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The possibility of using serum and salivary alkaline/acidic Ribonuclease as potential biomarkers for ductal carcinoma in situ detection among Iraqi patients

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Abstract:

Ribonucleases (RNases) play a crucial role in various biological processes. Many studies have affirmed their significant role in numerous types of cancer. Saliva is an absorbing fluid that has attracted the attention of researchers due to its essential role in pathological and physiological situations. Therefore, this study aims to explore the relevance of alkaline and acidic Ribonuclease activities in serum and saliva for the diagnosis of breast tumours as well as the potential of using saliva as an alternative diagnostic fluid for ductal carcinoma *in situ* (DCIS) patients and benign tumour. The study examined changes in RNase activities in the serum and saliva of some breast-tuomr patients. The result demonstrated a significant elevation ($P<0.0001$) in alkaline and acidic RNase activities and specific activities of ductal carcinoma *in situ* patients group in both fluids in addition to a significant correlation between serum and saliva in alkaline RNase activity of the ductal carcinoma *in situ* patients group ($P<0.002$). These findings suggest that alkaline RNase activity could be used as an alarm and prediction tool for ductal carcinoma *in situ* disease.

Key words: Acidic RNase, Alkaline RNase, Ductal carcinoma in situ (DCIS), Benign tumour, Saliva.

امكانية استخدام الرايبونيوكليز الحامضي والقاعدي في المصل واللعاب كعلامة حيوية محتملة لسرطان الثدي في الموقع في المريضات العراقيات

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الخلاصة

يلعب الرايبونيوكليز دورا حيوياً مهماً في العمليات البيولوجية المتنوعة .اكدت العديد من الدراسات دوره الفعال في العديد من انواع السرطانات. اللعاب هو سائل ماص جذب اهتمام الباحثين بسبب اهميته ودوره

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في الحالات المرضية والفسيولوجية.ليهدف هذا البحث الى استكشاف اهمية انشطة الرايبونيوكليز الحامضي والقاعديي في المصل واللعاب في تشخيص اورام الثدي ,اضافة الى امكانية استخدام اللعاب كسائل تشخيصي بديل لمرضى سرطان الاقنية في الموقع في المراحلة المبكرة والاورام الحميدة.قيس في الدراسة التغيرات في انشطة الرايبونيوكليز في كل من المصل واللعاب لعدد من مرضى اورام الثدي. اظهرت النتائج ارتفاعاً ملحوضاً (P<0.0001)في المصل واللعاب في فعالية الرايبونيوكليز القاعدي والحامضي لمرضى سرطان الاقنية في الموقع في كلا السائلين, بالاضافة الى ذلك,هنالك ارتباط ملحوظ بين مصل ولعاب الرايبونيوكليز القاعدي في مجوعة سرطان القنوات في الموقع (P<0.002).تشير النتائج الى ان الرايبونيوكليز القاعدي يمكن استخدامه كمنبه او أدة لتوقع مرض سرطان الاقنية في الموقع .

1. Introduction

Breast tumours are prevalent among women and are divided into two types: benign tumours and malignant tumours (breast cancer) [1]. Breast cancer is regarded as one of the most terrifying diseases, chiefly for women, because it is a fatal disease if left without treatment. It can also infect men [2]. Ductal carcinoma in situ (DCIS) (which is considered as zero stage of this cancer) is a term used to describe neoplastic lesions that ordinarily arise from uncontrolled cell division in the breast ducts and with no confirmation of invasion of these cancerous cells to the surrounding tissues [3].

Ribonuclease enzymes (RNases) belong to nuclease enzymes, which have the potential to degrade RNA molecules into smaller fragments, strictly through hydrolysis, the phosphodiester bonds that are present in RNA molecules. RNases have been isolated biochemically and identified from numerous organisms and mammal tissues. Several types of RNases (ribonucleases) are categorized based on their structures, activities, or functions. Two major types of RNA can be classified based on their site of action [4,5]: endoribonucleases and exoribonucleases

RNase plays a pivotal role in copious biological processes involving gene expression, RNA degradation, and regulation of RNA structure [6]. In this context, alterations in RNA metabolism and dysregulation of RNA processing are commonly observed, and they have been reported to raise the probability of the development and progression of cancer [7].

