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Collagen Degradation and Prolidase Activity and Relation to Mineral Homeostasis in Iraqi Vitiligo Patients

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Abstract

Vitiligo is a common depigmenting skin disorder classified as an autoimmune ailment. It is linked to immune mechanisms, oxidative stress, and disturbances in the cellular environment. Among the factors contributing to histological changes, proteolytic enzymes such as collagenase stand out, due to their role in degrading components of the extracellular matrix. Prolidase is a key enzyme in collagen metabolism, involved in the breakdown of proline-rich peptides. ***Objective*:** This study aims to investigate the mechanism of collagen decline in Iraqi vitiligo patients, prolidase activity and basic homeostasis to understand their roles in disease progression. This study was conducted as a cross-sectional comparative study, and 150 participants were included in the present study, divided equally into three groups as follows: patients treated with Narrowband Ultraviolet B (NB-UVB) (PUV), a newly diagnosed group (PN) and an apparently healthy control group (C). The sample size was determined based on statistical power analysis (0.80), ensuring adequate comparison between groups using G Power. The collagenase level was measured using the ELISA immunological method, and prolidase activity was measured using the modified Chinchard's method. Several biochemical parameters, including manganese (Mn^{2+}), zinc (Zn^{2+}), and calcium (Ca^{2+}), were measured using colourimetric analysis. The results showed significant increases in collagenase level and prolidase activity of PUV as well as PN compared with the control ($p < 0.001$). The results indicated no significant difference in Mn^{2+} and Zn^{2+} levels between PUN,PN and control. However, a significant reduction in Ca^{2+} levels was observed in PUV compared to the control. According to the results, high collagenase levels and prolidase activity in the serum contribute to the development of vitiligo by weakening the skin structure in affected areas.

Keywords: Vitiligo, Collagenase, Prolidase, Zinc, Manganese

تحلل الكولاجين ونشاط البرولايديز وعلاقته بالتوازن المعدني لدى مرضى البهاق العراقيين

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الخلاصة

الخلفية: البهاق اضطراب جلدي شائع يُسبب فقدانًا للتصبغ، ويُصنف كمرض مناعي ذاتي. يرتبط البهاق بآليات المناعة، والإجهاد التأكسدي، واضطرابات في البيئة الخلوية. من بين العوامل المساهمة في التغيرات

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النسيجية، تبرز الإنزيمات المحللة للبروتين، مثل الكولاجينيز ، نظرًا لدورها في تحلل مكونات المصفوفة خارج الخلوية. يُعد البرولايديز إنزيمًا رئيسيًا في أيض الكولاجين، حيث يشارك في تفكيك الببتيدات الغنية بالبرولين.الهدف تهدف هذه الدراسة إلى دراسة آلية انخفاض الكولاجين لدى مرضى البهاق العراقيين ونشاط البرولايديز والتوازن الداخلي الأساسي لفهم أدوارهما في تطور المرض. الطريقة: أجريت هذه الدراسة كدراسة مقارنة مقطعية، وشملت 150 مشاركًا تم تضمينهم في الدراسة الحالية مقسمين بالتساوي إلى ثلاث مجموعات على النحو التالي: المرضى الذين عولجوا بالأشعة فوق البنفسجية ضيقة النطاق(PUV) (NB-UVB) B ، والمجموعة التي تم تشخيصها حديثًا(PN) ، والتي تبدو بصحة جيدة كمجموعة ضابطة .(C) تم تحديد حجم العينة بناءً على تحليل القوة الإحصائية (0.80)، ضمان المقارنة المناسبة بين المجموعات باستخدام G Power. تم قياس مستوى الكولاجينيز بطريقة الاليزا المناعية ونشاط البرولايديز بطريقة تشينارد المعدلة بينما تم قياس العديد من المعايير الكيميائية الحيوية بما في ذلك المنغنيز (Mn^{2+}) والزنك (Zn^{2+}) والكالسيوم (Ca^{2+}) باستخدام التحليل اللوني. النتائج: ظهرت النتائج زيادات ملحوظة في مستوى الكولاجينيز ونشاط البرولايديز في PUV، بالإضافة إلى PN، مقارنةً بالمجموعة الضابطة ($p < 0.001$). وأشارت النتائج إلى عدم وجود فرق ملحوظ في مستويات Mn^{2+} وZn^{2+} بين PUN و PN والمجموعة الضابطة. ومع ذلك، لوحظ انخفاض ملحوظ في مستويات Ca^{2+} في PUV مقارنةً بالمجموعة الضابطة. الاستنتاجات: وفقًا للنتائج، يساهم ارتفاع مستوى الكولاجينيز ونشاط البرولايديز في المصل في تطور البهاق عن طريق إضعاف بنية الجلد في المناطق المصابة.

