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WJEM mainly publishes articles reporting research results and findings obtained in the field of experimental medicine and covering a wide range of topics including clinical laboratory medicine (applied and basic research in hematology, body fluid examination, cytomorphology, genetic diagnosis of hematological disorders, thrombosis and hemostasis, and blood typing and transfusion), biochemical examination (applied and basic research in laboratory automation and information system, biochemical methodology, and biochemical diagnostics), *etc*.

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ORIGINAL ARTICLE

Case Control Study

Altered expression of miR-125a and dysregulated cytokines in systemic lupus erythematosus: Unveiling diagnostic and prognostic markers

Tagreed Qassim Alsbihawi, Mojtaba Zare Ebrahimabad, Fakhri Sadat Seyedhosseini, Homa Davoodi, Nafiseh Abdolahi, Alireza Nazari, Saeed Mohammadi, Yaghoub Yazdani

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Abstract

BACKGROUND

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder impacting multiple organs, influenced by genetic factors, especially those related to the immune system. However, there is a need for new biomarkers in SLE. MicroRNA-125a (miR-125a) levels are decreased in T cells, B cells, and dendritic cells of SLE patients. MiR-125a plays a regulatory role in controlling the levels of tumor necrosis factor-alpha (TNF-α) and interleukin 12 (IL-12), which are crucial pro-inflammatory cytokines in SLE pathogenesis.

AIM
To assess the levels of miR-125a, IL-12, and TNF-α in SLE patients' plasma, evaluating their diagnostic and prognostic value.

METHODS
The study included 100 healthy individuals, 50 newly diagnosed (ND), and 50 SLE patients undergoing treatment. The patients were monitored for a duration of 24 wk to observe and record instances of relapses. MiR-125a expression was measured using real-time reverse transcription polymerase chain reaction, while ELISA kits were used to assess IL-12 and TNF-α production.

RESULTS
The results showed significantly reduced miR-125a expression in SLE patients compared to healthy individuals, with the lowest levels in ND patients. TNF-α and IL-12 expression levels were significantly elevated in SLE patients, especially in the early stages of the disease. Receiver operating characteristic curve analyses, and Cox-Mantel Log-rank tests indicated miR-125a, TNF-α, and IL-12 as proper diagnostic biomarkers for SLE. A negative correlation was found between plasma miR-125a expression and IL-12/TNF-α levels in SLE patients.

CONCLUSION
Decreased miR-125a levels may be involved in the development of SLE, while elevated levels of IL-12 and TNF-α contribute to immune dysregulation. These findings offer new diagnostic and prognostic markers for SLE. Moreover, the negative correlation observed suggests an interaction between miR-125a, TNF-α, and IL-12. Further research is necessary to uncover the underlying mechanisms that govern these relationships.

Key Words: Systemic lupus erythematosus; microRNA-125a; Interleukin-12; Tumor necrosis factor alpha; Biomarker

Core Tip: The aim of this study was to investigate the levels of microRNA-125a (miR-125a), interleukin 12 (IL-12), and tumor necrosis factor-alpha (TNF-α) in the plasma of systemic lupus erythematosus (SLE) patients, and assess the diagnostic and prognostic value of these biomarkers in SLE. The study included healthy individuals, newly diagnosed SLE patients, and SLE patients undergoing treatment. The results revealed decreased levels of miR-125a in SLE patients, particularly in newly diagnosed cases. On the other hand, elevated levels of IL-12 and TNF-α were observed in SLE patients, especially in the early stages of the disease. The study also identified miR-125a, TNF-α, and IL-12 as potential diagnostic biomarkers for SLE. The negative correlation observed between miR-125a and IL-12/TNF-α suggests an interaction between these factors. These findings provide insights into new diagnostic and prognostic markers for SLE, highlighting the importance of immune dysregulation in the disease.

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INTRODUCTION

Systemic lupus erythematosus (SLE) involves an overactive immune system causing organ damage and posing significant health risks[1]. SLE significantly reduces quality of life, increases susceptibility to premature death, and imposes substantial financial burdens[2]. Both genetic and environmental factors contribute to the development of SLE, leading to the production of autoantibodies that attack and harm the body's own tissues and organs[3,4]. Epigenetic changes like deoxyribonucleic acid methylation, histone modifications, non-coding RNAs, and chromatin remodeling are associated with SLE development[5,6].

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression and play a significant role in various biological processes, impacting disease development and progression[7,8]. miRNAs are crucial in regulating immune function and their imbalance is linked to autoimmunity in SLE. Specific miRNAs like miR-146a, miR-155, miR-148a, miR-21, and miR-125a are implicated in SLE[6,9,10]. The imbalance of these miRNAs may contribute to SLE mechanisms by fostering autoreactivity, where the immune system reacts against the body's own cells and tissues[11].

Research suggests reduced miR-125a levels in SLE patients' T cells, B cells, and dendritic cells, leading to increased activation, proliferation, and inflammatory cytokine production[12]. Restoring miR-125a levels shows potential as a treatment target, suppressing abnormal activation[13]. It directly regulates key SLE-related cytokines and serves as a diagnostic and prognostic marker, correlating with disease activity and organ damage[14,15]. Adjusting miR-125a

expression in animal models has shown promise in reducing autoantibody production and improving outcomes[16], although further research is crucial for human application and personalized treatment strategies[11].

Imbalanced and dysfunctional cytokine production and signaling significantly contribute to SLE onset. Dysregulation of cytokines like interleukin (IL)-6, interferon alpha (IFN-α), tumour necrosis factor alpha (TNF-α), IL-17, and IL-12 is linked to SLE development and progression[17]. IL-12, essential in Th1 cell differentiation and immune responses, is linked to SLE severity and lupus nephritis, exerting both pro-inflammatory and immune-regulatory effects. Therapeutic targeting of IL-12 in SLE yields mixed results, reflecting disease complexity and diverse signaling pathways[18-24]. TNF-α, a pro-inflammatory cytokine, exhibits disrupted regulation in SLE, promoting abnormal immune function and autoantibody production. Its role in inflammation includes immune cell recruitment, cytokine and chemokine production, and endothelial cell activation, potentially leading to tissue damage and fibrosis in affected organs. While TNF-α inhibitors hold promise for SLE therapy, identifying responsive patient subsets and optimizing treatment strategies remain essential for improving outcomes[25-29].

Common molecular biomarkers for diagnosing and monitoring SLE include antinuclear antibodies, anti-dsDNA antibodies, complement levels, C-reactive protein, erythrocyte sedimentation rate, IFN-α, and B-cell activating factor. Yet, these markers have limitations such as lack of specificity, sensitivity, and predictability, high costs, and invasive testing, prompting the search for more reliable alternatives[30,31]. MiR-125a regulates TNF-α and IL-12 by targeting their mRNA and influencing related signaling molecules like NF-κB, mitogen activated protein kinases, and signal transducer and activator of transcription proteins. Given the association of TNF-α and IL-12 dysregulation with inflammation, miR-125a holds potential as a therapeutic target. Adjusting miR-125a expression could restore cytokine balance and mitigate inflammation in conditions like rheumatoid arthritis and inflammatory bowel disease, although challenges persist in understanding its regulation[32-35].

To the best of our knowledge, no study has comprehensively examined the expressions of miR-125a, IL-12, and TNF-α in SLE patients collectively and evaluated their diagnostic and prognostic significance. Hence, the aim of this study was to analyze the levels of miR-125a, TNF-α, and IL-12 in the plasma of both SLE patients and healthy individuals, explore their potential correlations, and investigate their usefulness as diagnostic and prognostic biomarkers for SLE.

MATERIALS AND METHODS

Study participants and samplings

A total of 200 participants were enrolled in this study, including 50 newly diagnosed (ND) and 50 under-treatment (UT) SLE patients from the Rheumatology Clinic at Sayyad Shirazi Hospital in Gorgan. Additionally, 100 healthy individuals were included as controls. SLE cases were diagnosed based on the ACR criteria and UT group were receiving standard treatment for SLE without any immunomodulatory or immunosuppressive therapies that could impact the variables under investigation. The control group consisted of age, sex, and ethnicity-matched individuals with no history of autoimmune diseases. All participants were within the age range of 18-65 years and were excluded if they had other autoimmune diseases, active infections, or were pregnant. Under-treatment cases had a minimum disease duration of 6 mo, and their disease activity was recorded at the time of serum collection to minimize the influence of disease activity on the variables of interest. The study received ethical approval from the Committee of Ethics at Golestan University of Medical Sciences (GoUMS), Gorgan, Iran, and adhered to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants prior to their inclusion. Blood samples (5 per participant) were collected and sent to the Research Central Laboratory at GoUMS. Plasma was isolated from the whole blood through centrifugation and stored at -80°C to ensure sample integrity and prevent contamination. Table 1 presents the levels of miR-125a, IL-12, and TNF-α in relation to various clinical characteristics of SLE patients.

ELISA cytokine assay

The expression of cytokines was examined using commercially available ELISA kits from ZellBio (ZellBio GmbH, Germany; IL-12 Cat.NO. RK00072-96; TNF-α Cat.NO. RK00030-96). We strictly adhered to the manufacturer's instructions provided in the kit datasheet to ensure precise and reliable results. The optical density values from the samples and standards were measured using a StatFax 3300 ELISA reader (Awareness Technology, Inc., United States)[36]. Non-linear regression analysis was employed to generate standard curves and calculate the concentrations of IL-12, and TNF-α in each sample, measured in picograms per milliliter (pg/mL) for IL-12 and pg/dL for TNF- α[37].

RNA isolation, cDNA synthesis and real time reverse transcription polymerase chain reaction

Total RNA was extracted from the plasma samples using TRIzol reagent (Invitrogen, United States) following a previously described protocol[38]. The RNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, United States). The RNA was either stored at -80°C or used immediately for subsequent applications, ensuring the use of RNase-free reagents and equipment throughout the process. For cDNA synthesis, the Sinnaclon First Strand cDNA Synthesis Kit (Cinnagen, Iran; Cat. NO. RT5201) was employed. To convert mature miRNA molecules into cDNA for amplification and quantification through quantitative polymerase chain reaction (qPCR), the stem-loop method was utilized, along with a specific stem-loop primer and a common reverse primer (as listed in Table 2). The expression levels of miRNA were quantitatively analyzed using Sina Green HS-qPCR Mix (Cinnagen, Iran; Cat. NO. MM2042) along with specific primers. The qPCR reactions were conducted on a Step One Plus cycler (Thermo Fisher Scientific, Iran). In this study, the cycle threshold (Ct) values of miR-125a plasma expression levels were normalized using the internal control U6 (small nuclear RNA U6). The 2-dCt method, a commonly used

Table 1 The association of microRNA-125a, tumor necrosis factor-alpha, and interleukin 12 with major clinical symptoms of systemic lupus erythematosus patients

Characteristics		miR-125a		TNF-α		IL-12	
Lupus nephritis	Yes	0.199 ± 0.07	*P* = 0.576	53.42 ± 14.44	*P* = 0.153	122.37 ± 22.36	*P* = 0.104
	No	0.205 ± 0.09		45.60 ± 12.13		108.34 ± 29.87	
Malar rash	Yes	0.180 ± 0.06	***P* = 0.017**	54.60 ± 12.44	*P* = 0.360	128.45 ± 20.28	*P* = 0.065
	No	0.216 ± 0.09		43.37 ± 11.51		102.42 ± 28.94	
Hair loss	Yes	0.188 ± 0.06	***P* = 0.022**	52.63 ± 13.08	*P* = 0.401	126.87 ± 20.62	***P* = 0.049**
	No	0.211 ± 0.09		44.34 ± 11.98		103.19 ± 29.48	
SLEDAI	≤ 4	0.202 ± 0.08	***P* = 0.005**	44.00 ± 14.71	***P* = 0.001**	102.43 ± 23.38	***P* = 0.000**
	5-12	0.280 ± 0.08		30.49 ± 12.59		69.54 ± 32.43	
	≥ 12	0.199 ± 0.09		49.11 ± 13.51		113.26 ± 28.54	

Table 2 List of primers for microRNA-125a and U6 internal control

Primer	Sequence (5'>3')
miR-125a	**F:** GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGT
	R: GTCGTATCCAGTGCAGGGTCCGAGGTGCCGAGGATTTCCACCACCTG
U6	**F:** GCTTCGGCAGCACATATACTAAAAT
	R: CGCTTCACGAATTTGCGTGTCAT

approach for normalizing gene and miRNA expression levels, was utilized for data analysis[39].

Statistical analysis

Statistical analyses were performed using SPSS 22.0 (IBM Corporation, United States) and Prism 8.0 (GraphPad Software Inc, United States). The Shapiro-Wilk test was used to assess the normality of the data, and parametric or non-parametric tests were chosen accordingly. Independent Samples *t*-test or Mann-Whitney U test was employed for comparisons between two groups, while one-way ANOVA with Tukey's post-test or Kruskal-Wallis with Dunn-Bonferroni post-test was used for comparisons involving more than two groups. Pearson/Spearman tests were utilized for correlation analyses based on the distribution of the data. Receiver operating characteristic (ROC) curve analyses were conducted to evaluate the diagnostic utility of each variable, and logistic regression was employed for combined ROC curve analysis and prediction of variable performance. The Mantel-Haenszel (Mantel-Cox) log-rank test was utilized to assess the prognostic value of variables in predicting flare occurrence after a 24-wk follow-up. All experiments were performed in triplicates. The significance level for all statistical tests was set at 0.05, with a 95% confidence interval and a test power of 80%.

RESULTS

Expression levels of miR-125a, TNF-α, and IL-12 in SLE patients

This study analyzed the expression levels of miR125a, TNF-α, and IL-12 in SLE patients and normal subjects. The results indicated that the expression level of miR-125a was significantly decreased in SLE patients compared to normal subjects (*P* value < 0.0001) (Figure 1A). However, it was shown that miR-125a expression was lowest in ND SLE patients compared to those UT (*P* value < 0.01) (Figure 1B). TNF-α was significantly elevated in SLE patients compared to normal subjects (*P* value < 0.0001) (Figure 1C). The expression level of TNF-α was compared among SLE patients with different disease states (under treatment and newly diagnosed). The results showed that TNF-α expression was highest in ND SLE patients compared to UT patients (*P* value < 0.01) (Figure 1D). Independent samples t-test indicated that the expression level of IL-12 was significantly elevated in SLE patients compared to normal subjects (*P* value < 0.0001) (Figure 1E). The expression level of IL-12 was highest in ND SLE patients compared to those with longer disease durations (*P* value < 0.01) (Figure 1F).