Saliva comprises water, electrolytes, enzymes, antibodies, hormones, DNA fragments, RNA, proteins, and other molecules that can provide valuable information about an individual's health report. Its advantages have become a popular diagnostic fluid in research and clinical settings [8]. Many studies suggest that it can be used to diagnose infectious and malignant diseases, autoimmune disorders, endocrine disorders, and hereditary conditions [9]. One of the most important advantages of utilizing saliva as a diagnostic fluid is its non-invasive nature and its painless sample collection method. Additionally, saliva is easily obtainable compared to other fluids or tissues, and all these properties guide the researchers in studying the possibility of using it as an attractive option for obtaining biological samples [8]. This study aims to investigate whether RNase activity can be used as a predictive parameter for ductal carcinoma and to explore the potential of saliva as an alternative to blood for diagnostic purposes.

2. Materials and methods

2.1.Collection of saliva and serum samples

Breast tumour patients (n=59) who were recently diagnosed and were not on medication, these patients were attending AL- Elwea Hospital Early Detection For Breast Cancer, adjusted with individual control (n=27). In the samples used in the study before saliva collection, all participants rinsed their mouths with normal saline. About 3-4 millilitres (mLs) of saliva were collected and separated in a centrifuge (2400×g) for about 15 minutes. The

supernatant of the saliva samples was stored in a freezer at-20°C. At the same time, from the same individuals, a blood sample of 4-5 mL was collected and centrifuged (3000 × g) for 5 minutes, and the obtained serum was frozen at a temperature of -20°C.

To secure the accuracy of the study outcome and diminish risk factors, individuals who have any other health problems, smokers, or alcoholics, or those with some health problems, were taking any type of medicine excluded from this study.

2.2.Alkaline RNase Activity Assessment

The activity of alkaline RNase is determined following the method of Bardon method, based on Bardoń and Shugar (1980) and later modified by Hasan and Al-Issa (2011)

1-Instead of (0.2ml), which was used, a volume of (1mL) of Davis buffer was used.

2-The total volume of the reaction mixture was adjusted to 1.1mL.

3-A (1% w/v) RNA solution was applied.

$RNase\ Activity\ (U/L) = \Delta A\ /t \times Vt\ /Vs \times 1000 \times D.F$

ΔA= Sample absorbance at (260 nm.) – Blank absorbance at (260 nm.),Vt = the total volume Vs= the volume of serum used, t= the incubation time (min), and diluted factor.

2.3.Acidic RNase Activity Assessment

Similarly, alkaline RNase activity was determined using the Bardon [10] method (Bardoń and Shugar, 1980). Acidic RNase activity was also determined with some modifications, as described below, by adjusting the pH to 5 for the reaction mixture [11].

Practically, the same volumes and components as described above for alkaline RNase activity.

$RNase\ Activity\ (U/L) = \Delta A\ /t \times Vt\ /Vs \times 1000 \times D.F$

ΔA= Sample absorbance at (260 nm.) – Blank absorbance at (260 nm.),Vt = the total volume Vs= the volume of serum used, t= the incubation time (min), and diluted factor.

The protein concentration measurements were carried out by following both Lowry's method [12] and modified Lowry's method by Hartree in serum and saliva fluids [13]

The specific activity is expressed as = $enzyme\ activity$ (U/mL)/$protein\ concentration\ (mg/mL)$.

2.4.*Statistical analysis*

In this study, a program, SPSS version 26, was used to analyse all obtained data. A one-way ANOVA test was chosen to compare the obtained results, while the Pearson correlation coefficient was used to examine the correlation relationships between all variables. P value ($P < 0.0001$) was referred to as highly significant, while a value was significant at (0.05) and when the value was ($P > 0.05$), it was expressed as a non-significant [14].

3. Result

This study involved 59 women, with 54.24% having benign breast tumours, while 45.76% had malignant breast tumours DCIS, as illustrated in Table 1 below. It was important to note that the patients were newly diagnosed women at stage zero who received no medication.

Table 1: The number of cases and (Mean ± SD) of age for all studied groups

Group	N	Age (year)	Sig.	Note
Control	**27**	**45.07±15.36**		
Benign tumour	**32**	**47.18±13.95**	**P<0.03 a**	**Before taking any medication**
DCIS	**27**	**49.96±12.78**	**P<0.01 b**	

a: The difference in age between the benign group and the control group.
b: The difference in age between the DCIS group and the control group.