1. Introduction

Skin pigmentation disorder, known as vitiligo, is a non-contagious disease characterised by a progressive loss of skin pigmentation due to a disturbance in the function of melanocytes. It can affect mucous membranes, such as the lips, inside the mouth, and eyes, as well as in the hair [1]. Its severity and extent vary considerably, ranging from small, barely noticeable patches to large, widespread areas of lost skin colour [2]. Regardless of gender and race, this disorder affects between 0.38% and 2.9% of the arena's population. In some populations, inclusive of the ones in India, the prevalence is higher, affecting approximately 8% of the population [3]. Vitiligo is not just a benign cosmetic condition. Still, it is a disease that affects the patient's physical and emotional well-being, especially in darker skin tones, where the white patches can be more noticeable. Consequently, patients can undergo substantial alterations to their physical appearance [4, 5]. The immune dysfunction and oxidative stress play a major role in the development of vitiligo, as autoimmune responses destroy melanocytes. The accumulation of reactive oxygen species also contributes to cellular damage that promotes skin pigment loss[6-9]. Given the pivotal role of both oxidative stress and autoimmune responses in causing melanocyte damage and pigment loss in vitiligo patients, it has become imperative to study the cellular mediators that may contribute to the aggravation of these mechanisms. Among these factors, extracellular matrix-degrading enzymes are of particular interest due to their role in maintaining tissue homeostasis and regulating inflammatory responses. Collagenase (EC 3.4.24.7), one of the most important of these enzymes, belongs to the metalloprotease family and is capable of cleaving collagen, which plays important roles in both physiological and pathological states [10]. It is hypothesised that increased levels of collagenase might be related to skin disorders, including some autoimmune skin disorders, leading to alterations of dermal microscopical histological structure and skin pigmentation[11], which opens the way to investigate its possible role in vitiligo. Prolidase(EC 3.4.13.9) is an enzyme that plays a key role in the metabolism of peptides containing proline and hydroxyproline[12], two key components of collagen. Therefore, it plays a role in tissue repair and maintaining skin structural balance [13]. Despite its biological importance, its role in vitiligo has not yet been studied, making it a promising research target that could reveal new paths to understanding the disease. Therefore, investigating collagenase levels and prolidase activity in vitiligo patients may contribute to expanding understanding of the cellular interactions associated with disease

progression and fill a clear gap in the literature, especially in studies addressing local populations and their genetic and environmental specificities.

This study aims to investigate the role of collagenase levels and prolidase activity, along with their relationship to mineral balance, in the development of vitiligo. It also seeks to enhance scientific knowledge that may contribute to improving diagnostic and treatment methods.