The diagnostic utilities of miR-125a, TNF-α and IL-12

ROC curve analysis was conducted to evaluate the diagnostic utility of miR-125a, TNF-α and IL-12 to distinguish SLE patients from normal subjects, and ND SLE patients from UT. The area under the curve (AUC) for the expression of miR-

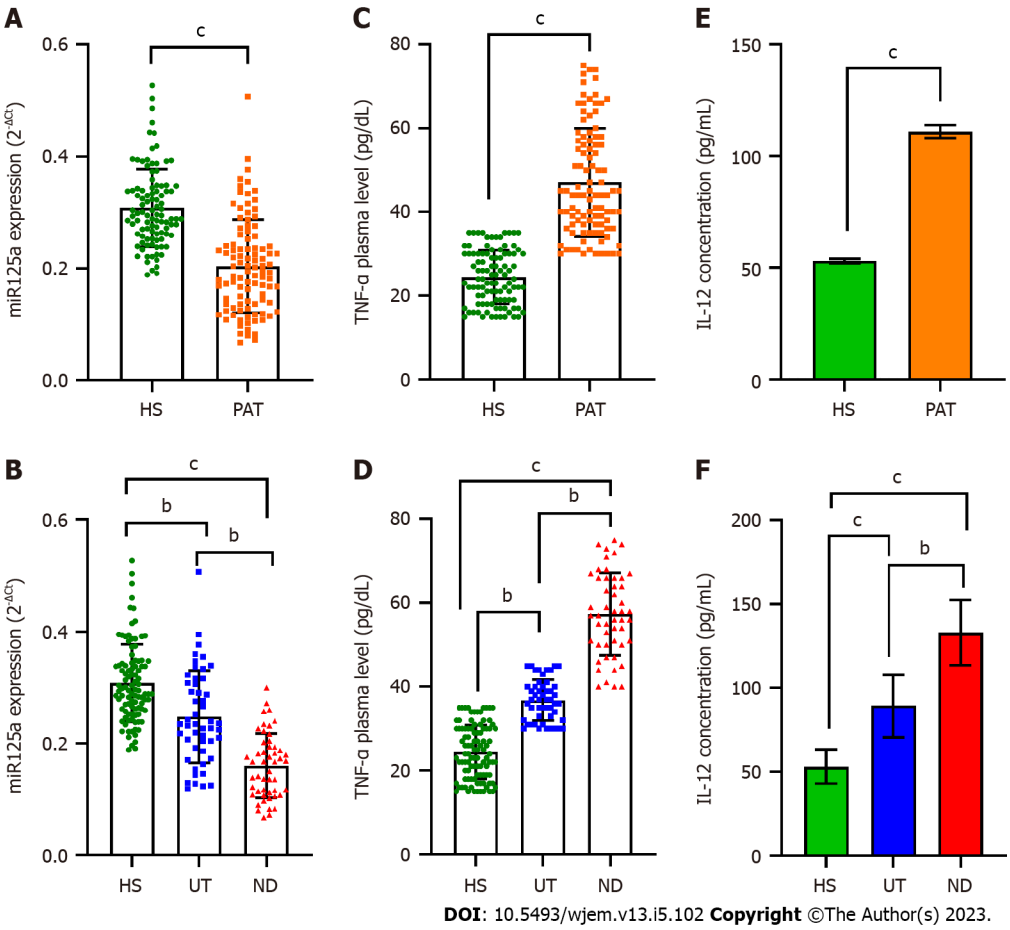

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Figure 1 The expression levels of microRNA-125a, tumor necrosis factor-alpha, and interleukin 12 in systemic lupus erythematosus patients and normal subjects. This study examined the expression levels of microRNA-125a (miR-125a), tumor necrosis factor-alpha (TNF-α), and interleukin 12 (IL-12) in systemic lupus erythematosus (SLE) patients and normal subjects. A: The findings revealed that miR-125a expression was significantly lower in SLE patients compared to normal subjects; B: With the lowest levels observed in newly diagnosed patients; C: TNF-α expression was higher in SLE patients compared to normal subjects; D: Its levels were highest in newly diagnosed patients; E: Similarly, the expression of IL-12 was significantly elevated in SLE patients compared to normal subjects; F: It was highest in newly diagnosed patients. The Independent Samples *t*-test or Mann-Whitney U test were employed for comparing two groups, while one-way ANOVA with Tukey's post-test or Kruskal-Wallis with Dunn-Bonferroni post-test were used for comparing more than two groups. The error bars represent means ± SD. Significance levels are denoted as $^{b}P < 0.01$ and $^{c}P < 0.0001$. HS: Humic subjects; PAT: Patients; UT: Under-treatment; ND: Newly diagnosed.

125a [humic subjects (HS) *vs* Patients (PAT)] (Figure 2A) was 0.8370 (95%CI: 0.7803 to 0.8936; $P < 0.0001$). The cut-off point was set at the fold change level of 0.2365 with the sensitivity of 72.00% (95%CI: 62.51% to 79.86%), the specificity of 88.00% (95%CI: 80.19% to 93.00%), and likelihood ratio (LR) of 6.0. The calculated AUC for miR-125a (ND *vs* UT) (Figure 2B) was 0.8102 (95%CI: 0.7279 to 0.8925; $P < 0.0001$). The cut-off value was set at the FC level of 0.1849 with the sensitivity of 70.0% (95%CI: 56.25% to 80.90%), the specificity of 78.0% (95%CI: 64.76% to 87.25%), and LR of 3.182.

The AUC for the expression of TNF-α (HS *vs* PAT) (Figure 2C) was 0.9668 (95%CI: 0.9476 to 0.9860; $P < 0.0001$). The cut-off point was set at the level of 34.50 with the sensitivity of 84.00% (95%CI: 75.58% to 89.90%), the specificity of 92.00% (95%CI: 85.00% to 95.89%), and LR of 10.50. Similarly, the calculated AUC for TNF-α (ND *vs* UT) (Figure 2D) was 0.9748 (95%CI: 0.9513 to 0.9983; $P < 0.0001$). The cut-off value was set at the level of 44.50 with the sensitivity of 88.00% (95%CI: 76.20% to 94.38%), the specificity of 92.00% (95%CI: 81.16% to 96.85%), and LR of 11.00. The AUC for the expression of IL-12 (HS *vs* PAT) (Figure 2E) was 0.9778 (95%CI: 0.9599 to 0.9957; $P < 0.0001$). The cut-off point was set at the level of 69.50 pg/mL with the sensitivity of 91.00% (95%CI: 83.77% to 95.19%), the specificity of 98.00% (95%CI: 93.00% to 99.64%), and LR of 45.5. Similarly, the calculated AUC for IL-12 (ND *vs* UT) (Figure 2F) was 0.9600 (95%CI: 0.9289 to 0.9911; $P < 0.0001$). The cut-off value was set at the level of 119.50 pg/mL with the sensitivity of 72% (95%CI: 58.33% to 82.53%), the specificity of 98% (95%CI: 89.50% to 99.90%), and LR of 36.0.

The correlations of miR-125a with TNF-α and IL-12

We examined the relationship between plasma levels of miR-125a and IL-12 as well as miR-125a and TNF-α. The results of a Pearson correlation analysis revealed a negative correlation between IL-12 and miR-125a ($r = -0.569$, $P < 0.0001$) (Figure 3A). Similarly, a negative correlation was observed between TNF-α and miR-125a ($r = -0.570$, $P < 0.0001$) (Figure 3B).

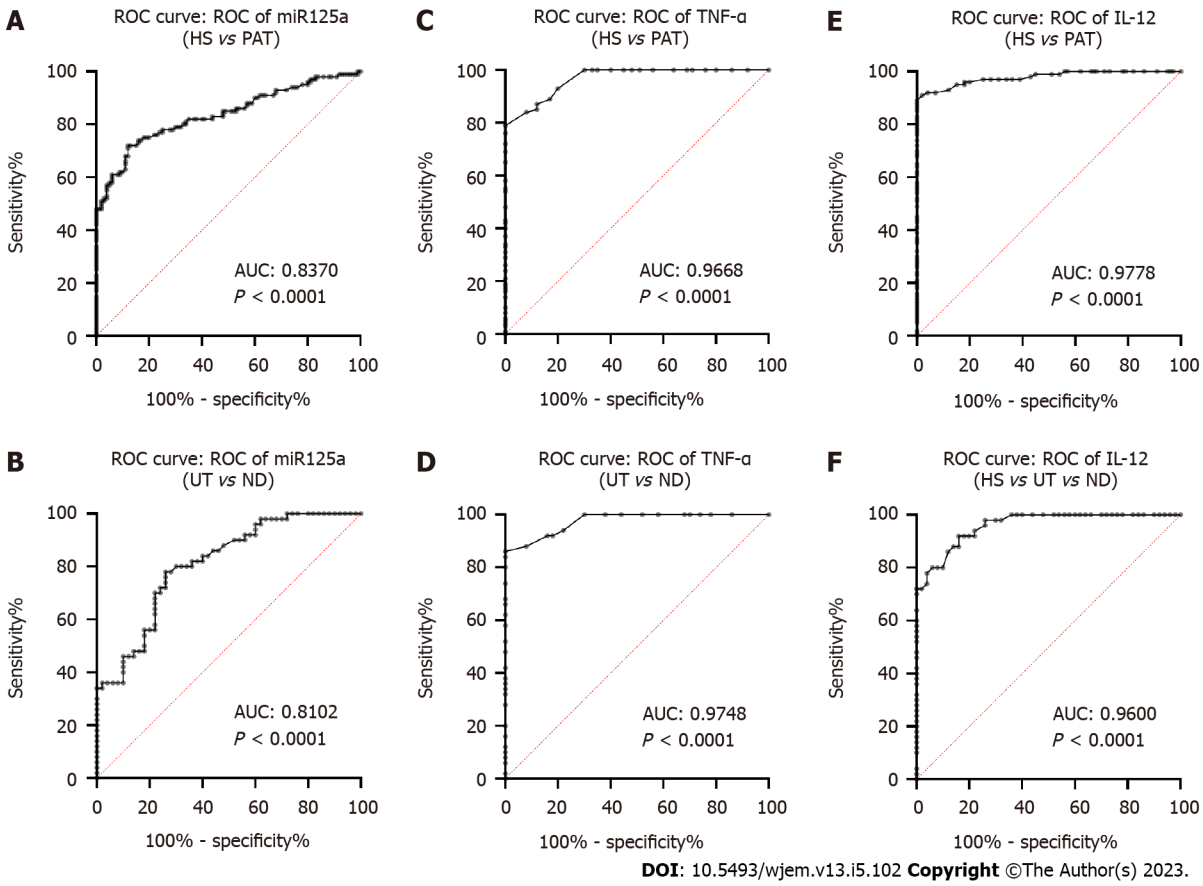

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Figure 2 Diagnostic utilities of microRNA-125a, tumor necrosis factor-alpha, and interleukin 12 in systemic lupus erythematosus patients. Receiver operating characteristic curve analysis was performed to assess the diagnostic accuracy of microRNA-125a (miR-125a), tumor necrosis factor-alpha (TNF-α), and interleukin 12 (IL-12) in distinguishing systemic lupus erythematosus (SLE) patients from normal subjects and newly diagnosed SLE patients from those under treatment. A: The area under the curve (AUC) values for miR-125a were 0.8370 (95%CI: 0.7803 to 0.8936; $P < 0.0001$) in SLE patients *vs* normal subjects; B: 0.8102 (95%CI: 0.7279 to 0.8925; $P < 0.0001$) in newly diagnosed *vs* under treatment SLE patients; C: For TNF-α, the AUC values were 0.9668 (95%CI: 0.9476 to 0.9860; $P < 0.0001$) in SLE patients *vs* normal subjects; D: 0.9748 (95%CI: 0.9513 to 0.9983; $P < 0.0001$) in newly diagnosed vs. under treatment SLE patients; E: Regarding IL-12, the AUC values were 0.9778 (95%CI: 0.9599 to 0.9957; $P < 0.0001$) in SLE patients *vs* normal subjects; F: 0.9600 (95%CI: 0.9289 to 0.9911; $P < 0.0001$) in newly diagnosed *vs* under treatment SLE patients. HS: Humic subjects; PAT: Patients; UT: Under-treatment; ND: Newly diagnosed.

The prognostic utilities of miR-125a, TNF-α and IL-12

The levels of miR-125a, TNF-α, and IL-12 were categorized as low or high based on the optimal cut-off points determined from ROC curve analyses. The predictive ability of these biomarkers for the outcome (Flare) of SLE patients was assessed using the log-rank test. The results showed that miR-125a did not significantly predict the outcome of SLE patients ($P < 0.7151$) (Figure 4A). TNF-α, on the other hand, had a predictive potential for the outcome of SLE patients, but the association was not statistically significant ($P = 0.4828$) (Figure 4B). In contrast, IL-12 demonstrated a significant predictive ability for the outcome of SLE patients ($P = 0.0508$) (Figure 4C) according to the log-rank test.

DISCUSSION

Recent research has focused on exploring the diagnostic and prognostic potential of various biomarkers in SLE. Our study investigated the levels of miR-125a, IL-12, and TNF-α in the plasma of individuals with SLE. The main objective was to evaluate the usefulness of these biomarkers in predicting SLE flares after 24 wk. By analyzing their diagnostic utilities and prognostic power, this study aimed to improve the detection and management of SLE.

Our study found a significant decrease in miR125a expression in SLE patients compared to normal individuals. Furthermore, miR125a expression was observed to be lowest in newly diagnosed SLE patients as compared to those who were already under treatment. These results are consistent with the findings of Zhao *et al*[15], who showed reduced expression of miR-125a and increased expression of its predicted target gene KLF13 in SLE patients. The study by Zhao *et al*[15] had limited samples, while our study had a larger sample size. Furthermore, while Zhao *et al*[15] did not categorize their SLE patients based on disease duration, our study did so and revealed that miR-125a expression is more reduced in newly-diagnosed SLE patients. Consequently, our findings suggest that treatment may have an impact on miR-125a expression levels in SLE patients. A study conducted by Zhang *et al*[40] also aimed to investigate the roles of circRNAs in SLE and their findings were consistent with ours. They utilized microarray analysis, which was verified by qPCR, to

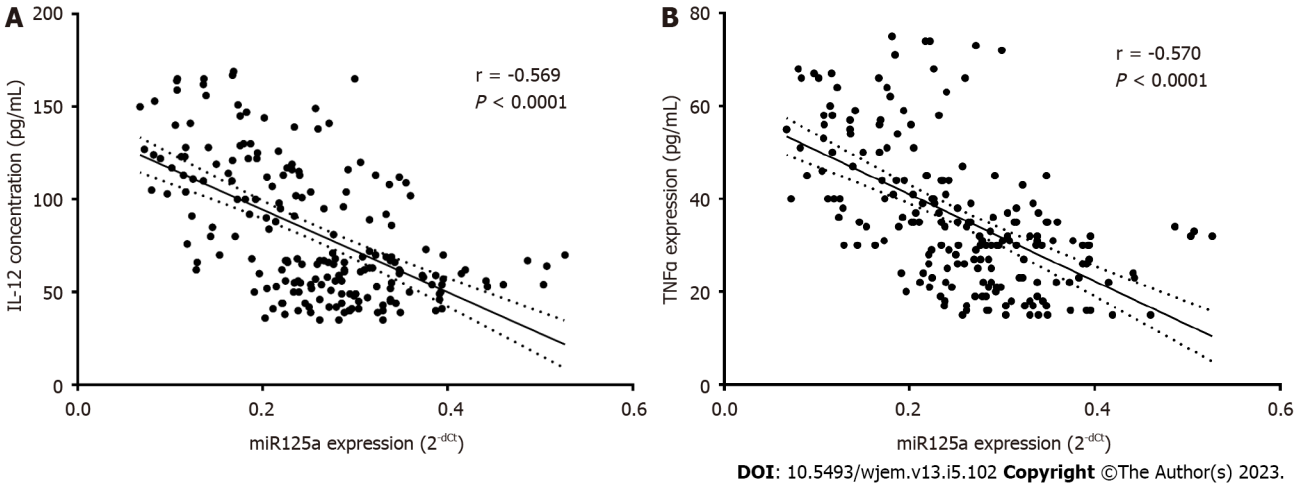

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Figure 3 The correlations of microRNA-125a with tumor necrosis factor-alpha and tumor necrosis factor-alpha. A: The relationship between plasma levels of microRNA-125a (miR-125a) and interleukin 12 was assessed, revealing a significant negative correlation (r = -0.569, P < 0.0001) as demonstrated by Pearson correlation analysis; B: Similarly, the plasma levels of miR-125a and tumor necrosis factor-alpha showed a significant negative correlation (r = -0.570, P < 0.0001) based on the Pearson correlation study.