In this study, the activity of RNase was observed to increase in the patient group, as illustrated below in **Table (2).**

Table 2: The (Mean ± SD) of alkaline RNase activity and specific activity in serum samples.

Group	Mean ± SD (Activity)(U/L)	Mean ± SD(Specific activity)(U/mg)	Sig.
Control	4.464± 0.859286	2.795±0.740	P < 0.0001 a** ,b**, c**
Benign tumour	74.737± 13.211	82.730± 9.577	
DCIS	110.545 ± 21.274	121.899± 13.602	

*The mean difference is significant at the 0.05 level. **The difference is highly significant at the P< 0.0001
a: Refers to differences in serum RNase activity and specific activity of the benign group compared to the control group.
b: Refers to differences in sera RNase activity and specific activity of the DCIS group compared to the control group.
c: Refers to differences in sera RNase activity and specific activity of the DCIS group compared to the benign group.

As shown in Table 2, there is a significant difference in alkaline RNase activity and specific activity in the serum between the benign tumor group and the control group (P < 0.0001). Additionally, significant difference between activity and specific activity in the serum of the **DCIS** group in comparison to the control group and benign group (P <0.0001).

Table 3: The Mean ± SD of alkaline RNase activity and specific activity in saliva samples.

Group	Mean ± SD (Activity) (U/L)	Mean ± SD (Specific activity) (U/mg)	Sig.
Control	**5.065± 1.535**	**4.703 ± 1.433**	**P <0.0001 a** b** c****
Benign tumour	**170.683± 27.717**	**143.774 ±23.399**	
DCIS	**268.562± 63.747**	**216.706 ±51.412**	

*The mean difference is significant at the 0.05 level .**The difference is highly significant at the p< 0.0001.
a: Refers to differences in saliva RNase activity and specific activity of the benign group compared to the control group.
b: Refers to differences in saliva RNase activity and specific activity of the DCIS group compared to the control group.
c: Refers to differences in saliva RNase activity and specific activity of the DCIS group compared to the benign group.

The results in **Table (3)** illustrate the presence of a significant difference (P <0.0001) in the activity of alkaline RNase as well as in the specific activity in the saliva of the benign tumour group in comparison to the control group. In addition, the results showed a significant difference(P <0.0001) in the activity of alkaline RNase and specific activity in the saliva of the **DCIS** group compared to the control group.

Table 4: The (Mean ± SD) of acidic RNase activity and specific activity in serum samples

Group	Mean ± SD (Activity(U/L))	Mean ± SD (Specific activity)	Sig.
Control	**40.441± 12.688**	**5.685± 1.804**	**P <0.0001 a**,b** ,c****
Benign tumour	**606.546± 61.497**	**73.049±7.660**	
DCIS	**1458.966± 440.570**	**166.105±49.256**	

* The mean difference is significant at the 0.05 level. **The difference is highly significant at the P< 0.0001

a: Refers to differences in serum RNase activity and specific activity of the benign group compared to the control group.

b: Refers to differences in sera RNase activity and specific activity of the DCIS group compared to the control group.

c: Refers to differences in sera RNase activity and specific activity of the DCIS group compared to the benign group.

A significant difference in Table (4) illustrates the activity of acidic RNase and specific activity in the serum of the benign tumour group in comparison to the control group (P <0.0001) as well as the results showed a significant difference between the activity of acidic RNase and specific activity in serum of the DCIS group in comparison to the control group (P <0.0001).

Table 5: The (Mean ± SD) of acidic RNase activity and specific activity in saliva samples.

Group	Mean± SD (Activity) (U/L)	Mean± SD (Specific activity)(U/mg)	Sig.
Control	**6.939± 2.199**	**6.437±2.031**	P <0.0001 a**,b** P <0.241 c P <0.805 d
Benign tumour	**218.407± 40.886**	**183.932±34.315**	
DCIS	**233.256± 43.037**	**188.239±34.658**	

*The mean difference is significant at the 0.05 level. **The difference is a highly significant at the P< 0.0001

a: Refers to differences in saliva acidic RNase activity and specific activity of the benign group compared to the control.

b: Refers to differences in saliva acidic RNase activity and specific activity of the DCIS group compared to the control.

c: Refers to the differences in acidic saliva RNase activity of the benign group compared to the DCIS group.

d: Refers to differences in the saliva acidic RNase specific activity of the benign group compared to the DCIS group

The results presented in Table 5 reveal a significant difference in both activity and specific activity of alkaline RNase in the saliva between the benign tumor group and the control group (P < 0.0001). Similarly, significant differences were observed in the saliva of the DCIS group in comparison to the control group (P <0.0001). Still, there was no significant difference between the benign group and the DCIS (P <0.241) in the activity of acidic RNase as well as in the specific activity of acidic RNase(P <0.805).