2. Materials and Methods

This study was conducted from October 2024 to February 2025, involving 150 participants (27-40 years old) who attended the Dermatology and Venereology Department at Baghdad Teaching Hospital, located in the Medical City Complex, and Al-Imamain Al-Kadhimiya Teaching Hospital in Iraq. The participants were divided into three groups. The first group included 50 patients treated with Narrowband Ultraviolet B (NB-UVB) (PUV). The second group included 50 patients newly diagnosed (PN), while the third group consisted of 50 apparently healthy individuals (C). The sample size was determined based on a statistical power analysis, where a power value of 0.8 was adopted, which is the scientifically accepted value to ensure a sufficient probability of detecting statistically significant differences when they exist by using G Power (version 3.1) using a three-group ANOVA test [14]. For information, the number of phototherapy sessions for patients treated ranged from 50 to 100 sessions. The number of women and men was almost equal in all groups under study. After receiving their consent, all participants underwent a personal interview utilising a specifically created questionnaire format that included their complete medical history and all pertinent details. The protocol was approved by the Ethics Committee of the College of Science/ University of Baghdad (Ref: CSEC/1224/0123). The exclusion criteria of samples include autoimmune diseases, psoriasis, eye cataract, skin cancer, history of cutaneous photosensitivity, pregnancy, lactation, diabetes, and anaemia. Ten millilitres of room-temperature venous blood were collected as samples, and the tube was centrifuged for 5 minutes at 150 xg. Before being examined, the serum was placed in an Eppendorf tube and kept in a freezer at -20°C. Serum prolidase activity was determined using the Chinard colourimetric method, which is based on the reaction of the resulting proline with the ninhydrin reagent to form a colored complex that is measured spectrophotometrically at 515 nm using a spectrophotometer. Standard reaction and incubation steps were followed according to the original protocol [15]. The collagenase level in the samples was measured by an immunological method using the Human Collagenase ELISA Kit (Cat. ELK9586) from ELKBiotechnology. The detection sensitivity of the kit was 0.1 ng/mL, with a detection range of 0.16 to 10 ng/mL. Laboratory tests included Ca^{2+}, Zn^{2+}, and Mn^{2+} measurements using commercial colourimetric assays from different companies (Linear, Centronic GmbH, and Rakiro, respectively). The manufacturers' instructions were followed carefully, and the absorbance was read using a spectrophotometer. SPSS version 26 was used to properly characterise the data, utilising licensed materials. Using one-way ANOVA to compare mean± standard deviation (mean± SD), between three groups, because it is suitable for analysing differences between more than two groups and correlation between variables was determined by Pearson correlation coefficients (r)[16]. Confidence intervals (95%) were calculated to estimate the accuracy of the results, and effect sizes were calculated to demonstrate the practical significance of the differences, which enhances the reliability of statistical interpretation and contributes to clinical evaluation[17]. The Receiver operating characteristic (ROC) curve is an analysis of statistics that determines the optimal specificity and sensitivity of a diagnostic test by plotting the association between sensitivity and 1-specificity. This is used to evaluate the diagnostic performance of enzymes [18]. A p-value of <0.05 was considered statistically significant.

3. Results

The general anthropometrics of the participants are represented in Table 1. The mean age (± SD) was comparable across the PUV, PN, and C groups, ranging from 27 to 40 years. Body mass index (BMI), an anthropometric measure of weight relative to height, is presented in Table 1. A non-significant variance ($p > 0.05$) in (mean ± SD) of age, height, weight and BMI between the three groups was indicated; the results showed that individuals were overweight (BMI>26) for the three groups.

Table 1: The demographic characteristics of the three groups (n=50 in each group)

Characteristics	PUV group	PN group	C group	*p*-value
Sex				
Male	25(50%)	25(50%)	25(50%)	1.0
Female	25(50%)	25(50%)	25(50%)	
Vitiligo types				
Generalized	36(72%)	8(16%)	-	<0.001
Segmental	14(28%)	42(84%)	-	
Vitiligo stability				
Active	16(32%)	38(76%)	-	<0.001
Stable	34(68%)	12(24%)	-	
family history				
Yes	22(44%)	19(38%)	0(0%)	<0.001
No	28 (56%)	31(62%)	50(100%)	
Age (year)	32.8±3.9	32.23±4.17	32.35±4.16	0.764
Height (cm)	166.25±10.93	168.03±10.65	166.4±8.89	0.690
Weight (kg)	69.5±8.08	73.15±11.61	70.25± 9.55	0.221
BMI (kg/m²)	26.13±1.66	26.75±1.65	26.35±2.73	0.410

Categorical variables are presented as number (percentage), and continuous variables are presented as mean ± SD.

Table 2 shows the comparison of serum collagenase level and prolidase activity across the study groups. The results showed significant increases in prolidase activity and collagenase level in PUV and PN compared to the control. PUV patients revealed a higher mean value of prolidase activity than PN patients, and the results were significant (<0.001), whilst the collagenase level was also higher, but the results were non-significant. The results show no significant difference in Mn^{2+} and Zn^{2+} levels of PUN and PN compared to the control. Whilst results of Ca^{2+} showed a significant decrease in Ca^{2+} of PUV when compared with control no significant changes were seen between the PN and control groups, as well as PUV with PN.