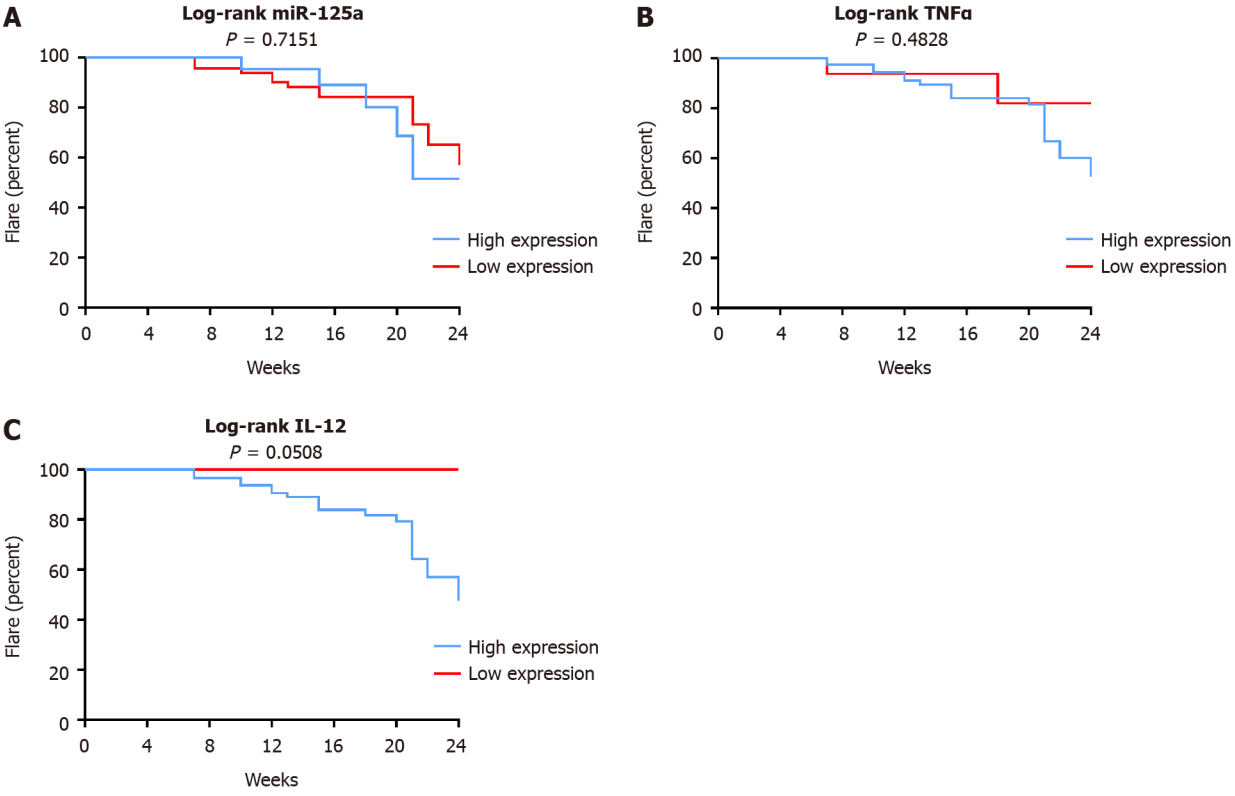

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Figure 4 The prognostic utilities of microRNA-125a, tumor necrosis factor-alpha, and interleukin 12 to predict flare in systemic lupus erythematosus patients. Based on the optimal cut-off points obtained from receiver operating characteristic curve analyses, the levels of microRNA-125a (miR-125a), tumor necrosis factor-alpha (TNF-α), and interleukin 12 (IL-12) were categorized as low or high. The predictive ability of these biomarkers for the outcome (Flare) in systemic lupus erythematosus (SLE) patients was assessed using the log-rank test. A: The results showed that miR-125a was not significantly predictive of the outcome (P < 0.7151); B: TNF-α showed a potential for predicting the outcome, but the association was not statistically significant (P = 0.4828); C: In contrast, IL-12 demonstrated a significant predictive capability for the outcome in SLE patients (P = 0.0508) based on the log-rank test results.

demonstrate that miR-125a was downregulated in SLE patients as compared to healthy controls, and this reduction was linked to SLE characteristics. However, their sample size was limited (n = 3), which may have affected the generalizability of their results. Our results were consistent with those reported by Nascimento *et al*[41], who observed downregulation of miR-125 in peripheral blood mononuclear cells (PBMCs) of childhood-onset systemic lupus erythematosus (cSLE) patients. However, their study was limited to cSLE patients and PBMC samples, while our investigation focused on adult

females and plasma samples. While our findings regarding reduced miR-125a expression in SLE patients are similar to those reported by Eissa *et al*[42], there is a difference in the focus of our studies. Eissa *et al*[42] evaluated the plasma expression of miR-125a specifically in juvenile SLE patients, whereas our study did not differentiate between adult and juvenile SLE patients. In summary, our study provides additional evidence to support the idea that levels of miR-125a are lower in patients with SLE and decrease further in more severe cases of the disease.

The expression of miR-125a was found to be significantly different between individuals with SLE and those without the disease. To determine whether miR-125a could be a useful diagnostic tool for detecting SLE and distinguishing between newly diagnosed patients and those already undergoing treatment, we analyzed ROC curves. The results showed that miR-125a has an AUC of 0.8370 and 0.8102 for distinguishing SLE patients from normal individuals and newly diagnosed patients from those already under treatment, respectively. This suggests that miR-125a is effective in identifying SLE patients and differentiating them from healthy individuals or those at different stages of treatment. Biomarkers used for diagnosis can be classified based on their AUC values, which determine how accurately they can differentiate between two groups, such as healthy and diseased individuals. An AUC value of 0.9-1.0 indicates an excellent biomarker, meaning that it is highly dependable in identifying the presence or absence of a target condition. A biomarker with an AUC value between 0.8-0.9 is considered good, indicating reasonably accurate diagnostic ability. An AUC value of 0.7-0.8 is classified as fair, meaning that it has moderate diagnostic accuracy but may not be as reliable. Biomarkers with an AUC value below 0.7 should be considered poor and should not be relied upon for diagnoses[43]. Therefore, miR-125a is classified as an effective diagnostic biomarker for SLE. While Zhang *et al*[40] have suggested that miR-125a could serve as a diagnostic biomarker for SLE based on its significant reduction in SLE patients, and several studies have introduced miR-125a as diagnostic biomarkers for malignancies such as Pancreatic Cancer[44], and Cervical Cancer[45], our study is, to the best of our knowledge, the first to propose miR-125a as an effective diagnostic biomarker for both discriminating between SLE patients and healthy subjects, as well as distinguishing under-treatment patients from newly diagnosed ones. In this study, patients were also divided into high and low expression groups based on the suggested miR-125a cut-off point. These individuals were then monitored for 24 wk to determine if they experienced a flare or not. Based on the results of the log-rank test, it was concluded that miR-125a is not a predictor of SLE patient outcomes after 24 wk. It should be noted that, to date, no other research has explored the utility of miR-125a as a prognostic marker for SLE flare outcome. Further research is required to confirm these findings.

Regarding the function of miR-125a in SLE pathogenesis, Zhang and his colleagues suggested that miR-125a may play a role in the development of SLE. They speculated that miR-125a could help maintain self-tolerance by limiting the activity of T cells that promote inflammation, but they found that its expression is lower in T cells from individuals with SLE. Based on these findings, they proposed that increasing levels of miR-125a might be a promising strategy for treating SLE[46]. In their study, Zhao and colleagues discovered that the expression of miR-125a was lower in individuals with SLE. They also observed an increase in KLF13, a gene that miR-125a is predicted to target. When miR-125a was overexpressed, it led to a significant decrease in the expression of RANTES and KLF13. The researchers found that miR-125a inhibited KLF13 expression in a dose-dependent manner using gain- and loss-of-function methods. Additionally, introducing miR-125a into T cells from SLE patients reduced the high levels of RANTES expression. Notably, the expression of miR-125a in T cells increased in a dose- and time-dependent manner[15]. However, further research is needed.

Our study analyzed the expression level of TNF-α in SLE patients and normal subjects. The results showed that the expression level of TNF-α was significantly elevated in SLE patients compared to normal subjects. Additionally, TNF-α expression was highest in newly diagnosed SLE patients compared to those with longer disease durations. ROC curve analysis was used to assess the diagnostic accuracy of TNF-α in distinguishing SLE patients from normal subjects and newly diagnosed SLE patients from those under treatment. The AUC for TNF-α expression was high in both cases, indicating excellent diagnostic utility. However, Log-rank test results revealed that TNF-α was not capable of predicting the outcome (Flare) of SLE patients, after 24 wk. The role of TNF-α in the pathogenesis of SLE is well established, and our findings are supported by several confirmatory studies, further strengthening their significance. According to the results of their research, Idborg and colleagues carried out a study aimed at determining potential biomarkers for identifying disease activity in patients with SLE by evaluating a large group of cytokines and basic laboratory tests. They discovered that TNF-α had the most significant ability to differentiate between SLE patients and healthy individuals, with higher levels detected in SLE patients. The researchers also observed a strong association between TNF-α and measures of disease activity, particularly in the subgroup of patients with nephritis[47]. In research conducted by Ma and colleagues, it was observed that SLE patients had higher levels of plasma TNF-α than healthy individuals, which is consistent with our own results. Additionally, the study found that TNF-α levels were elevated in SLE patients who were experiencing active symptoms compared to those who were not, as well as compared to healthy controls, which agrees with previous findings of increased TNF-α expression in patients who have recently been diagnosed with SLE[48]. In addition to their other findings, Rana and colleagues discovered that the TNF-α gene was highly expressed in most patients with SLE. Furthermore, they observed a strong association between these expression levels and both renal involvement and disease activity as measured by SLE Disease Activity Index (SLEDAI) scores[49]. According to Sabry and colleagues, SLE patients with active hematological disease exhibited elevated levels of TNF-α as compared to those with inactive disease or healthy individuals. Additionally, they found a strong positive correlation between the TNF-α levels and the SLEDAI score. The results of their study indicated that increased levels of TNF-α may contribute to the onset of anemia among Egyptian patients with Lupus Nephritis[50]. According to our research, a study conducted by Sabry *et al*[27] came up with similar results. They found that patients who had active SLE had significantly higher levels of TNF-α compared to those who had inactive SLE or were healthy. They concluded that measuring the levels of TNF-α in the blood could be a useful way to predict disease activity and distinguish between individuals with SLE and those without. The elevated levels of TNF-α and its possible role as a diagnostic marker was also confirmed in other studies by Aringer *et al*[51],

Umare *et al*[52], and Zhu *et al*[53].

Regarding the association between TNF-α and diverse clinical manifestations in SLE, notably, patients with CNS involvement exhibit markedly elevated TNF-α levels in contrast to those without CNS involvement. Furthermore, TNF-α levels are significantly heightened in lupus patients overall, particularly in those with NPLE. Additionally, a substantial TNF-α level increase is observed in patients presenting with multiple focal pattern hypoperfusion, the predominant SPECT pattern in individuals with NPLE[54]. Diffusion tensor imaging (DTI) relies on assessing water diffusion within cellular compartments. In this context, DTI holds the potential to serve as an imaging biomarker for neuropsychiatric systemic lupus erythematosus and a valuable tool for correlation with TNF-α levels[55].

We also analyzed the expression level of IL-12 in SLE patients and normal subjects. The results revealed that the expression level of IL-12 was significantly higher in SLE patients than in normal subjects. Additionally, among SLE patients, those who were newly diagnosed had the highest levels of IL-12 expression compared to those who had been under treatment for longer periods. The diagnostic ability of IL-12 to distinguish SLE patients from normal subjects and newly diagnosed SLE patients from those under treatment was evaluated through ROC curve analysis, suggesting that IL-12 may be a useful diagnostic marker for SLE, particularly in distinguishing between newly diagnosed and under-treated patients. Following the patients for 24 wk, Log-rank test results revealed that IL-12 was capable of predicting the outcome (Flare) of SLE patients. Similarly, the role of IL-12 in SLE pathogenesis is established in previous studies, and there are several studies highlighting the elevation of IL-12 in SLE patients. According to our discoveries, Capper and colleagues demonstrated that the levels of IL-12 were noticeably elevated in individuals with SLE when compared to those who did not have the condition. This difference was observed irrespective of whether the SLE patients were having an active episode of the disease or not[56]. Lauwerys and colleagues also noted similar results, where they observed an increase in IL-12 p40 levels in the blood of SLE patients. They further demonstrated that the administration of immunosuppressive therapy resulted in a significant decrease in serum IL-12 levels, which verified the lower levels of IL-12 found in individuals without SLE in our study[20], supported in the studies conducted by Qiu *et al*[57], and, Uzrail *et al*[58].

Despite the significant role of IL-12 in SLE pathogenesis, few studies assessed its diagnostic and prognostic accuracy for SLE. In a study by Ye *et al*[22] SLE patients had significantly higher plasma levels of IL-12, and the area under the curve (AUC) for IL-12 was 0.756, indicating its potential as biomarkers for SLE diagnosis. Through our study on a larger sample size, we have discovered that IL-12 can serve as an excellent biomarker for SLE. While Ye *et al*[22] identified it as a fair biomarker, our findings indicate its potential as a stronger indicator of the disease. Additionally, our study is the first to evaluate the occurrence of flares in SLE patients after 24 wk of follow-up. Thus, our results provide valuable insights into the long-term management and prognosis of SLE.

While our study yielded promising results regarding the potential diagnostic and prognostic utilities of miR-125a, TNF-α, and IL-12 in SLE patients, we acknowledge that there were limitations to our research. Despite having a larger sample size compared to previous studies, the generalizability of our findings could be further improved by increasing the sample size even more. Furthermore, as our study only assessed the plasma levels of these molecules, it may be worthwhile to explore their expression in different types of cells or tissues to gain a more comprehensive understanding of their role in SLE pathogenesis. Moreover, it is important to note that our study had a cross-sectional design which limits our ability to establish a cause-and-effect relationship between the levels of these molecules and disease progression. Therefore, we suggest conducting a longitudinal cohort study to further investigate the potential causal relationships between miR-125a, TNF-α, and IL-12 levels and SLE progression.

CONCLUSION

Our study has shed light on the potential of miR-125a, TNF-α, and IL-12 as biomarkers for SLE diagnosis and management. We found that the downregulation of miR-125a is an effective diagnostic tool for distinguishing between SLE patients and healthy individuals, as well as newly diagnosed patients from those under treatment. Furthermore, TNF-α and IL-12 levels were elevated in SLE patients, with TNF-α serving as a useful diagnostic marker and IL-12 being both a diagnostic and prognostic marker for SLE flare outcome. These findings could contribute to improved patient outcomes and better management of SLE. However, further research is needed to fully understand the mechanisms by which these biomarkers are involved in SLE pathogenesis and their potential as therapeutic targets.

ARTICLE HIGHLIGHTS

Research background

Systemic lupus erythematosus (SLE) is a long-lasting autoimmune disorder that impacts multiple organs and significantly increases the risk of morbidity and mortality. MicroRNA-125a (miR-125a) levels are decreased in T cells, B cells, and dendritic cells of SLE patients. MiR-125a plays a regulatory role in controlling the levels of tumor necrosis factor-alpha (TNF-α) and interleukin 12 (IL-12), which are crucial pro-inflammatory cytokines in SLE pathogenesis.

Research motivation

Recent research has focused on exploring the diagnostic and prognostic potential of various biomarkers in SLE. Since the levels of miR-125a, TNF-α, and IL-12 are altered in SLE, these molecules could be introduced as novel biomarkers.

Research objectives

The aim of this study was to analyze the levels of miR-125a, TNF-α, and IL-12 in the plasma of both SLE patients and healthy individuals, explore their potential correlations, and investigate their usefulness as diagnostic and prognostic biomarkers for SLE.

Research methods

The study included 100 healthy individuals, 50 newly diagnosed (ND), and 50 SLE patients undergoing treatment. The patients were monitored for a duration of 24 wk to observe and record instances of relapses. MiR-125a expression was measured using real-time reverse transcription polymerase chain reaction, while ELISA kits were used to assess IL-12 and TNF-α production.

Research results

The results showed significantly reduced miR-125a expression in SLE patients compared to healthy individuals, with the lowest levels in ND patients. TNF-α and IL-12 expression levels were significantly elevated in SLE patients, especially in the early stages of the disease. Receiver operating characteristic curve analyses, and Cox-Mantel Log-rank tests indicated miR-125a, PDCD4, and IL-10 as proper diagnostic biomarkers for SLE. A negative correlation was found between plasma miR-125a expression and IL-12/TNF-α levels in SLE patients.

Research conclusions

Decreased miR-125a levels may be involved in the development of SLE, while elevated levels of IL-12 and TNF-α contribute to immune dysregulation. These findings offer new diagnostic and prognostic markers for SLE. Moreover, the negative correlation observed suggests an interaction between miR-125a, TNF-α, and IL-12.

Research perspectives

Further research is necessary to uncover the underlying mechanisms that govern the relationships between miR-125a, TNF-α, and IL-12 in SLE pathogenesis.

