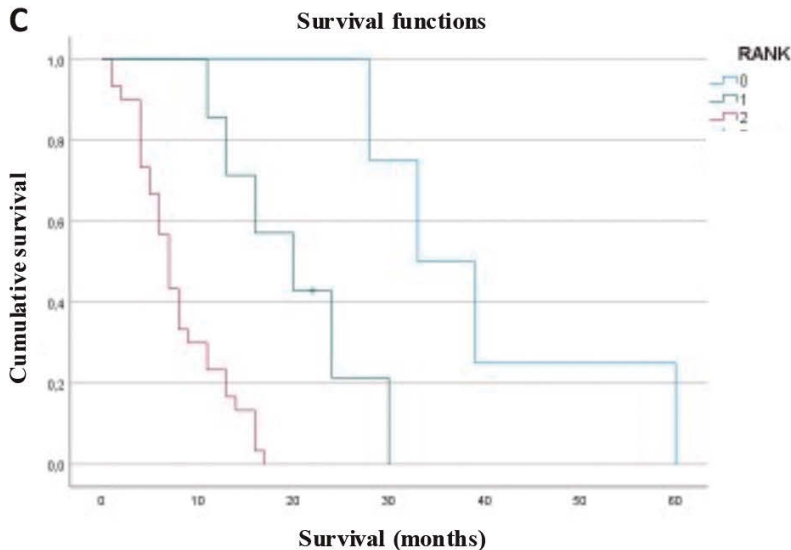
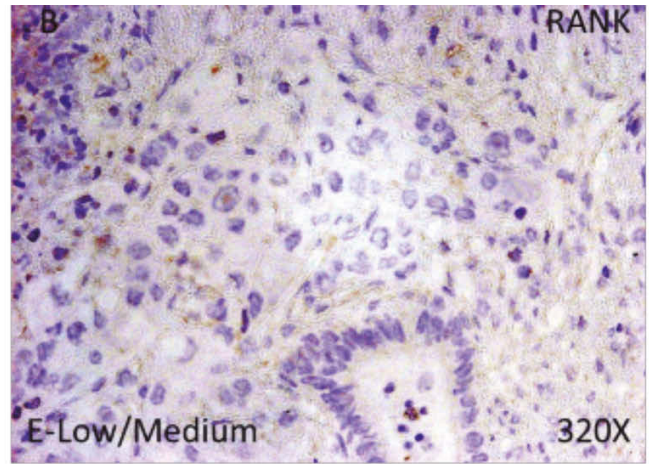
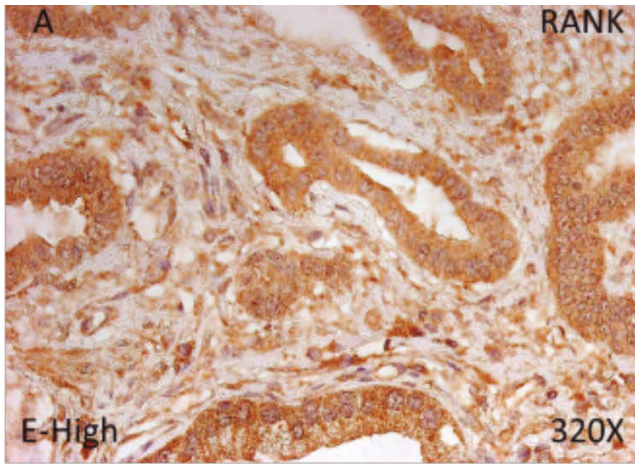
**Fig. 1. A.** Images showing high protein expression of RANKL in patients diagnosed with pancreatic cancer. **B.** Images showing low/medium protein expression of RANKL in patients diagnosed with pancreatic cancer. **C.** The survival time based on tumor expression of RANKL was assessed using Kaplan-Meier curves. The blue curve represents tissue expression classified as negative, the green curve represents tissue expression classified as low/medium, and the red curve represents tissue expression classified as high.

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findings indicate that patients exhibiting elevated levels of both RANK and RANKL show a notably diminished survival rate when compared with those expressing lower to moderate levels of these molecules. Furthermore, a substantial disparity in survival rates was observed between individuals lacking any expression of RANK and RANKL and those with detectable levels of these markers.

Both RANK and osteoprotegerin (OPG) are two transmembrane receptors belonging to the tumor necrosis factor (TNF) receptor (TNFR) family, which mediate different biological effects by competing for RANKL binding (Nelson et al., 2012). In more detail, RANKL is a ligand involved in osteoclast generation through its binding with RANK, whereas OPG is a decoy receptor for RANKL (Yasuda, 2021). Because of this, different studies have defined these interactions as the RANKL/RANK/OPG system, having been recognized as critical mediators of bone physiology and

disease (Boyce and Xing, 2007a,b). However, previous works identified a significant role of RANK/RANKL signaling in other tissues like the breast (mediating mammary development and lactation), the immune system (modulating dendritic cells activation and osteoimmunity), hair follicle formation, temperature regulation in the body, muscle metabolism, and tumor development (Boyce and Xing, 2007a; Ono et al., 2020). To date, little is known about the role of RANK/RANKL in pancreatic cancer. Prior works explored the relevance and association of high expression levels of RANK and RANKL with poor prognosis and reduced survival in breast and prostate cancer patients (Ohtaka et al., 2017; Casimiro et al., 2021). Similarly, our results show a direct association between high RANK/RANKL expression and poor survival in pancreatic cancer patients. Notwithstanding, to the best of our knowledge, this is the first study demonstrating this association. Another work also found that OPG was upregulated in