Table 2: Comparison of serum collagenase level, prolidase activity, Mn^{2+}, Zn^{2+} and Ca^{2+} in sera of the three studied groups.

Parameter	PUV group (mean±SD)	PN group (mean±SD)	C group (mean±SD)	*p*-value (ANOVA)	η^2
Collagenase level (ng/ml)	3.39 ± 0.95[a]	3.03 ± 1.05[b]	0.57 ± 0.21	<0.0001	0.696
Prolidase activity (U/L)	4.52 ± 0.98[a,c]	3.46 ±0.95[b,c]	1.64 ± 0.65	<0.0001	0.625
Mn^{2+} (µg/L)	1.94 ± 0.89	1.82 ± 0.76	1.74 ± 0.70	0.527	0.012
Zn^{2+} (nmol/µL)	0.45 ±0.12	0.44 ±0.11	0.44 ±0.09	0.968	0.019
Ca^{2+} (mg/dL)	6.03 ± 0.45[a]	6.32 ± 0.77	6.39 ± 0.46	0.016	0.068

(a): indicated significant difference between PUV and C.(b): indicated significant difference between PN and C.(c): indicated significant difference between PUV and PN.(Tukey post hoc analysis, $p < 0.05$). η^2:effect size

The results revealed a significant increase in the mean collagenase level in vitiligo patients compared to the control group (Table 3). The mean in the PUV group was 3.39 ng/mL, 95% CI: 3.09–3.70, and in the PN group was 3.03 ng/mL, 95% CI: 2.69–3.37, whereas in the control group it was 0.57 ng/mL, 95% CI: 0.48–0.65. Prolidase activity was highest in the PUV group (4.52 U/L, 95% CI: 3.15–3.76), followed by the PN group (3.46 U/L, 95% CI: 4.21–4.84), compared to the control group (1.64 U/L, 95% CI: 1.73–1.92).

Table 3: Statistical Analysis of 95% Confidence Intervals for Mean of Collagenase Level and Prolidase Activity in the studied groups.

Parameter	95% Confidence Interval for Mean		
	PUV group	PN group	C group
Collagenase level (ng/ml)	3.090-3.703	2.697-3.374	0.485-0.657
Prolidase activity (U/L)	3.155-3.764	4.213-4.845	1.736-1.921

The correlation between prolidase activity, collagenase level and other factors studied for both patient groups (PUV and PN) was examined as summarised in Table 4

Table 4 : Correlation between prolidase activity and collagenase level with other studied parameters for the PUV and PN groups.

Parameter		PUV group		PN Group	
		Prolidase activity	Collagenase level	Prolidase activity	Collagenase level
Collagenase level	**r**	-0.314	1	0.052	1
	P-value	0.049		0.75	
Prolidase activity	**r**	1	-0.314	1	0.052
	P-value		0.049		0.752
Mn^{2+}	**r**	-0.086	-0.234	0.070	0.498
	P-value	0.596	0.147	0.668	0.001
Zn^{2+}	**r**	-0.456	0.404	0.287	0.273
	P-value	0.003	0.01	0.072	0.088
Ca^{2+}	**r**	-0.051	-0.081	-0.042	0.054
	P-value	0.754	0.619	0.797	0.742

P-value less than 0.05 (significant).

Prolidase activity showed a weak negative correlation with collagenase level and a medium negative correlation with Zn^{2+}, while collagenase level showed a weak positive correlation with

Zn^{2+} in the PUV group. The collagenase level showed a medium positive correlation with Mn^{2+} in the PN group.

A diagnostic test's optimal specificity and sensitivity are determined via statistical analysis (ROC), which plots the association between sensitivity and 1-specificity. The following Figure (1and 2) represents the ROC curve of prolidase activity & collagenase level in PUV and PN.