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FOOTNOTES

Author contributions: Alsbihawi TQ, Seyedhosseini F, Davoodi H, Mohammadi S, and Yazdani Y contributed to conceptualization (Mohammadi S and Yazdani Y), contributed to methodology; Alsbihawi TQ, Ebrahimabad MZ, and Mohammadi S contributed to software; Alsbihawi TQ, Mohammadi S, and Yazdani Y contributed to validation; Alsbihawi TQ, and Mohammadi S contributed to formal analysis; Alsbihawi TQ, Ebrahimabad MZ, and Mohammadi S contributed to investigation; Alsbihawi TQ, Mohammadi S, and Yazdani Y contributed to resources; Alsbihawi TQ, Mohammadi S, and Yazdani Y contributed to data curation; Alsbihawi TQ, Ebrahimabad MZ, Seyedhosseini FS, Davoodi H, Abdolahi N, Mohammadi S, and Yazdani Y contributed to writing – original draft preparation; Alsbihawi TQ, Ebrahimabad MZ, Seyedhosseini FS, Davoodi H, Abdolahi N, Mohammadi S, and Yazdani Y contributed to writing – review and editing; Mohammadi S and Yazdani Y contributed to supervision; Mohammadi S and Yazdani Y contributed to project administration; Mohammadi S and Yazdani Y contributed to funding acquisition; All authors have read and approved the final version of the manuscript.

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Informed consent statement: All participants provided informed consent prior to their involvement. Utmost confidentiality and privacy were ensured throughout the study, and precautionary measures were implemented to prevent any form of undue influence or coercion.

Conflict-of-interest statement: All the authors declare that there are no conflicts of interest to disclose.

Data sharing statement: The data supporting the findings of this study will be made accessible upon reasonable request by the corresponding author, Dr. Saeed Mohammadi (s.mohammadi@goums.ac.ir).

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ORIGINAL ARTICLE

Retrospective Cohort Study

Red cell distribution width: A predictor of the severity of hypertriglyceridemia-induced acute pancreatitis

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Abstract

BACKGROUND

Compared with patients with other causes of acute pancreatitis, those with hypertriglyceridemia-induced acute pancreatitis (HTG-AP) are more likely to develop persistent organ failure (POF). Therefore, recognizing the individuals at risk of developing POF early in the HTG-AP process is a vital for improving outcomes. Bedside index for severity in acute pancreatitis (BISAP), a simple parameter that is obtained 24 h after admission, is an ideal index to predict HTG-AP severity; however, the suboptimal sensitivity limits its clinical application. Hence, current clinical scoring systems and biochemical parameters are not sufficient for predicting HTG-AP severity.

AIM

To elucidate the early predictive value of red cell distribution width (RDW) for POF in HTG-AP.

METHODS

In total, 102 patients with HTG-AP were retrospectively enrolled. Demographic and clinical data, including RDW, were collected from all patients on admission.

RESULTS

Based on the Revised Atlanta Classification, 37 (33%) of 102 patients with HTG-AP were diagnosed with POF. On admission, RDW was significantly higher in patients with HTG-AP and POF than in those without POF (14.4% *vs* 12.5%, $P < 0.001$). The receiver operating characteristic curve demonstrated a good discrim-

inative power of RDW for POF with a cutoff of 13.1%, where the area under the curve (AUC), sensitivity, and specificity were 0.85, 82.4%, and 77.9%, respectively. When the RDW was ≥ 13.1% and one point was added to the original BISAP to obtain a new BISAP score, we achieved a higher AUC, sensitivity, and specificity of 0.89, 91.2%, and 67.6%, respectively.

CONCLUSION
RDW is a promising predictor of POF in patients with HTG-AP, and the addition of RDW can promote the sensitivity of BISAP.

Key Words: Red cell distribution width; Bedside index for severity in acute pancreatitis; Persistent organ failure; Hypertriglyceridemia-induced acute pancreatitis

Core Tip: Red cell distribution width (RDW) reflects systemic inflammation, which is significantly associated with the severity of acute pancreatitis. However, the relationship between RDW and hypertriglyceridemia-induced acute pancreatitis (HTG-AP) remains unclear. Herein, RDW exhibited a potent discriminatory power for predicting persistent organ failure in patients with HTG-AP. Furthermore, the addition of RDW is able to promote the sensitivity of bedside index for severity in acute pancreatitis.

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INTRODUCTION

Hypertriglyceridemia is the most common etiology of acute pancreatitis (AP)[1-3]. Compared with patients with other causes of AP, those with hypertriglyceridemia-induced AP (HTG-AP) are more likely to suffer from persistent organ failure (POF)[4,5]. POF is a major determinant of mortality in early HTG-AP and correlates with the magnitude of inflammatory responses[1], thereby indicating the prognosis of patients. Given this, early recognition of HTG-AP patients at risk of POF is necessary for improving patients' outcomes.

Red cell distribution width (RDW) reflects the size of circulating erythrocytes[6]. Studies[7,8] have reported that high RDW is significantly associated with AP severity. The mechanism underlying this association may be attributed to the following: (1) Inflammation blocks anti-apoptosis or promotes erythrocyte death[9]; (2) inflammatory cytokines can desensitize bone marrow erythroid progenitors to erythropoiesis, thereby inhibiting erythrocyte maturation[10]; and (3) during AP, erythrocytes cannot absorb vital materials, including vitamin B12, iron, and folic acid[11].

However, the relationship between RDW and HTG-AP was not well elucidated in previous studies; therefore, our study aimed to evaluate the predictive value of RDW for POF in patients with HTG-AP.

MATERIALS AND METHODS

Patient selection

In this retrospective study, consecutive patients with HTG-AP who were hospitalized at the Affiliated Baiyun Hospital of Guizhou Medical University from January 2017 to February 2021 were enrolled. AP and POF (lasting > 48 h) were diagnosed based on the Revised Atlanta Classification[12]. This study was reviewed and approved by the Science and Research Office of the Affiliated Baiyun Hospital of Guizhou Medical University. Patients' consent for inclusion was waived owing to the retrospective nature of the study.

HTG-AP was diagnosed if serum triglyceride levels were > 11.3 mmol/L or between 5.56 and 11.30 mmol/L accompanied by chylous effusion, with the exclusion of other etiologies[13,14].

The exclusion criteria were as follows: (1) Patients who were admitted > 24 h after AP onset, (2) those who were < 18 years of age; and (3) those with other etiology-induced AP, recurrent AP, gestational AP, AP with anemia (woman < 110 g/L, man < 120 g/L), chronic pancreatitis, and cancer history. The primary endpoint was in-hospital POF.

Data collection

All data were collected from our hospital's electronic medical database. For each patient, age, sex, and medical history were collected as baseline demographic data. Furthermore, information on vital signs was collected from all patients on admission, and the results of laboratory tests, radiological imaging, and clinical outcomes were collected within 24 h of

admission. The bedside index for severity in acute pancreatitis (BISAP)[15] and the Acute Physiology and Chronic Health Evaluation II (APACHE II) score[16] were calculated within 24 h of admission.

The measurement of RDW

On admission, 2-3 mL of blood was collected, placed in EDTA-K2 anticoagulation tubes, and stored at 4-8℃. The whole blood samples were directly loaded into a hematology analyzer (XN-2000, Japan Sysmex) for detection. RDW (%) = SD of red blood cell volume/mean corpuscular volume x 100. The reference range of RDW was 11.0%-16.0%.

Statistical analysis

Data were presented as median (interquartile range) or mean ± SD. Categorical data were presented as percentages (%). Univariate analysis was performed using the Student's *t*-test, Mann-Whitney U test, and chi-squared test, as appropriate. Admission variables that were significant in univariate analysis were subjected to multivariate regression analysis.

A receiver-operating characteristic (ROC) curve was used to estimate predictive accuracy. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC) were calculated. Pearson's analysis was performed to determine the correlations between two variables. GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, United States) and SPSS 25.0 (IBM Corp., Armonk, NY, United States) were used to perform statistical analyses. A P value < 0.05 was considered statistically significant.

RESULTS

Patient demographics and clinical characteristics

Table 1 summarizes the demographic, laboratory, and clinical characteristics of patients with and without POF. In total, 102 patients were included in this retrospective study. The age range was 20-58 years and 34 (33%) of 102 patients developed POF; no patients died.

Clinical outcomes

Univariate analysis identified several risk factors of POF on admission (P < 0.05). Multivariate analysis revealed that RDW (P = 0.002) and BISAP (P = 0.001) were associated with POF (Table 1). RDW was positively correlated with BISAP (r = 0.457, P < 0.001) and APACHE II (r = 0.440, P < 0.001) (Figure 1).

ROC analysis indicated that the optimal cutoff value for RDW was 13.1%. The sensitivity, specificity, PPV, and NPV were 82.4%, 77.9%, 65.1%, and 89.8%, respectively. Using the cutoff for RDW, the 102 patients with HTG-AP were divided into two groups: RDW < 1 3.1% and RDW ≥ 13.1% groups. More POF patients were presents in the RDW ≥ 13.1% group (P < 0.001) (Figure 2 and Table 2).

When RDW was ≥ 13.1%, one point was added to the BISAP score to obtain a new BISAP score (BISAP plus RDW: BR score). When the cutoff value of BR score was set as 2 points, the AUC, sensitivity, specificity, PPV, and NPV for predicting POF was 0.89, 91.2%, 67.6%, 58.5%, and 93.9%, respectively (Figure 3 and Table 2). Compared with BISAP alone (AUC = 0.82, sensitivity = 64.7%, specificity = 82.4%, PPV = 64.7%, and NPV = 82.4%) and APACHE II (AUC = 0.74, sensitivity = 73.5%, specificity = 60.3%, PPV = 48.1%, and NPV = 82.0%), BR score exhibited a higher predictive accuracy (Figure 3 and Table 2).

DISCUSSION

Herein, our study suggested that RDW on admission was a promising indicator for POF and that the BR score could efficiently predict POF in patients with HTG-AP. This is the first study to uncover the association between RDW and POF in patients with HTG-AP.

Compared with other AP etiologies, HTG-AP is more likely to causes POF, which results in an unfavorable prognosis for patients[12,17,18]. Therefore, the predictive value of RDW is of significance because the application of this biomarker can facilitate the discrimination of patients who have a high risk of developing POF and the design of treatment regimens. Moreover, RDW examination is reproducible, inexpensive, and convenient, and thus can be widely implemented in almost every hospital.

The production of inflammatory cytokines and corresponding inflammatory cascade have long been considered a critical pathogenic mechanism of POF in AP patients[1,8]. RDW reflects systemic inflammation, and several studies[6-8] have proposed the RDW can predict in-hospital mortality and severity in AP. However, these studies only included gallstones- and alcohol-induced AP but not HTG-AP. Consequently, our study focused on the association between RDW and POF in patients with HTG-AP. The results indicated that RDW was positively correlated with BISAP and APACHE II scores, which were commonly used indicators of POF. Meanwhile, RDW exhibited a promising value in predicting POF.

Although the mechanism underlying increased RDW in HTG-AP patients developing POF, it may be attributed to factors as follows: inflammatory cascade triggers triglyceride-mediated lipotoxicity; in turn, increased triglyceride levels augment the intensity of the inflammatory cascade, which contributes to the elevation of RDW[17,18]. Notably, we excluded patients with anemia, which would affect RDW; this might promote the predictive value of RDW[6,9,19].

Table 1 Demographic and clinical characteristics of the patients, *N* (%)

Variable	Non-POF (*n* = 68)	POF (*n* = 34)	Univariate *P*	Multivariate *P*
Age (yr)	40 ± 8	40 ± 9	0.967	
Sex (M/F)	56/12	29/5	0.785	
Hypertension	16 (24)	7 (21)	0.876	
Diabetes	33 (49)	22 (65)	0.144	
Fatty liver disease	39 (57)	16 (47)	0.679	
Alcohol	28 (41)	15 (44)	0.833	
Smoking	45 (66)	21 (62)	0.667	
WBC (x10^9/L)	12.4 (10.9, 15.9)	14.9 (13.2, 17.1)	0.009	0.172
MPV (fL)	10.42 ± 1.76	11.54 ± 2.06	0.012	0.085
RDW (%)	12.5 (12.0, 13.0)	14.4 (13.4, 14.9)	< 0.001	0.002
Amylase (U/L)	124 (66, 258)	239 (83, 917)	0.036	0.383
Calcium (mmol/L)	2.35 (2.25, 2.42)	2.29 (2.10, 2.95)	0.064	
Triglyceride (mmol/L)	16.42 (11.58, 20.31)	19.57 (15.23, 24.70)	0.010	0.101
ALT (U/L)	36.0 (25.0, 47.4)	35.2 (20.8, 46.6)	0.412	
AST (U/L)	25.9 (19.1, 31.7)	25.4 (17.9, 33.6)	0.879	
Albumin (g/L)	46.1 (44.1, 48.4)	44.8 (42.1, 48.3)	0.078	
BUN (mmol/L)	4.30 (3.60, 5.18)	3.85 (2.90, 5.00)	0.057	
Creatinine (μmol/l)	60.50 (47.50, 76.55)	54.90 (41.70, 71.05)	0.158	
C-reaction protein (mg/L)	24.89 (11.14, 57.54)	63.10 (23.57, 99.91)	0.008	0.185
BISAP	1 (0, 1)	2 (1, 2)	< 0.001	0.001
APACHE II	4 (2, 5)	5 (4, 8)	< 0.001	0.119

Data are presented as *n* (%) prevalence, and mean ± SD or median (interquartile range). WBC: White blood cells; MPV: Mean platelet volume; RDW: Red cell distribution width; ALT: Alanine aminotransferase; AST: Aspartate transaminase; BUN: Blood urea nitrogen; BISAP: Bedside index for severity in acute pancreatitis; APACHE II: Acute Physiology and Chronic Health Evaluation II; POF: Persistent organ failure.

Table 2 Overall accuracy of red cell distribution width (RDW), bedside index for severity in acute pancreatitis (BISAP), acute physiology and chronic health evaluation II, and BISAP plus RDW score for predicting persistent organ failure in patients with hypertriglyceridemia-induced acute pancreatitis on admission

Variable	AUC	Cut-off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
RDW	0.85	13.1	82.4	77.9	65.1	89.8
BISAP	0.82	2	64.7	82.4	64.7	82.4
APACHE II	0.74	5	73.5	60.3	48.1	82.0
BR	0.89	2	91.2	67.6	58.5	93.9

HTG-AP: Hypertriglyceridemia-induced acute pancreatitis; AUC: Area under the curve; PPV: Positive predictive value; PPV: Negative predictive value; RDW: Red cell distribution width; BISAP: Bedside index for severity in acute pancreatitis; APACHE II: Acute Physiology and Chronic Health Evaluation II; BR: BISAP plus RDW.

Several multiparameter predictors, including Ranson criteria, BISAP, and APACHE II, have been adopted to evaluate POF in HTG-AP patients[20-22]. However, Ranson criteria can only be used after 48 h of hospitalization and the calculation of APACHE II is complicated[16]. Although BISAP is widely used because of its simplicity of calculation, its low sensitivity limits its clinical application[16]. Li *et al*[21] compared the differences in scoring systems for predicting the prognosis of HTG-AP patients. They suggested that BISAP was the best indicator for predicting the HTG-AP severity. However, BISAP had a suboptimal sensitivity (66.7%) for predicting POF. Similarly, Yang *et al*[20] reported that BISAP exhibited low sensitivity (54%) for predicting HTG-AP severity. Consistent with previous findings, our results revealed

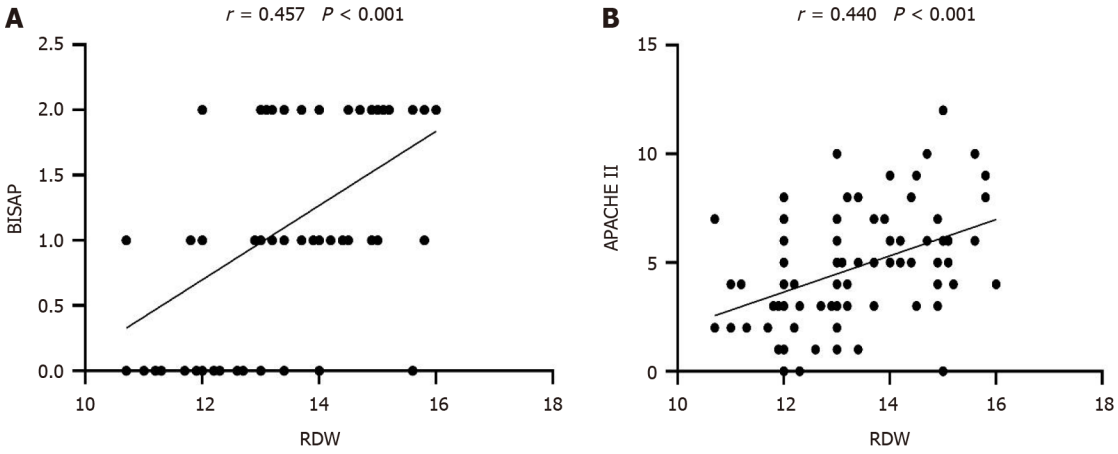

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Figure 1 Correlation between red cell distribution width and bedside index for severity in acute pancreatitis and acute physiology and chronic health evaluation II. A: Positive correlation between red cell distribution width (RDW) and bedside index for severity in acute pancreatitis; B: Positive correlation between RDW and acute physiology and chronic health evaluation II. RDW: Red cell distribution width; BISAP: Bedside index for severity in acute pancreatitis; APACHE-II: Acute Physiology and Chronic Health Evaluation II.