Correlation

Table 6: shows the Pearson correlation (A)between serum and saliva of acidic and alkaline RNase activity (B) Pearson correlation between serum and saliva of acidic and alkaline RNase Specific activity.

Saliva / Serum	Correlation between serum and saliva of activity of RNase (A)	Correlation between serum and saliva of sp. activity of RNase (B)
Acidic RNase Control	r= 0.058, P>0 .776	r= -0.005 , P>0 .980
Acidic RNase Benign	r=0.083, P>0.653	r=0.017, P>0.927
Acidic RNase DCIS	r=-0.078, P>0.706	r=-0.079 , P>0.702
Alkaline RNase Control	r=0.435* , P<0.024	r=0.485* , P<0.010
Alkaline RNase Benign	r=0.072, P=0.696	r=0.078 , P>0.676
Alkaline RNase DCIS	r=0.559**, P<0 .002	r=0.637**, P<0.0001

Table (6) presents a non-significant correlation between serum and saliva samples in acidic RNase activity for the control group, benign group, and the DCIS. On the other hand, there was a significant correlation in serum and saliva samples in alkaline RNase in the control group and the DCIS groups, except for the benign patient group, which was not correlated.

Table 7: The area under the curve and cut-off value from the ROC curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Cut off value	specificity	sensitivity
Alkaline RNase in saliva	0.968	.017	0.000	163.35	100%	100%
Acidic RNase in saliva	0.767	.050	0.000	137.31	100%	100%

Table (7) shows a high significance for the alkaline RNase test and (area under the curve) AUC=0.968, which indicates an excellent value for considering the alkaline RNase test a diagnostic or prediction test and a cut value of 163.35 in the DCIS disease group in saliva samples compared with acidic RNase.

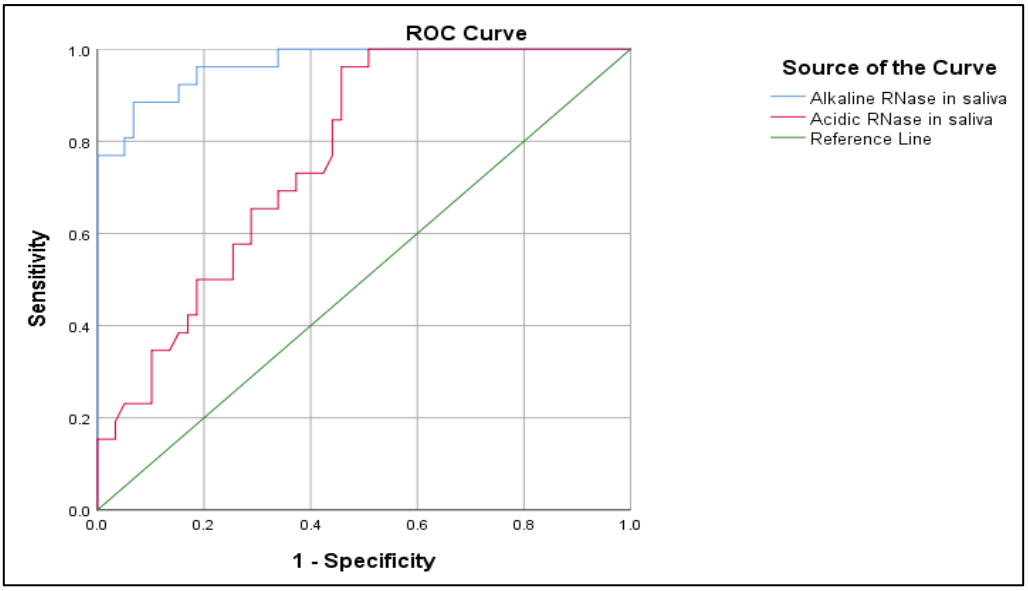

Figure 1: The ROC curve for alkaline and acidic RNase in the saliva of the DCIS group.

Table 8: The AUC and cut-off value from the ROC curve.