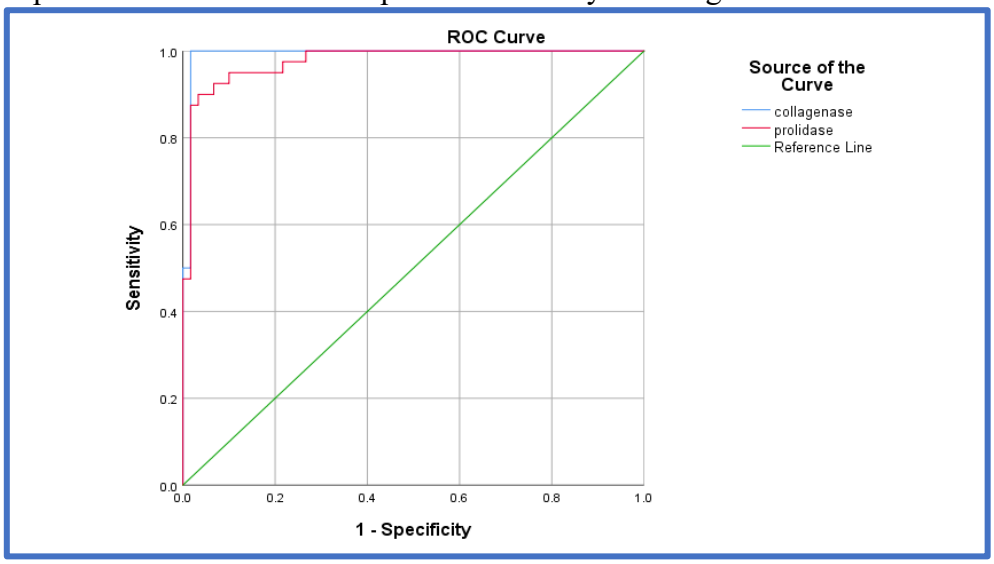

Figure 1: ROC curve of collagenase level & prolidase activity in PUV and control groups.

Table 5: Measurements of AUC, P-value, Lower Bound, Upper Bound, Cut-Off Value, Sensitivity and Specificity for collagenase level & prolidase activity in PUV and control groups.

Parameter	AUC	SE	P-value	Asymptotic 95% Cl		Cut-Off Value	Sensitivity	Specificity
				Lower Bound	Upper Bound			
Collagenase level	0.992	0.008	<0.001	0.975	1.000	2.02	90.0%	98.3%
Prolidase activity	0.976	0.013	<0.001	0.951	1.000	2.88	95.0%	81.7%

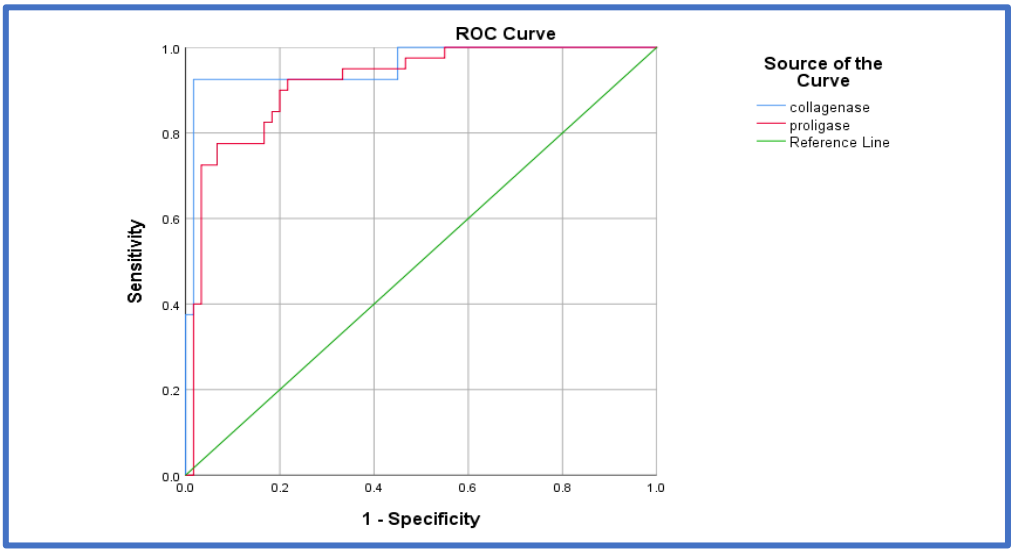

Figure 2: ROC curve of collagenase level & prolidase activity in PN and control groups.