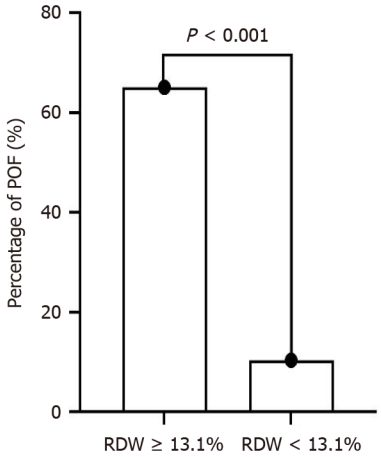

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Figure 2 Percentage of patients with hypertriglyceridemia-induced acute pancreatitis between the red cell distribution width (RDW) < 13.1% group and the RDW ≥ 13.1% group. POF: Persistent organ failure; RDW: Red cell distribution width.

that BISAP also exhibited a low sensitivity (64.7%) for predicting POF.

Several studies have demonstrated the improved predictive value by combining BISAP and other biomarkers. Zheng *et al*[22] found that BISAP plus C-reactive protein levels for predicting POF in AP had increased AUC, sensitivity, and specificity compared with using BISAP alone (0.873, 81.6%, and 85.2% *vs* 0.856, 80.8%, and 84.1%, respectively). Zhou *et al* [19] (BISAP plus RDW) also suggested that while the addition of RDW promoted the predictive value of BISAP, it decreased the sensitivity. In the present study, the results indicated that the BR score exhibited higher AUC (0.89 *vs* 0.82) and sensitivity (91.2% *vs* 64.7%) than BISAP alone for predicting POF in HTG-AP patients, whereas the specificity (67.6% *vs* 82.4%) dropped.

Fluid therapy is a long-established cornerstone to prevent organ hypoperfusion within 24 h of AP onset[16,23]. Recent studies[17,23] have indicated that aggressive fluid therapy in AP may result in poor outcomes, particularly in older patients. Since HTG-AP patients tend to have a younger age, aggressive fluid therapy is a relatively safe treatment. However, our results suggested that more attention should be paid to patients with RDW ≥ 13.1%.

Our study has some limitations. First, no patient died in our cohort; therefore, the ability of RDW to predict mortality could not be investigated. However, considering the intimate association between POF development and unfavorable prognosis at the early stage of AP[12], we inferred that the mortality prediction performance of RDW might be superior in HTG-AP than in POF. Second, this study was limited by its retrospective design and small sample size, making it impossible to draw definitive conclusions regarding the diagnostic value of evaluated biomarkers for predicting POF in HTG-AP. Third, the definition of HTG-AP differed between countries[17], which limited the extension of our findings to other countries. Moreover, we did not perform a subgroup analysis to exclude the influence of metabolic disorders on RDW. Furthermore, BR score had a high false positive rate, indicating that the BR score was not an optimal indicator for

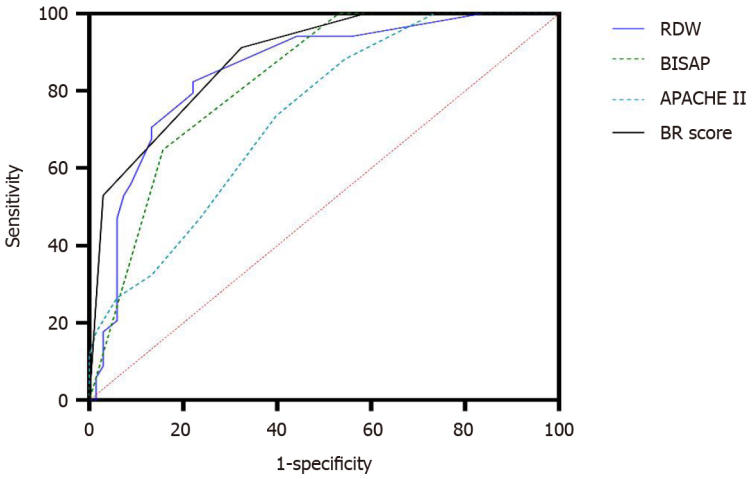

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Figure 3 Receiver operating characteristic curves for red cell distribution width (RDW), bedside index for severity in acute pancreatitis (BISAP), Acute Physiology and Chronic Health Evaluation II, and BISAP plus RDW score for predicting persistent organ failure in hypertriglyceridemia-induced acute pancreatitis. HTG-AP: Hypertriglyceridemia-induced acute pancreatitis; RDW: Red cell distribution width; BISAP: Bedside index for severity in acute pancreatitis; APACHE II: Acute Physiology and Chronic Health Evaluation II; BR: BISAP plus RDW; POF: Persistent organ failure.

predicting POF. Nevertheless, the BR score had a relatively high sensitivity, so it could be a screening indicator for POF in HTG-AP patients. Additional new biomarkers and scoring systems should be developed to better predict POF.

CONCLUSION

RDW on admission is an independent predictor of POF in patients with HTG-AP. The addition of RDW improves the low sensitivity of BISAP. Large-scale, multicenter prospective studies are required to verify the results.

ARTICLE HIGHLIGHTS

Research background

In clinical settings, compared with patients with other causes of acute pancreatitis (AP), those with hypertriglyceridemia-induced acute pancreatitis (HTG-AP) commonly suffer from severe acute pancreatitis; therefore, it is critical to identify severe HTG-AP early. However, current clinical scoring systems and biochemical parameters are not efficient for predicting HTG-AP severity.

Research motivation

Red cell distribution width (RDW) may be closely associated with the mortality of patients with AP. Meanwhile, clinical application of the bedside index for severity in acute pancreatitis (BISAP) is limited by its low sensitivity. Therefore, new parameters or scoring systems are warranted for determining HTG-AP severity early.

Research objectives

To determine whether RDW can be used as a potential biomarker for predicting POF in HTG-AP.

Research methods

We explored the relationship between RDW and POF in patients with HTG-AP and determined the cutoff value of RDW using ROC analysis. BISAP plus RDW improved the suboptimal sensitivity of BISAP when RDW was ≥ 13.1% and one point was added to the BISAP.

Research results

On admission, RDW was significantly higher in patients with HTG-AP and POF. Compared with BISAP and Ranson criteria, BISAP plus RDW had a higher accuracy for predicting POF in HTG-AP patients.

Research conclusions

BISAP plus RDW exhibited a promising predictive value for POF in HTG-AP patients, despite its low specificity.

Research perspectives

The combination of BISAP and other indicators may better predict HTG-AP severity. However, additional large-scale, multicenter prospective studies are required to verify the results.

FOOTNOTES

Author contributions: Lv YC and Lei JJ designed the study; Lv YC, Yao YH, Zhang J, and Wang YJ, participated in the acquisition, analysis, and interpretation of the data; Lv YC wrote the manuscript; Lei JJ revised the article.

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ORIGINAL ARTICLE

Observational Study

Ground level utility of Access, Watch, Reserve classification: Insights from a tertiary care center in North India

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Abstract

BACKGROUND

The overuse and misuse of antimicrobials contribute significantly to antimicrobial resistance (AMR), which is a global public health concern. India has particularly high rates of AMR, posing a threat to effective treatment. The World Health Organization (WHO) Access, Watch, Reserve (AWaRe) classification system was introduced to address this issue and guide appropriate antibiotic prescribing. However, there is a lack of studies examining the prescribing patterns of antimicrobials using the AWaRe classification, especially in North India. Therefore, this study aimed to assess the prescribing patterns of antimicrobials using the WHO AWaRe classification in a tertiary care centre in North India.

AIM

To study the prescribing patterns of antimicrobials using WHO AWaRe classification through a cross-sectional study in All India Institute of Medical Sciences Rishikesh.

METHODS

A descriptive, cross-sectional study was conducted from July 2022 to August 2022 at a tertiary care hospital. Prescriptions containing at least one antimicrobial were included in the study. Data on prescriptions, including patient demographics, departments, types of antimicrobials prescribed, and duration of treatment, were collected. A questionnaire-based survey was also conducted to assess the knowledge and practices of prescribing doctors regarding the utility of AWaRe classification.

RESULTS

The study involved a total of 123 patients, each of whom received at least one antimicrobial prescription. Most prescriptions were for inpatients, evenly distributed between Medicine (Internal medicine, Pediatrics, Dermatology) and Surgical departments (General surgery and specialties, Otorhinolaryngology,

Ophthalmology, Obstetrics and Gynecology). Metronidazole and ceftriaxone were the most prescribed antibiotics. According to the AWaRe classification, 57.61% of antibiotics fell under the Access category, 38.27% in Watch, and 4.11% in Reserve. Most Access antibiotics were prescribed within the Medicine department, and the same department also exhibited a higher frequency of Watch antibiotics prescriptions. The questionnaire survey showed that only a third of participants were aware of the AWaRe classification, and there was a lack of knowledge regarding AMR and the potential impact of AWaRe usage.

CONCLUSION

This study highlights the need for better antimicrobial prescribing practices and increased awareness of the WHO AWaRe classification and AMR among healthcare professionals. The findings indicate a high proportion of prescriptions falling under the Access category, suggesting appropriate antibiotic selection. However, there is a significant difference between the WHO Defined Daily Dose and the prescribed daily dose in the analysed prescriptions suggesting overuse and underuse of antibiotics. There is room for improvement and educational interventions and antimicrobial stewardship programs should be implemented to enhance knowledge and adherence to guidelines, ultimately contributing to the containment of AMR.

Key Words: Antimicrobial resistance; AWaRe classification; Access; Watch; Reserve; Daily defined dose; Questionnaire based survey

Core Tip: With the rise in antimicrobial resistance (AMR) particularly in developing countries it is the need of the hour to adopt better prescribing practices. It is important to raise knowledge and awareness about AMR and improve adherence to guidelines. The research highlights areas of improvement in prescribing practices.

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INTRODUCTION

The discovery of antimicrobials is regarded as one of the most crucial advancements of the 20th century. It is imperative to prescribe antimicrobials judiciously, emphasizing their use in circumstances where they can offer significant advantages to patients with maximum efficiency, all the while mitigating the potential for exacerbating antimicrobial resistance (AMR)[1]. However, there is mounting evidence indicating that the overuse and misuse of antimicrobials have become leading factors in the complex causative web of AMR[2]. It poses a global public health problem, capable of spreading and causing significant human and economic burdens[3]. The situation in India is particularly concerning, as the country experiences some of the highest rates of AMR among bacteria commonly causing infections in both community and healthcare settings[4].

It is imperative to address the emergence of Multi-drug resistant organisms (MDR), which threaten the progress made in medical advancements over the past century. Conversely, in many parts of the world, there is not only overuse and misuse of antimicrobials but also inadequate access. Pneumonia remains a leading cause of childhood deaths globally, with over 2 million estimated deaths per year, primarily due to limited access to antimicrobials[5]. Thus, promoting access to these life-saving medicines for those in need should also be a priority[1].

Antimicrobial stewardship aims to bridge the gap between excessive and inadequate antimicrobial use[6]. However, assessing the impact of antimicrobial stewardship programs has proven challenging, as the commonly used metrics, such as defined daily doses (DDD) per 1000 inhabitants per day and days of therapy, provide limited information about the quality of antibiotic use[7]. The existing defined daily dose method used in adult antibiotic surveillance is unsuitable for neonates and children due to their widely variable bodyweights[8].

To address these issues, the World Health Organization (WHO) updated its Model List of Essential Medicines in 2017, introducing the Access, Watch, Reserve (AWaRe) classification system[9]. The AWaRe system categorizes antimicrobials based on their appropriateness, safety, and potential impact on AMR. The Access group includes antibiotics of choice for the 25 most common infections, with favourable safety profiles and a low likelihood of exacerbating AMR. These antimicrobials should be consistently available, affordable, and quality-assured. The Watch group comprises critically important antimicrobials that require strict monitoring and limited use due to their higher potential for negatively impacting AMR. The Reserve group includes last-resort antimicrobials effective against MDR bacteria, and their use should be minimized as they represent a valuable, non-renewable resource. The fourth category is of the discouraged antimicrobials which mostly includes antimicrobial combinations. Some fixed dose combinations of antibiotics, do not have any reasonable

indications for the treatment of infectious diseases in humans and may negatively impact AMR and patient safety[1]. The AWaRe system is also represented as a traffic-light approach: Access = green, Watch = orange and Reserve = red. The overall goal was to reduce the use of Watch Group and Reserve Group, and to increase the use of Access antibiotics to > 60% by 2023[5].

Unlike previous measures of antibiotic consumption, the AWaRe classification allows for the quantification of antibiotic use in each category, providing insights into the overall quality of antibiotic use within a country[8]. For instance, A 10-Year Study on urinary tract infections, their Epidemiology and Antibiotic Resistance Based on the WHO AWaRe classification was done[10]. Therefore, the WHO AWaRe categories can serve as a valuable tool for monitoring antibiotic consumption and optimizing antibiotic use, complementing antibiotic stewardship efforts at the national level.

Given the ongoing struggle to identify appropriate measures for hospitals and their antimicrobial stewardship programs, this study aims to audit antimicrobial prescriptions and compare the utility of the AWaRe classification with other process measurements of antimicrobial utilization. Additionally, the study seeks to assess the knowledge and attitudes of prescribing physicians regarding AMR and the AWaRe classification. This study is particularly important in the context of North India, where the burden of resistance is increasing, and limited research has been conducted in this region. Furthermore, the study aims to examine the prescribing practices of antibiotics in a tertiary care center and compare them with the WHO Defined Daily Dosage to determine whether common antibiotics are being under or over-dosed.

MATERIALS AND METHODS

Study design

Cross sectional study conducted on patient's prescriptions of various departments in a tertiary care hospital from July 2022 to August 2022.

Setting

This study was carried out in a tertiary care centre in North India after approval by the Institutional Ethics Committee [All India Institute of Medical Sciences, Rishikesh, India (Reference number -AIIMS/IEC/22/252)].

Study population

Consenting treating physicians (faculty, junior and senior residents) and their prescriptions having at least one antimicrobial were studied. Universal sampling was used to select prescriptions for a duration of 2 mo.

Inclusion criteria

Prescriptions containing at least one antimicrobial in both outpatient and inpatient settings and the physician prescribing it.

Exclusion criteria

Treating physician not giving consent.

Observations

After obtaining an informed consent from the prescribing doctor, a questionnaire about their knowledge and practices on AWaRe classification was asked through online or offline modes. The questionnaire was self-structured after searching medical literature for comparable studies and adapting questions designed in other physicians' surveys previously carried out[1,11]. The questionnaire consisted of two sets of questions: one designed to assess the knowledge of physicians and the other intended to gauge their attitudes towards AMR and the AWaRe classification. Pre-validation of questionnaire for its contents and relevance was done by experts (one clinician, one pharmacologist, one biostatistician). Data on prescriptions, including patient demographics, departments, types of antimicrobials prescribed, and duration of treatment, were collected.