**Fig. 2.** **A.** Images showing high protein expression of RANK in patients diagnosed with pancreatic cancer. **B.** Images showing low/medium protein expression of RANK in patients diagnosed with pancreatic cancer. **C.** The survival time based on tumor expression of RANK was assessed using Kaplan-Meier curves. The blue curve represents tissue expression classified as negative, the green curve represents tissue expression classified as low/medium, and the red curve represents tissue expression classified as high.

patients with pancreatic cancer, the levels of this protein being inversely correlated with the overall survival of these patients (Shi et al., 2014). Therefore, the RANKL/RANK/OPG system might also be an interesting point of study in pancreatic cancer, offering a promising therapeutic target that should be evaluated in these patients.

The biological mechanisms underlying the link between exacerbated RANK/RANKL expression and poor survival in pancreatic cancer could be explained by revising the available literature. Prior works described that RANK and RANKL could exert a potential immunomodulatory role in the pancreas, particularly through the generation and activation of potent regulatory T cells (Tregs) (Peters et al., 2019). Tregs are pivotal players in the pancreatic tumor microenvironment. In more detail, these cells are critically involved in different carcinogenic mechanisms, mainly influencing tumor growth, tumor-promoting inflammation, and therapeutic regimes in these tumors (Reyes et al., 2022). Despite their prognostic role, Tregs in cancer have shown some controversial results (Shang et al., 2015); it seems that increased tumor-infiltrating Tregs have been positively correlated with poor prognosis and survival rates in pancreatic tumors (Saleh and Elkord, 2020). In addition, this is not the only carcinogenic mechanism that may help explain the relationship between RANK and RANKL and poorer survival. RANKL is a product that can be released by both tumoral and stromal cells, leading to the activation of M2 macrophages and Tregs. In turn, Tregs can enhance RANKL production and act in cancerous cells, promoting tumor proliferation, epithelial-mesenchymal transition (EMT), cell migration, and chemoattraction while influencing endothelial cells, favoring angiogenesis, extravasation, and metastasis (Renema et al., 2016). All these mechanisms are involved in cancer development, progression, and dissemination, which may help us understand the association between the observed increased expression of RANK and RANKL in our study and poor clinical outcomes, although further studies are needed to unravel the biological role of both components in pancreatic tumor cells. Interestingly, our results show that despite RANKL being overexpressed in a smaller subset of patients when compared with RANK (80.49% versus 90.24%), patients with positive RANKL expression present lower median survival rates in those patients with high (6 versus 7 months) and low/medium expression (13 versus 20 months). Future works could corroborate these results and delve deeper into the carcinogenic role of RANKL in pancreatic cancer.

On the other hand, previous works evidenced a relationship between RANK/RANKL overexpression and bone metastasis, especially in the case of breast and prostate tumors (Zhang et al., 2022). According to the available literature, bone metastasis is remarkably less common in pancreatic cancer patients, accounting for 5 to 20% of all patients (Borad et al., 2009). Despite

previous works having failed to find any association between bone metastasis and overall survival in pancreatic cancer patients, it represents an important clinical challenge to face, being equally associated with important comorbidities like fractures and poor quality of life (Puri et al., 2021). Considering these results, it would be interesting to correlate RANK and RANKL expression with the occurrence and prognosis of bone metastasis in pancreatic cancer patients.

Finally, we were not able to find any significant association between our clinical variables and the expression levels of RANK and RANKL. Previous works demonstrated a direct association between different clinical and sociodemographic variables included in this study and the expression level of RANKL and RANK. For instance, cigarette consumption and the protein expression of both components in different tissues such as bones and muscle (Nogueira and Breen, 2021). Similar results were found in association with long-term alcohol consumption (Wang et al., 2021) and chronic diseases such as obesity, type 2 diabetes, and others (Boyce and Xing, 2007a,b; Kalkan and Becer, 2019; Xing et al., 2023). Besides, RANKL expression seems to be higher in males than in females (Kodrič et al., 2019). Interestingly, many of these factors associated with the increased expression of RANK and RANKL are directly associated with increased mortality in pancreatic cancer patients (Hu et al., 2021). However, to the best of our knowledge, no studies have considered the association between these clinical factors and RANK/RANKL expression in pancreatic tissue. Future works could be directed toward evaluating a possible association between these factors and RANK/RANKL expression in pancreatic cancer patients, as this could represent a more potential therapeutic target for certain groups of pancreatic cancer patients with increased risk of mortality.

### Conclusions

Our study evidenced a direct association between expression levels of RANK and RANKL and poorer survival in patients with pancreatic cancer. Future studies should delve deeper into the carcinogenic role of both components in this type of tumor, as well as their possible translational use as predictive biomarkers or therapeutic targets.

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*Conflicts of Interest.* The authors declare no conflict of interest.

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