Table 6: Measurements of AUC, P-value, Lower Bound, Upper Bound, Cut-Off Value, Sensitivity and Specificity for collagenase level & prolidase activity in PN and control groups.

Parameter	AUC	SE	P-value	Asymptotic 95% Cl		Cut-Off Value	Sensitivity	Specificity
				Lower Bound	Upper Bound			
Collagenase level	0.957	0.022	<0.001	0.915	0.999	2.14	87.5%	98.3%
Prolidase activity	0.917	0.029	<0.001	0.861	0.973	2.08	92.5%	78.3%

4. Discussion

Descriptive data revealed clear homogeneity in demographic characteristics across the studied groups, thereby enhancing the power of statistical comparison and reducing the likelihood of bias. No significant differences were observed in age, gender, or BMI between patients (PUV and PN) and the control group, enhancing the reliability of subsequent analyses results. Studies have demonstrated that genetic factors and a family history of the disease play a significant role in its development [19]. Collagenase enzymes play a crucial role in collagen degradation and modification of the extracellular matrix, influencing skin structure and melanocyte function in vitiligo [10]. Prolidase is important in the degradation of collagen peptides[20], but previous studies have not investigated its role in vitiligo, easuring its activity in this study a new step in understanding the structural changes associated with the disease. According to the current study, significant increases in serum collagenase levels were observed in PUV and PN compared with the control group (Table 2). Various studies have suggested that connective tissue is altered in vitiligo patients. Collagenase in the skin formed during vitiligo causes serious damage to the extracellular matrix in these foci, destroying collagen fibrils and affecting the neighbouring keratinocytes in the process [20]. Injury and rupture of the skin lead to the absorption of tissue collagen breakdown products, mainly collagenase [21]. Fibroblasts secrete collagenase and can efficiently degrade the dermal collagen. This enzyme also affects the number and activity of melanocytes [22]. Meanwhile, collagenase is capable of digesting collagen and other elements of the extracellular matrix and inducing melanocyte damage that may be due to the release of pigment granules, accumulation of pigment, and the formation of melanin in keratinocytes, or induction of melanocyte apoptosis in some conditions[22]. The current study found significant increases in the serum prolidase activity in PUV and PN compared with the control group. PUV patients revealed a higher mean value of prolidase activity than PN patients. Increased prolidase activity can be considered a marker of increased collagen turnover. Elevated prolidase levels and impaired collagen metabolism have been observed in a wide variety of diseases, including radiation exposure, scurvy, celiac disease, diabetes mellitus, liver cirrhosis, cancer, and hypertension[23]. We consider that serum prolidase activity was increased in patients with vitiligo for the following reasons: epithelial redox homeostasis was disrupted; increased oxidative stress accumulated as a result of inhibiting the antioxidant system to remove the reactive oxygen species that oxidized the skin protein and targeted the melanocytes by releasing more proline-containing dipeptides through collagen and elastin degradation[24]. As a result of the detected increase in prolidase activity in this study, it can be assumed that patients receiving UVB therapy may have an additional source of Pro-Hyp. Increased prolidase activity is attributed to prolidase being expressed in greater amounts rather than increased activity of prolidase, despite a high association between serum prolidase activity and levels [25]. Prolidase activity typically increases in environments with oxidative stress, as well as in response to tissue damage [13]. According to current studies, there is no significant difference in Mn^{2+} and Zn^{2+} levels of PUN and PN compared with the control. Ca^{2+} was measured in this study, and the results appeared to show a significant decrease in Ca^{2+} of PUV when compared with control, whilst no significant changes were seen between the PN and Control groups, as well as PUV with PN. Zinc, which is found in all cells and makes up less than 0.005% of body weight, is essential for the proper operation of the