Comparator

The relevance of antimicrobial prescriptions was checked by measuring appropriateness (right drug, right dose and right duration) according to WHO guidelines, Infectious Disease Society of America guidelines and disease specific guidelines if needed. Simultaneously sub-group comparison on AWaRE classification was also performed.

Outcome

Proportion of prescribed antimicrobials based on AWaRe classification was calculated. Then the knowledge and practices of prescribing doctor about the utility of AWaRe was determined. The appropriateness of AWaRe classification with days of therapy and defined daily doses of antimicrobial utilization was also estimated.

Statistical analysis

Data was collected on predefined proforma, google form, and excel sheet (Microsoft excel spreadsheet software, office 2016) and analysed by estimating the proportion of various variables including antimicrobial utilizations as per AWaRe

classification. Univariate analysis was performed and the data was arranged as percentages of total. Mean daily dosing for a particular antimicrobial was determined and compared with WHO DDD using the Students *T* test. *P* value less than 0.05 was considered statistical significant.

RESULTS

The research was carried out in accordance with the methodology presented in Figure 1. A total of *n* = 123 patients were enrolled in this study, with each of them receiving antibiotic prescriptions. The majority of these prescriptions were issued to inpatients (75.4%), and both the Medicine and Surgical departments were equally represented, accounting for 49.6% and 50.4%, respectively. Among the healthcare providers responsible for prescribing antibiotics, 72% were Junior Residents, 18.7% were Senior Residents, and 9.3% were Consultants. These findings have been summarized in Table 1.

The prescriptions included 27 different antibiotics, with metronidazole being the most prescribed (19%) followed by ceftriaxone (17%). The mean number of antibiotics used per patient was 1.84 ± 0.83. The mean duration of antibiotics prescribed was 6.63 ± 3.83 days. The maximum number of antibiotics prescribed per patient was five. According to the AWaRe classification, 57.61% of antibiotics fell under the Access, 38.27% in Watch, and 4.11% in Reserve categories, suggesting appropriate antibiotic selection according to these criteria. The distribution of antibiotics prescribed according to the WHO AWaRe categories is presented in Figure 2. The difference in prescribing frequencies amongst departments can be noted. Most of the antibiotics prescribed in the Access category were from the Medicine department (75.4%), followed by Surgery (24.6%). For Watch antibiotics, Medicine had a higher proportion (63.4%) compared to Surgery (36.6%). In terms of seniority, Junior Residents prescribed the highest number of antibiotics for both Access and Watch categories in Medicine and Surgery departments. Senior residents and Consultants prescribed a lower number of antibiotics in all categories and departments. Only a few antibiotics were prescribed in the Reserve category, with most prescriptions being from the Medicine department.

The study also evaluated the Knowledge and Awareness of Healthcare professionals towards the WHO AWaRe classification through a questionnaire survey. A total of 93 participants responded to the survey. Among them, most participants were Junior Residents (69.9%), followed by Senior Residents (25.8%) and Faculty (4.3%). When enquired if they knew about the WHO AWaRe classification only 33.3% of the participants responded positively. Of those who were aware of the AWaRe classification, the most common source of information was the internet (31.2%), followed by the antimicrobial policy of their institution (15.1%) as seen in Table 2.

The survey results on the knowledge and awareness of AMR among healthcare professionals are also presented in Tables 3 and 4. Out of the 93 participants, 68 (73.1%) agreed that the emergence of AMR is inevitable, while only 13 (14.0%) disagreed that AWaRe usage will result in the inability to treat serious infections. Additionally, 58 (62.4%) agreed that it will lead to lengthier hospital stays, 43 (46.2%) agreed that the success of chemotherapy and major surgery will be hampered, and the majority also agreed that its use will lead to increased cost of treatment and increased mortality rates. Regarding the utilization of AWaRe in the hospital summarized in Tables 4 and 5, 35.5% of the participants agreed that it should be used, while only 2.2% disagreed. Additionally, 34.4% agreed that AWaRe reduces adverse effects of inappropriate prescription. However, 37.6% of the participants considered that AWaRe threatens a clinician's autonomy and 30.1% thought that its use can delay treatment.

Additionally, the DDD of each drug was also evaluated. The usage of various antimicrobial drugs in a hospital setting, along with their daily doses and DDD according to the WHO's Anatomical Therapeutic Chemical classification system was calculated. Some of the important findings include high usage rates of ceftriaxone and metronidazole, and relatively low usage rates of drugs like colistin and clindamycin. Additionally, some drugs had wider ranges than others. Comparison of WHO defined DDD with Daily Drug dose (Mean) in the studied prescriptions is represented in the Clustered Bar chart in Figure 3.

Finally, the Mean Daily Drug Dose for prescribed drugs was compared with WHO defined DDD for each drug using a Student's *T* test. The mean daily drug dose of amoxy/clav was significantly higher than the WHO DDD (1.8 *vs* 1.50, $P = 0.014$), while the mean daily drug dose of metronidazole and doxycycline were significantly lower than the WHO DDD ($P < 0.001$ and $P = 0.008$, respectively). The mean daily drug dose of piperacillin/tazobactam, amikacin, clindamycin, and levofloxacin did not show significant differences compared to the WHO DDD ($P > 0.05$).

DISCUSSION

The findings of this study highlight several important issues related to antimicrobial prescribing practices and awareness among healthcare professionals. This study, conducted in a tertiary care institute in North India included prescriptions having at least one antibiotic in both inpatient (75.4%) and outpatient (24.6%) settings collected over a duration of 2 months by universal sampling. A total of 123 patient prescriptions were included which included 243 individual antibiotics. The mean number of antibiotics used per patient was 1.84 ± 0.83 which coincides with a study conducted in Brazil[9] with a mean of 2.4 antibiotics per patient. This finding is in line with the WHO prescribing indicators which state that each prescription should contain an average of 1.6–1.8 antibiotics[7]. The use of multiple antibiotics can increase the risk of adverse effects, drug interactions, and development of AMR. The mean duration of antibiotics prescribed was 6.63 ± 3.83 d. A study conducted in a tertiary care hospital in India reported a mean duration of 5.7 ± 3.1 d[12], while another in Ethiopia reported a mean duration of 10.2 d[13].These variations could be attributed to differences in patient demographics, disease prevalence, prescribing habits, and hospital policies.

Table 1 Descriptive data representing the antibiotic usage patterns categorized according to the designation of prescriber, department, outpatient *vs* inpatient and AWaRe classification

Category	Counts	% of total
Total number of patients	123	
Total prescriptions	123	
No. of different antibiotics used	27	
Most common antibiotic	Metronidazole	19
Designation		
Senior resident	42	18.7
Junior resident	162	72.0
Consultant	21	9.3
OPD/IPD		
OPD	55	24.6
IPD	169	75.4
Department		
Medicine	112	49.6
Surgery	114	50.4
WHO AWaRe classification		
Access	140	57.61
Watch	93	38.27
Reserve	10	4.11
	Antibiotics used in a patient	**Duration of antibiotics**
Mean	1.84	6.63
Median	2	6.00
Standard deviation	0.833	3.83
Minimum	1	1.00
Maximum	5	19.0

Second half of the table represents the descriptive analysis of antibiotics used and duration of individual antibiotics. OPD: Outpatient; IPD: Inpatient; WHO: World Health Organization.

In the present study, prescriptions included 27 different antibiotics of which Metronidazole was the most prescribed antibiotic, which could be due to its activity against anaerobic bacteria, which are commonly implicated in infections of the gastrointestinal tract, genitourinary tract, and skin. It is prescribed mostly in combination with amoxicillin in obstetrics and gynaecological care (mainly in post-delivery prophylaxis), and occasionally in caesarean section. Similar results were found study at Ghana Police Hospital[14]. This was followed by Ceftriaxone, a third-generation cephalosporin from the Watch group. The high proportion of cephalosporins in the prescriptions is consistent with the WHO analysis of South-East Asian countries which also found a very high level of consumption of the same in all states in India [15].

The WHO AWaRe classification system was used to analyse the antibiotics prescribed in this study, and the results showed that most antibiotics fell under the Access (57.61%) category. These antibiotics have a narrow spectrum of activity, lower cost, a good safety profile and generally low resistance potential and should be widely available and affordable. While this is a positive finding, it is also important to note that a significant proportion of antibiotics prescribed were from the Watch category (38.27%), which includes antibiotics that are at risk of becoming ineffective due to overuse and misuse. The small percentage of antibiotics prescribed in the Reserve category (4.11%) is reassuring, as these antibiotics are reserved for use as a last resort and should only be used in highly specific circumstances. The overall prescribing of antibiotics in our study is lower than the WHO recommendations, which state that more than 60% of all prescribed antibiotics must be from the Access group by 2023. Figures in our study are consistent with another Indian study which had Access (53.31%), Watch (40.09%) from, and Reserve (3.40%) category[16], and study done in Zambia with (n = 384) which had Access (55.5%), Watch (43.1%) and Reserve (1.4%)[7]. Our findings differ from another study in India[17] in which Watch group antibiotics accounted for 53.19 % of the total antibiotics and a Bangladesh study in which

Table 2 Representation of knowledge of World Health Organization Access, Watch, Reserve classification among healthcare professionals

Category	Counts	% of total
Position		
Junior resident	65	69.9
Senior resident	24	25.8
Faculty	4	4.3
Do you know about WHO AWaRe classification		
No details	22	23.7
Yes	31	33.3
Never heard	21	22.6
Little Idea	19	20.4
How did you hear about AWaRe?		
The internet	29	31.2
The WHO website	10	10.8
The antimicrobial policy of our institution	14	15.1
Other sources	19	20.4
No idea about it	21	22.6

AWaRe: Access, Watch, Reserve; WHO: World Health Organization.

Table 3 Knowledge (Score) on World Health Organization Access, Watch, Reserve classification

	Total score	Junior resident (*n* = 21)	Senior resident (*n* = 9)	Consultant (*n* = 3)	Medicine (*n* = 24)	Surgery (*n* = 9)
n	33	-	-	-	-	-
Mean	3.91	3.81	4.22	3.67	3.79	4.22
Standard deviation	2.17	2.25	2.33	1.53	2.08	2.49
Minimum	1	1	1	2	1	1
Maximum	8	8	8	5	8	8

WHO: World Health Organization.

64.0% of the patients were treated with antibiotics from the Watch group, 35.6% were treated with antibiotics from the Access group, and only 0.1% were treated with antibiotics from the Reserve group. The higher proportion of Access category antibiotics prescribed in our study could be attributed to the fact that these antibiotics are commonly used for the treatment of common infections encountered in the hospital, such as urinary tract infections, respiratory tract infections, and skin and soft tissue infections. The low proportion of Reserve category antibiotics prescribed in our study is consistent with the recommended sparing use of these antibiotics by WHO and other guidelines, which recommend their use only as a last resort in the treatment of severe infections[18]. In terms of departments and designations, our study found that the Medicine department prescribed the majority of antibiotics in both the Access and Watch categories, followed by the Surgery department. This finding is consistent with previous studies conducted in India and other low- and middle-income countries, where the Medicine department was found to be the highest prescriber of antibiotics[19].

To assess knowledge and attitude of prescribing doctor about the utility of AWaRe a Questionnaire based survey was carried out. Although (73.1%) agreed that the emergence of AMR is inevitable the lack of awareness about the programmes and measures being taken to curb it is concerning. In contrast to this study, a study conducted in the United States found that healthcare professionals had a high level of knowledge about AMR and the appropriate use of antibiotics[20]. This difference could be attributed to variations in healthcare systems and education programs in different countries. Moreover, in a study conducted in Germany, the majority of physicians agreed that the rational use of antibiotics is important for the prevention of AMR[21].

Despite the WHO AWaRe classification being included in the antibiotic policy of our institution only 33.3% of the responders knew about it which shines a light on the gap between the measures being taken and their implementation.

Table 4 Representation of awareness towards World Health Organization Access, Watch, Reserve classification among healthcare professionals

Statement	False	True	No idea	*n*
Emergence of Antimicrobial Resistance is inevitable	24	68	1	93
It will result in inability to treat serious infections	13	80	0	93
Lengthier hospital stays will be a result	35	58	0	93
Success of chemotherapy and major surgery will be hampered	50	43	0	93
Cost of treatment will be increased	52	41	0	93
MMR and IMR will increase	62	31	0	93

AWaRe: Access, Watch, Reserve; WHO: World Health Organization; MMR: Maternal mortality rate; IMR: Infant Mortality Rate

.

Table 5 Representation of attitude towards World Health Organization Access, Watch, Reserve classification among healthcare professionals

Question	Strongly disagree	Disagree	Neutral	Agree	Strongly agree
Should AWaRe be used in the hospital?	18 (19.4%)	2 (2.2%)	21 (22.6%)	33 (35.5%)	19 (20.4%)
AWaRe reduces adverse effects of inappropriate prescription	12 (12.9%)	5 (5.4%)	25 (26.9%)	32 (34.4%)	19 (20.4%)
AWaRe threatens a clinician's autonomy	9 (9.7%)	35 (37.6%)	35 (37.6%)	11 (11.8%)	3 (3.2%)
It can delay treatment	16 (17.2%)	37 (39.8%)	28 (30.1%)	8 (8.6%)	4 (4.3%)

AWaRe: Access, Watch, Reserve.

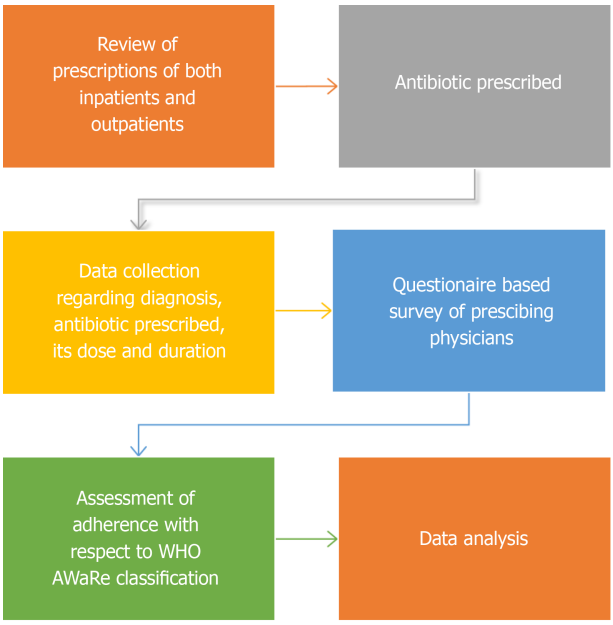

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Figure 1 Representation of the study flow. WHO: World Health Organization.