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Cut off value	specificity	sensitivity
Alkaline RNase in serum	1.000	0.000	0.000	851.58	100%	100%
Acidic RNase in serum	1.000	0.000	0.000	927.66	100%	100%

Table 8: shows a high significance for the alkaline RNase test and acidic test, with an AUC= 1, which indicates an excellent value for considering the alkaline RNase test and acidic RNase test as a diagnostic or prediction test. The cut-off values are 851.58 and 927.66, respectively, in the DCIS disease group in serum samples.

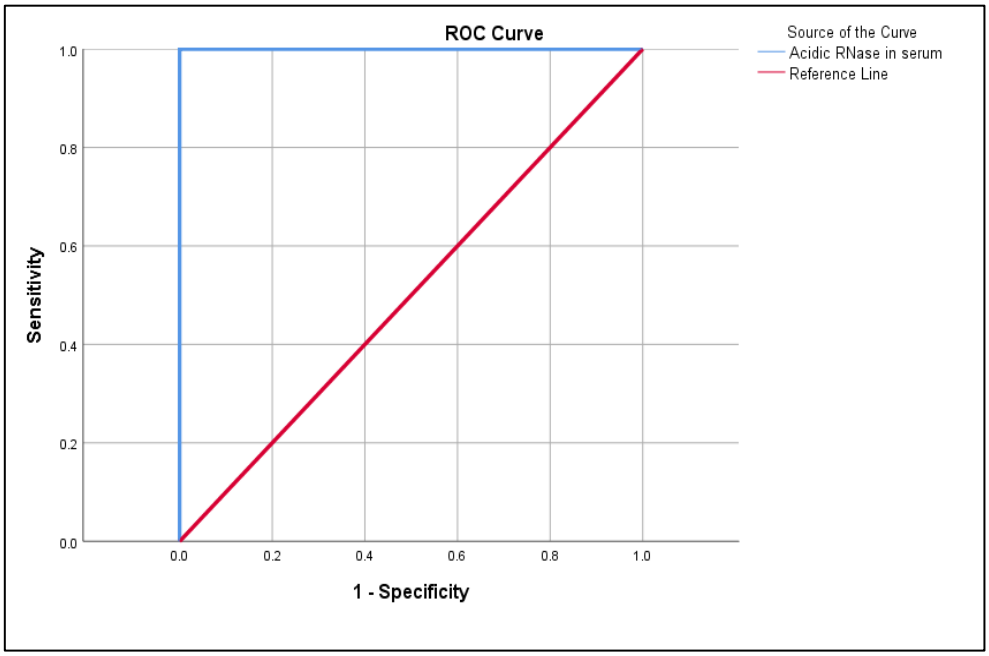

Figure. 2: The ROC curve for acidic RNase in serum of the DCIS group.