body's tissues, organs, and cells [26]. A study conducted by Bayram, M et al. reported no significant difference in the serum Zn^{2+} level among patients with vitiligo, suggesting that serum Zn^{2+} level may not be a distinguishing biochemical marker for the disease in the studied population[27]. There was no correlation between Mn^{2+} levels in the body and their concentrations in specific tissues[28]. Mn^{2+} was a potent innate immune stimulator by itself, also more strongly activating inflammasomes [29]. Several skin processes, including keratinocyte development, skin barrier formation, and permeability barrier homeostasis, are regulated by calcium ions (Ca^{2+}) and the concentration gradient of these ions in the epidermis. According to recent research, the endoplasmic reticulum (ER) and other intracellular Ca^{2+} stores are the main constituents of the epidermal Ca^{2+} gradient, and the homeostasis of the ER Ca^{2+} is essential for controlling the differentiation of keratinocytes, the formation of intercellular junctions, the antimicrobial barrier, and the permeability barrier [30]. Although collagenase requires zinc, its gene expression and regulation can be influenced by other factors, such as inflammation, oxidative stress, and inflammatory cytokines (such as TNF-α and IL-6), which may be elevated in vitiligo patients [31]. The study results showed that the effect sizes for both collagenase level and prolidase activity were 0.696 and 0.625, respectively, indicating a medium to large effect. These values reflect a clear and noticeable difference in the activity of these enzymes between the studied groups, supporting the hypothesis of their effective role in the structural changes associated with vitiligo. In contrast, the effect sizes for the minerals (Mn^{2+},Zn^{2+} and Ca^{2+}) were less than 0.1, and the differences were not significant. This indicates a weak relationship between these elements and the disease under the conditions of the current study, and they may be influenced by other factors that were not taken into account. In addition to the statistical significance, the confidence intervals for the mean collagenase level and prolidase activity in this study further supported the reliability of the differences between groups. The confidence intervals for both collagenase level and prolidase activity were higher for the PUV and PN groups than for the control group, with no overlap between them, reflecting the consistency of the differences and their lack of randomness. This clear separation in the confidence intervals supports the findings of the p-values and reinforces the strength of the effect sizes, which were within the medium to high range for both enzymes. The correlation results in Table 4 indicate weak negative correlation prolidase activity with collagenase level and medium negative correlation with Zn^{2+}, whilst Collagenase level showed weakly positive with Zn^{2+} in the PUV group and showed a medium positive correlation with Mn^{2+} in the PN group. The results of the Roc curve showed the diagnostic susceptibility of prolidase activity and collagenase level to PUV and PN. In PUV Patients Roc cut off point for collagenase level and prolidase activity was calculated. Collagenase level shown cut off point was (2.02) and the AUC was (0.992). The optimal cut-off point has a 98.3% specificity and a 90.0% sensitivity. The optimal cut-off point for protease activity demonstrated a sensitivity of 95.0% and specificity of 81.7%. The cut-off point was 2.88 and the AUC was 0.976). The cutoff value for the collagenase level in PN patients was 2.14, and the AUC was 0.957. The best cut-off point has a 98.3% specificity and an 87.5% sensitivity. According to the prolidase activity, the AUC was 0.917 and the cutoff value was 2.08.The best cut-off point shows a sensitivity of 92.5% and specificity of 78.3%. ROC curve results, Collagenase level and Prolidase activity may help diagnose PUV and PN. Note that if the area under the curve is less than 0.5, this test is not used in diagnosing the disease. A major limitation of this study is its cross-sectional design, which limits the possibility of inferring causal relationships. Furthermore, the sample size was limited, and enzymes were measured only in serum, not in the skin, which may affect the accuracy of representing local changes. Furthermore, the study's limited geographical scope may limit the generalizability of
the results.

Conclusion

The findings of this study reveal a significant and biologically significant relevant increase in collagenase levels and prolidase activity in vitiligo patients (PN and PUV), suggesting that these enzymes may play a role in the structural changes linked to melanocyte loss. The high effect size values and non-overlapping confidence intervals enhance the reliability of these results. In contrast, mineral elements (Mn^{2+}, Zn^{2+}, and Ca^{2+}) did not show significant differences or a clear effect, indicating a weak association with the pathogenesis in the studied sample. These findings support the importance of investigating the role of collagenases and prolidase as contributing factors in the development of vitiligo and call for broader future studies that include local analysis in skin tissues.

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Ethical

The Iraqi ministries of the environment, health, higher education, and scientific research have approved the Research Ethics Committee for scientific research (Ref: CSEC/1224/0123).

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