The institute antibiotic policy authorizes only the Senior Residents and Consultants to prescribe antibiotics from Watch and Reserve groups but in this study Junior Residents prescribed the highest number of antibiotics for both Access and Watch categories which coincides with previous studies conducted in India and other countries. In a study conducted in a tertiary care hospital in India, junior residents prescribed the majority (68.7%) of antibiotics[19]. The higher proportion of antibiotics prescribed by Junior Residents in our study could be attributed to their relatively higher workload and less clinical experience compared to Senior Residents and Consultants. Overall, the findings of this study suggest that there is

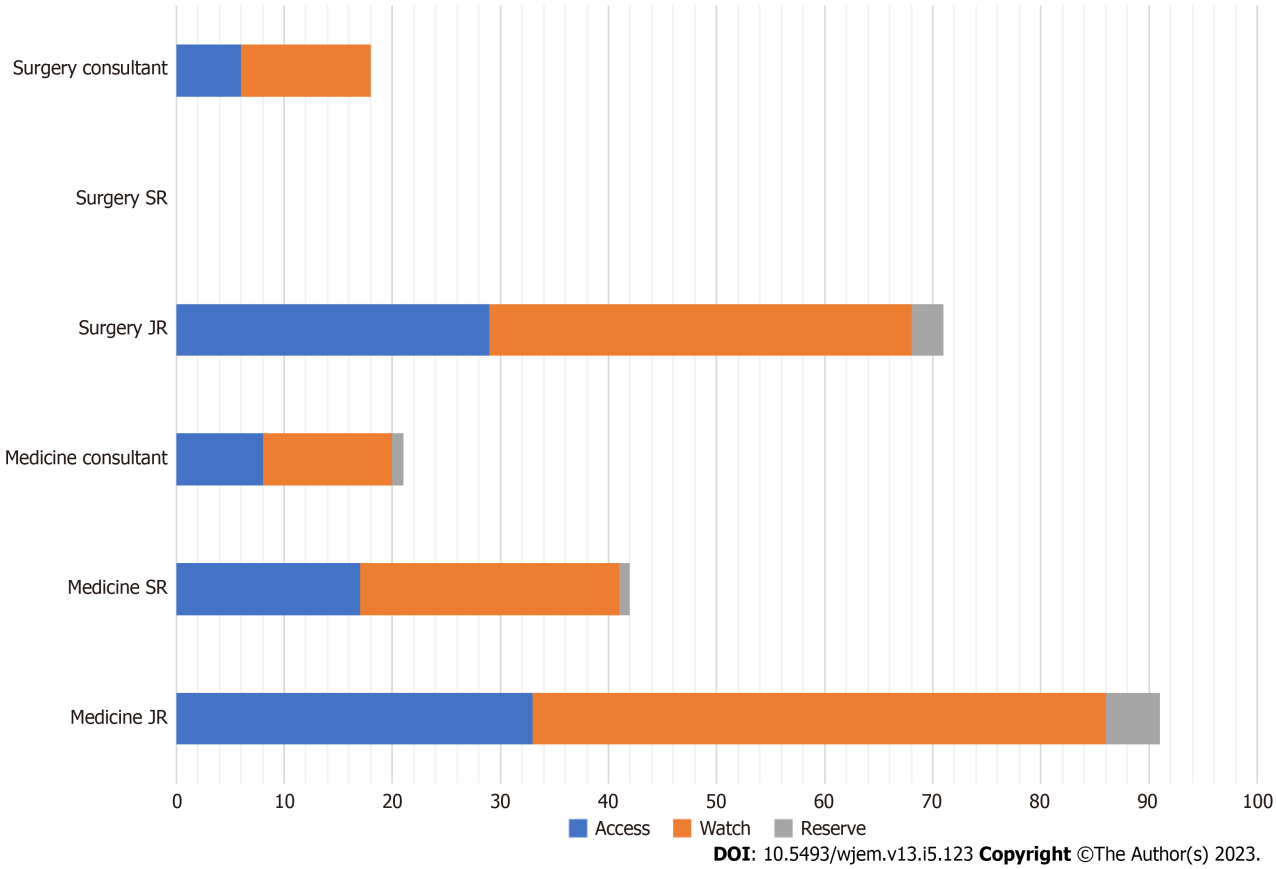

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Figure 2 Frequencies of World Health Organization Access, Watch, Reserve category by designation and department.

a moderate level of awareness among healthcare professionals about AMR, there is a need for further education and awareness programs to ensure the appropriate use of antibiotics in the hospital setting. It must be noted that most of the responders of the survey were also Junior Residents and hence education programmes can hence play a vital role in improving the results by working at grassroot levels.

Another analysis of the study depicts that there is a significant difference between the WHO DDD and the prescribed daily dose in the analysed prescriptions. Several studies conducted in the West have compared the mean daily drug dose of prescribed drugs with the WHO defined DDD. In a study conducted in a hospital in Italy found that the mean daily dose of antibiotics was generally lower than the WHO DDD for most antibiotics[22]. These findings are similar to the present study, which found higher mean daily doses of amoxicillin/clavulanic acid and piperacillin/tazobactam, and a lower mean daily dose of levofloxacin. The WHO DDD is a standardized measure of drug consumption used to compare the drug consumption between different countries and regions. It represents the assumed average maintenance dose per day for a drug used for its main indication in adults. On the other hand, the prescribed daily dose refers to the actual dose that a physician prescribes to a patient.

The difference between the WHO DDD and the prescribed daily dose can have several implications. Firstly, it can lead to overuse or underuse of medications, which can affect the therapeutic outcomes of patients. For example, if the prescribed daily dose is lower than the WHO DDD, the patient may not receive the optimal therapeutic effect of the medication. Conversely, if the prescribed daily dose is higher than the WHO DDD, the patient may be at risk of adverse effects or toxicity. Secondly, the difference between the WHO DDD and the prescribed daily dose can affect the comparability of drug consumption data between different regions and countries. If different regions or countries use different prescribed daily doses, it can be difficult to compare their drug consumption patterns. Therefore, adherence to the WHO DDD is important to ensure that drug consumption data are standardized and comparable across different regions and countries. In conclusion, the difference between the WHO DDD and the prescribed daily dose is an important issue that needs to be addressed in the prescribing practices of physicians. Adherence to the WHO DDD can help to ensure optimal therapeutic outcomes for patients and comparability of drug consumption data between different regions and countries.

Overall, the findings of this study emphasize the importance of improving antimicrobial prescribing practices and increasing awareness among healthcare professionals regarding the WHO AWaRe classification system and the threat of AMR. Effective antimicrobial stewardship programs that promote appropriate antibiotic use can help reduce the risk of AMR and improve patient outcomes. Future research should focus on implementing such programs in hospital settings and evaluating their effectiveness in reducing inappropriate prescribing practices.

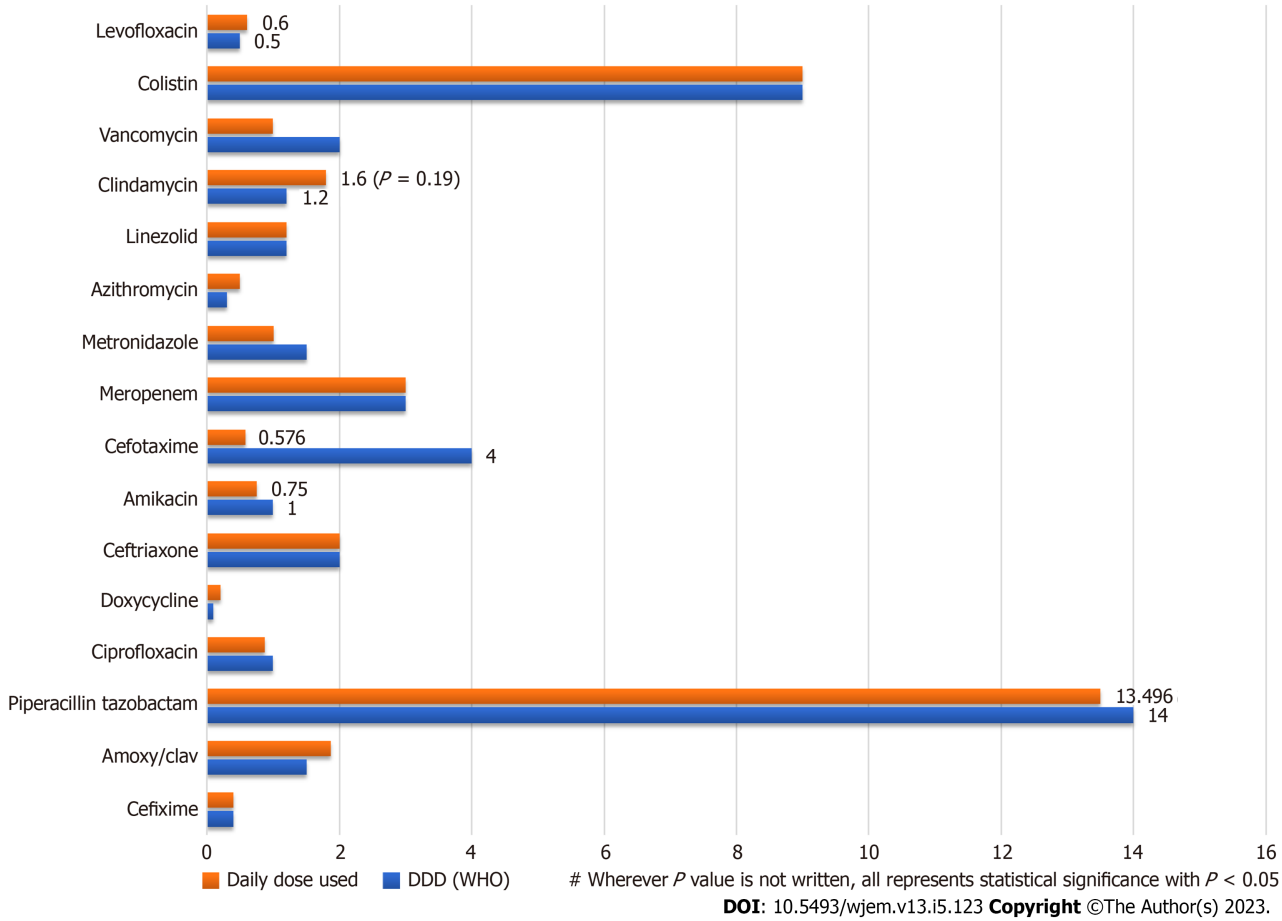

Figure 3 Comparison of drug utilization to World Health Organization defined daily doses.

CONCLUSION

This research indicates an appropriate proportion of prescriptions falling under the Access category (57.61%), suggesting appropriate antibiotic selection, a significant proportion also belongs to the Watch category (38.27%), emphasizing the need for greater caution to prevent the escalation of AMR. There is a moderate level of awareness among healthcare professionals about AMR and the steps being taken to tackle it, highlighting the gap in implementation of policies and need for more steps to be taken in spreading the knowledge about the subject. However, there is a significant difference between the WHO DDD and the prescribed daily dose in the analysed prescriptions suggesting overuse and underuse of antibiotics.

ARTICLE HIGHLIGHTS

Research background

India has particularly high rates of antimicrobial resistance (AMR), posing a threat to effective treatment. The World Health Organization (WHO) Access, Watch, Reserve classification system was introduced to address this issue and guide appropriate antibiotic prescribing. However, there is a lack of studies examining the prescribing patterns of antimicrobials using the AWaRe classification, especially in North India.

Research motivation

This study aimed to assess the prescribing patterns of antimicrobials using the WHO AWaRe classification in a tertiary care centre in North India.

Research objectives

(1) To audit the prescribing patterns of antimicrobials among clinicians using WHO's AWaRe classification in a tertiary care centre; (2) To assess knowledge and practices of prescribing doctor about the utility of AWaRe by Questionnaire based assessment; and (3) To compare the appropriateness of AWaRe classification with days of therapy and defined daily doses of antimicrobial utilization.

Research methods

A descriptive, cross-sectional study was conducted from July 2022 to August 2022 at a tertiary care hospital. Prescriptions containing at least one antimicrobial were included in the study. A questionnaire-based survey was also conducted to assess the knowledge and practices of prescribing doctors regarding the utility of AWaRe classification.

Research results

The study involved a total of 123 patients, each of whom received at least one antimicrobial prescription. Most prescriptions were for inpatients, metronidazole and ceftriaxone were the most prescribed antibiotics. According to the AWaRe classification, 57.61% of antibiotics fell under the Access category, 38.27% in Watch, and 4.11% in Reserve. The questionnaire survey showed that only a third of participants were aware of the AWaRe classification, and there was a lack of knowledge regarding AMR and the potential impact of AWaRe usage.

Research conclusions

This study highlights the need for better antimicrobial prescribing practices and increased awareness of the WHO AWaRe classification and AMR among healthcare professionals.

Research perspectives

The findings indicate a high proportion of prescriptions falling under the Access category, suggesting appropriate antibiotic selection. There is room for improvement and educational interventions and antimicrobial stewardship programs should be implemented to enhance knowledge and adherence to guidelines, ultimately contributing to the containment of AMR.

FOOTNOTES

Author contributions: Negi G, KB A, and Panda PK gave the concept, designed the project, collected the data, analysed, wrote the manuscript, critically reviewed, and approved the manuscript.

Institutional review board statement: The study was reviewed and approved by Institutional Ethics Committee of All India Institute of Medical Sciences, Rishikesh, India.

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ORIGINAL ARTICLE

Basic Study

In vitro study on the transmission of multidrug-resistant bacteria from textiles to pig skin

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Abstract

BACKGROUND

The survival of microorganisms on textiles and specifically on healthcare professionals' (HCP) attire has been demonstrated in several studies. The ability of microorganisms to adhere and remain on textiles for up to hours or days raises questions as to their possible role in transmission from textile to skin *via* HCP to patients.

AIM

To evaluate the presence, survival and transmission of different multidrug-resistant bacteria (MDRB) from HCP attire onto skin.

METHODS

Three MDRB [methicillin-resistant *Staphylococcus aureus* (MRSA); vancomycin-resistant *Enterococcus faecium* (VRE); carbapenem-resistant *Klebsiella pneumoniae*, CRKP)] were inoculated on textiles from scrubs (60% cotton-40% polyester) and

white coat (100% cotton) at concentrations of 10^8 colony-forming units (CFU), 10^5 CFU, and 10^3 CFU per mL. The inoculation of swatches was divided in time intervals of 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h. At the end of each period, textiles were imprinted onto pig skins and each skin square was inverted onto three different selective chromogenic media. Growth from the pig skin squares was recorded for the 3 MDRB at the three above concentrations, for the whole length of the 6-h experiment.

RESULTS

MRSA was recovered from pig skins at all concentrations for the whole duration of the 6-h study. VRE was recovered from the concentration of 10^8 CFU/mL for 6 h and from 10^5 CFU/mL for up to 3 h, while showing no growth at 10^3 CFU/mL. CRKP was recovered from 10^8 CFU/mL for 6 h, up to 30 min from 10^5 CFU/mL and for 1 min from the concentration of 10^3 CFU/mL.

CONCLUSION

Evidence from the current study shows that MRSA can persist on textiles and transmit to skin for 6 h even at low concentrations. The fact that all MDRB can be sustained and transferred to skin even at lower concentrations, supports that textiles are implicated as vectors of bacterial spread.

Key Words: Textiles; Attire; Multidrug-resistant bacteria; Methicillin-resistant *Staphylococcus aureus*; Vancomycin-resistant *Enterococcus faecium*; Extended-spectrum b-lactamase; Pig skin; Skin; Transmission

Core Tip: The current study aimed to evaluate the transmission of multidrug-resistant bacteria (MDRB) from attire (scrubs, white coats) onto skin. Three MDRB types [methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium*, carbapenem-resistant *Klebsiella pneumoniae*] were inoculated on textiles over various time intervals for up to 6 h and then imprinted onto pig skin. All MDRB were able to be sustained and transferred to skin, but at different concentrations and time. MRSA had the longest presence on textile and highest transmission potential even at lower concentrations. Evidence suggests that textiles can be implicated as vectors of MDRB spread in the healthcare setting.

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INTRODUCTION

It is well established that textiles can carry micro-organisms, a fact with raises concerns for their ability to transmit them either onto skin or to other textiles[1]. Previous studies and systematic reviews have demonstrated the ability of pathogens, including multidrug-resistant bacteria (MDRB), to survive on healthcare professionals' (HCP) attire, devices and surfaces in hospitals and long-term care facilities, thus raising the need to study their potential to contribute to transmission[1-4].

Cyprus is considered a high-prevalence country for MDRB. In fact, in healthcare-associated infections in Cyprus hospitals in 2020, methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) was detected in 49.1% of invasive infections by *S. aureus*, vancomycin-resistant *Enterococcus faecium* (*E. faecium*) (VRE) accounted for 44.1% of enterococci, and isolates of *Klebsiella pneumoniae* (*K. pneumoniae*) exhibited multidrug resistance in 18.2% and carbapenem resistance in 19.8%[5]. Further to this, in a previous study, our group demonstrated the presence of several different MDRB on HCP uniforms in hospitals and long-term care facilities in Cyprus, including MRSA, VRE, extended-spectrum b-lactamase (ESBL)-producing bacteria and carbapenem-resistant bacteria[1]. These findings suggest that MDRB may be transmitted in different ways within healthcare settings, thus raising the need to identify additional areas for targeted interventions and improvement.