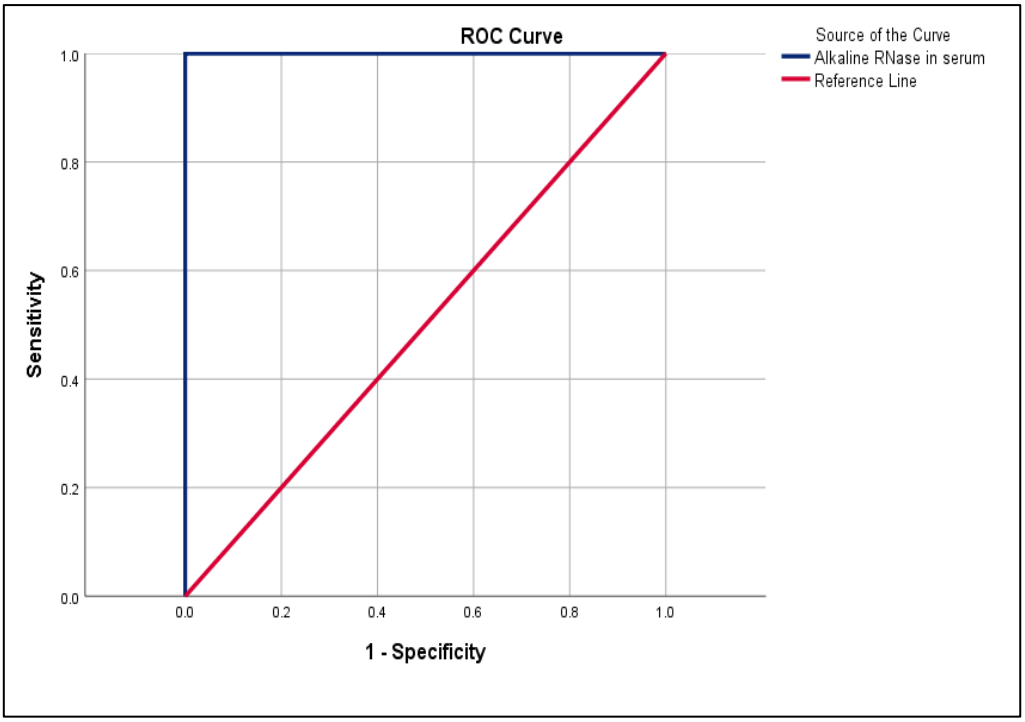

Figure. 3:The ROC curve for alkaline RNase in serum of the DCIS group.

4.Discussion

This study highlights the main differences between alkaline and acidic RNase activity in non-cancerous growth (benign tumour), which is regarded as common among women and cancerous growth in Iraqi patient groups. On the other hand, this study investigated the possibility of using saliva as a diagnostic fluid for this disease instead of blood or serum samples and using RNase activity as a prognostic and predictive biomarker for abnormal growth cases.

As shown in Tables 3 and 5, there was a high increase in saliva activity of ductal carcinoma in situ compared with the benign tumour group and control group in alkaline and acidic RNase These results agreed with the result of Al-Issa and Hasan in a study that addressed breast cancer in saliva only in 2011 [11]. RNase activity in Tables 2 and 4 in serum samples agreed with a study by Tawfeq *et al*. about breast tumours in serum samples only in 2019 [15]. Moreover, Moreover, this study agreed with Hussain et al.'s research on ovarian cancer in 2021 and Hasan and AL-Shammaree [16,17].

RNase activity in many studies shows a contrastive sensitivity (activation or inhibition) of specific signalling pathways in the cell that lead to different cellular responses to RNase activity to degraded or stabled RNA molecules [18,19].

RNases show different roles of their biological function in the context of cancer research because various types of RNases differ in molecular weight and source as the acidic RNase source is the liver or spleen [20], in contrast to alkaline RNase source is the pancreas, and liver, and the widely found in cytosol and mitochondria [21]. Some types of RNase were suggested to be used as anti-tumors that induce cell death, like RNase A and RNase H [22], in contrast with RNase 8, which is closely associated with tumor development that promotes cell growth and metastasis [23].

Practically, this study used both serum and saliva of the same patients and showed a high increase in the activity of RNase (alkaline and acidic) in both serum and saliva of the benign tumour group and DCIS group (which is abnormal tissue) in comparison that of the control group (normal tissue) ($P<0.0001$), the activity level of RNase was increased in serum of both the benign and the DCIS patients as this study proved that RNase strongly associated with this disease and have a notable role in this disease.

One suggestion for such a high serum RNase elevating level might be that the cancer cell is characterized by distinctive uncontrolled cell growth and speed division, which results in an increased expression and production of various types of proteins where one of its strategies will change the properties of the cell membrane, permeability, cell signalling pathways, receptors, enzymes, cell-cell interaction all these changes will combine with redundancy in protein synthesis [24-26]. Subsequently, due to all changes caused by malignant tumours, some enzymes will be affected, including RNase; RNase may be delivered to the peripheral blood from the tumour cells or surrounding tissues rather than being excreted in the urine [27].

Cancer is an inflammatory disease, and damaged tissues trigger the activation of the immune cells, which release RNase as part of the defence system response. These could explain the leading causes of high-level RNase activity in breast tumours [28]. Some types of RNase were considered responsive genes to various types of stress, which were discharged by necrotic cells or secreted by epithelial or immune cells as signals of abnormal events or hazardous occurrences, such as early stages of cancer [29]. In part of the possibility of using saliva as an alternative fluid to serum and blood, as a result presented in our opinion, there was a correlation only in the control and the ductal carcinoma in situ of alkaline RNase activity.

Conclusion

Alkaline RNase could potentially serve as a surveillance parameter or an early-stage predictive tool for breast cancer. To confirm the use of RNase activity to detect the stage of this disease and other types of cancer, to find out the variability of RNase activity, and to follow up on the progression of cancer. This study could be applied to patients with different stages, larger numbers, and comparisons with different ages and types of cancer.

5. Conflict of interest: None

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