A demonstrable means to evaluate the role of attire and textiles in the transmission cycle, is by studying the degree of transmission of micro-organisms from fabrics to skin. To achieve this, it is necessary to use an alternative to human skin with similar properties, such as pig skin, in order to allow accurate *in-vitro* experimentation[6]. The similarities between human and pig skin lie in the structure of epidermis and thickness ratios between dermis and epidermis, as well as in hair follicle and blood vessel patterns. In contrast to the skin of other animals used in laboratory studies, the dermal collagen and elastic content of the pig skin are more similar to the skin of humans. Furthermore, Schmook *et al*[7] demonstrated in an *in-vitro* study of percutaneous absorption, that in the absence of human tissue, pig skin was the most suitable model.

Based on current literature, including experimental and observational studies, we hypothesize that MDRB can carry the ability to survive on textiles to a considerable extent to be isolated and to transmit onto skin. Our aim was to evaluate the survival potential of different MDRB on different types of textiles and to evaluate their potential for transmission onto skin, using representative strains such as MRSA, VRE and carbapenem-resistant *K. pneumoniae* (CRKP).

MATERIALS AND METHODS

Aims

The aim of the current study was to determine the viability of MDRB over a 6-h period, which mimics the usual duration of a hospital shift, on two types of textiles and to evaluate whether these inoculated textiles can colonize pig skin using three MDRB (MRSA, VRE and CRKP) at different concentrations. Study approval was not required as it did not involve human subjects or animals. The strains were retrieved from a previous study that was approved by the Cyprus National Bioethics Committee (decision number "ΕΕΒΚ ΕΠ 2018.01.155").

Materials

Fresh MRSA, VRE and CRKP cultures were grown from previously isolated organisms from HCP uniforms from a previous study, that were stored in -20 °C. After 24 h of incubation, the organisms were prepared at 0.5 MacFarland standard each. Their precise concentrations were calculated: 1.18×10^8 CFU/mL MRSA, 1.6×10^8 CFU/mL for *E. faecium*, and 1.14×10^8 CFU/mL for *K. pneumoniae*. All 3 organisms were serially diluted from 10^8 CFU down to 10^2 CFU. The concentrations of 10^8, 10^5, and 10^3 CFU per mL were used during the process.

Textile swatches were cut in squares of 1.5 cm × 1.5 cm and sterilised in 100 °C in dry oven for 2 h. The swatches were taken from uniforms and scrubs used in healthcare settings in order to mimic txextiles used in real-life conditions, with a composition of 60% cotton and 40% polyester (T1, taken from a used scrub uniform of a nurse, who had worn it for 6 mo), and 100% cotton (T2, taken from a white coat used by a physician for 6 mo). Aluminium foil squares of 1.5 cm × 1.5 cm were cut and heat sterilised in the same manner as the cloth swathes. These would be used as a barrier when pressure would be applied on cloth or skin.

Fresh pig skin (commercially retrieved 24 h after slaughtering), cleared from most of the fat under the dermis, was scrubbed and washed with chlorhexidine soap and then with alcohol. The cleaned skin was then dried in examination paper and kept covered to avoid drying. When the experiment was ready to begin, square pieces of 1.5 cm × 1.5 cm of skin were cut using sterilized blades. Negative control skin samples were incubated on the 3 chromogenic media to ensure the absence of the 3 microorganisms used. The specialized chromogenic media used were CHROMO agar, MRSA medium, chromo VRE, and chromo ESBL.

Experimental design

The current experimental study was designed in duration and environmental conditions that mimic the working conditions within a 6-h HCP shift. Temperature of the laboratory room was kept constant at 22.9-23.5 °C and humidity of 49%-53% and the experiment was conducted on the bench.

Each microbial concentration had 10 stations allocated at time intervals of 1 min, 5 min, 15 min, 30 min, 1 h, and then every hour for a total of 6 h and at three different concentrations. Each station had in place: 2 cloth swatches (T1 & T2), 2 respective skin squares (S1 & S2) and chromogenic plates, for each of the 3 dilutions of the microorganisms (Figure 1).

Firstly, 2 cloth swatches in each station were inoculated with 50 μL of each dilution in each column. The inoculation started from 6 h up to 1 min, guided by stopwatches. At the end of each time slot, each cloth swatch was placed on top of the skin square. The cloth was applied onto skin with a pressure of 100 Gr (friction asset) for 5 s and foil was placed over it for protection. Afterwards and following the same technique, each skin square was pressed onto the chromogenic agar. The plates were then incubated at 37 °C. After 24 h, the incubated plates were checked and incubated for an additional 24 h. When the 48-h incubation period ended, the chromogenic plates were inspected, and the presence of growth was recorded. We did not perform a concentration count at each station; the results indicated presence or not. Thus, the experiment was performed based on the assumption that even a small amount of bacteria could be transmitted and possibly cause infection.

RESULTS

Over a period of 6 h and at different time points, a total of 3 MDRB (MRSA, VRE, CRKP) were inoculated in 3 different concentrations (10^8 CFU/mL, 10^5 CFU/mL, 10^3 CFU/mL) on 2 different cloth textiles (T1, T2) and then each cloth was applied on a pig skin square (S1, S2 respectively). The skin inoculates were then cultured on special medium for 48 h and MDRB growth was recorded.

All findings demonstrating the recovery of the 3 MDRB from skin and textiles are presented in Table 1. Recovery times for all 3 MDRB were the same in both skin swatches S1 and S2 that were in contact with T1 (60% cotton and 40% polyester) and T2 (100% cotton), respectively. Overall, no differences were observed in terms of growth for each MDRB between the two types of textiles. MRSA exhibited the highest recovery. In specific, recovery of MRSA was successful at all time intervals and for all 3 concentrations (10^8 CFU/mL, 10^5 CFU/mL and 10^3 CFU/mL). MRSA remained detectable and could be transmitted throughout the 6-h experiment duration. VRE was recovered from the highest concentration of

Table 1 Growth indicated in skin swatches S1 from textile T1 (cotton-polyester cloth) and S2 from T2 (100% cotton cloth), when imprinted on chromogenic agars at three concentrations

Time	MRSA (CFU/mL)						VRE (CFU/mL)						CRKP (CFU/mL)					
	S1 (10^8)	S2 (10^8)	S1 (10^5)	S2 (10^5)	S1 (10^3)	S2 (10^3)	S1 (10^8)	S2 (10^8)	S1 (10^5)	S2 (10^5)	S1 (10^3)	S2 (10^3)	S1 (10^8)	S2 (10^8)	S1 (10^5)	S2 (10^5)	S1 (10^3)	S2 (10^3)
1 min	G	G	G	G	G	G	G	G	G	G	/	/	G	G	G	G	G	G
5 min	G	G	G	G	G	G	G	G	G	G	/	/	G	G	G	G	/	/
15 min	G	G	G	G	G	G	G	G	G	G	/	/	G	G	G	G	/	/
30 min	G	G	G	G	G	G	G	G	G	G	/	/	G	G	G	G	/	/
60 min	G	G	G	G	G	G	G	G	G	G	/	/	G	G	/	/	/	/
2 h	G	G	G	G	G	G	G	G	G	G	/	/	G	G	/	/	/	/
3 h	G	G	G	G	G	G	G	G	G	G	/	/	G	G	/	/	/	/
4 h	G	G	G	G	G	G	G	G	/	/	/	/	G	G	/	/	/	/
5 h	G	G	G	G	G	G	G	G	/	/	/	/	G	G	/	/	/	/
6 h	G	G	G	G	G	G	G	G	/	/	/	/	G	G	/	/	/	/

CFU: Colony-forming units; CRKP: Carbapenem-resistant *Klebsiella pneumoniae*; G: Growth; MRSA: Methicillin-resistant *Staphylococcus aureus;* VRE: Vancomycin-resistant *Enterococcus faecium*.

10^8 CFU/mL for the whole duration of the 6-h period and for up to 3 h from the 10^5 CFU/mL concentration. No recovery of VRE was recorded from the lowest concentration of 10^3 CFU/mL. CRKP was also recovered from the highest concentration of 10^8 CFU/mL for the total duration of 6 h (duration of the experiment) and for 30 min from the second highest concentration (10^5 CFU/mL), whereas it was recovered for only up to 1 min from the lowest concentration of 10^3 CFU/mL.

Conclusively, our results support the sustainability of MRSA for the maximum of the duration of the study in all concentrations. The other gram-positive coccus (VRE) also remained for the whole duration of the study but only in the highest concentration, whereas VRE was not isolated for the lowest concentration. In contrast, the gram-negative bacterium (CRKP) remained for less time in the concentration of 10^5 CFU/mL and for only 1 min at the lowest concentration.

DISCUSSION

In our study, involving the transfer and presence of MDRB as a result of contact between textiles and pig skin, MRSA exhibited the longest persistence out of the 3 studied MDRB and over the duration of 6 h of the experiment. VRE and CRKP were both detected at the highest concentration of 10^5 CFU/mL, for up to 3 h and 30 min, respectively. The presence of MDRB, as recorded on both textile types that were used in the study, was also confirmed on pig skin, which provides evidence of potential for transfer of bacteria and MDRB onto skin from contaminated textiles and furthermore, suggesting this as a transmission mode to patients. The study results show that gram-positive cocci, such as staphylococci and enterococci, are more resilient on textiles and in the environment over time, whereas CRKP also showed prolonged presence at higher concentrations.

To our knowledge, only few previous studies have evaluated the transferability of bacteria onto skin. Butler *et al*[8] conducted a study on the transfer of bacteria onto pigskin by use of white coats. Specifically, MRSA, VRE and *Acinetobacter baumannii* (*A. baumannii*) (reported as pan-resistant) were inoculated on cloth swatches and rubbed on sanitized pigskin. All 3 MDRB exhibited ability to transfer from cloth to pig skin at time intervals of 1, 5 and 30 min, whereas *A. baumannii* was transferred up to a dilution of 1:1000. Desai *et al*[9] also confirmed sustainability and transmission of MRSA from cotton towels and bedsheets to pig skin for long periods reaching up to 14 d, whereas Sattar *et al*[10] reported transfer of MRSA from textiles (cotton and polycotton) to other textiles and to finger skin. The transfer was fivefold higher when the textile was moist and when there was friction. Varshney *et al*[11] in a study transfer between textiles of *Acinetobacter calcoaceticus, Escherichia coli* (*E. coli*) and *S. aureus,* also reported that cell transfer varied between 5%-61% when friction was applied compared to non-friction, which suggested the importance of the role of rubbing between same textiles. Malnick *et al*[12] examined the transfer of bacteria between fabric and surrogate skin taking into consideration the effect of surface energy and surface roughness of fabrics, while using *E. coli* and *S. aureus* against 100% cotton, 100% polyester and a 50-50 blend of both. Their conclusion was that the 100% polyester attracted the highest number of bacteria.

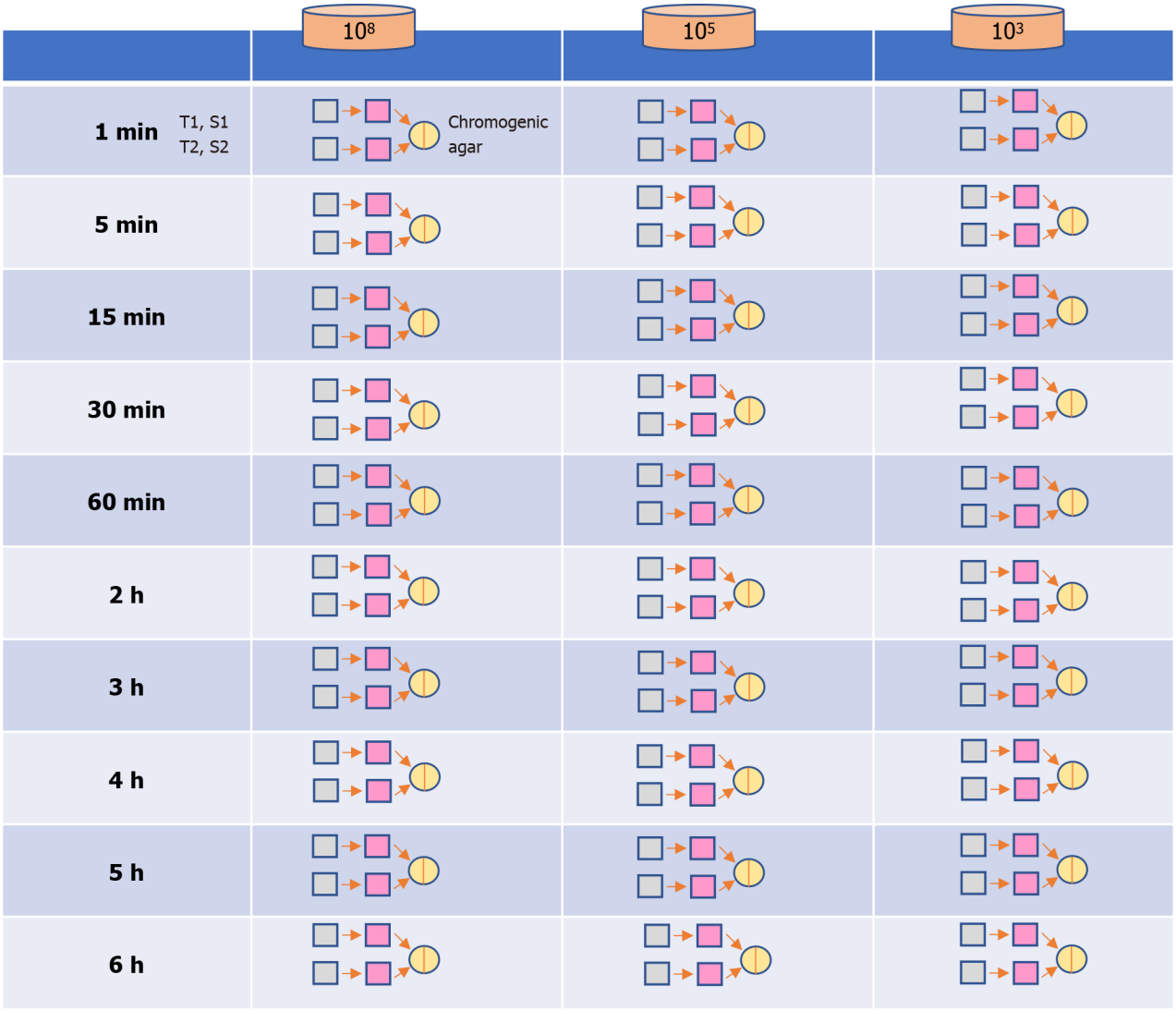

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Figure 1 Experimental set up: Each station contains 2 cloth swatches (T1 & T2), 2 pig skins (S1 & S2), and one chromogenic agar.

The survival ability of microorganisms on textiles has been previously addressed by several studies. Koca *et al*[13] used different fabrics, such as cotton, cotton-polyester, silk, and wool. To assess persistence of a variety micro-organisms, including yeasts, fungi, MRSA, VRE and ESBL-producing bacteria. Results showed that VRE had its longest survival on cotton-polyester fabrics (51 d, and 49 d on 100% cotton, silk and wool). MRSA had the longest survival period of 41 d on wool and 37 d on cotton silk and cotton-polyester. Respectively *E. coli* showed its longest survival for 45 d on cotton, silk and wool, and on cotton-polyester 37 d. These findings were confirmed by Malnick *et al*[12] who showed that 50% of VRE-swabbed pyjamas and bed sheets yielded micro-organisms overnight. Similarly, Neely and Maley[3] studied the survival of strains of MRSA and VRE on five different types of materials used commonly in hospitals: 100% cotton (which represented cloths and towels), cotton-polyester, 100% polyester, and 100% polypropylene plastic (represented which splash aprons). MRSA had the longest survival on polypropylene plastic (> 51 d), followed by 40 d (polyester), and 21 d (cotton). VRE exhibited long survival periods of > 80 d on polypropylene plastic and polyester, and 22 d on the other materials.

In agreement with the above findings, a literature review supported that contaminated textiles can harbour bacteria and consequently transmit infection for weeks[14]. Current evidence supports survival and persistence on textiles not only of bacteria, but also mycobacteria, fungi and viruses. Specifically, VRE can survive for more than 90 d, MRSA for up to 56 d and *K. pneumoniae* for 11 d. Survival at room temperature favoured polyester against cotton. Even though there is only a limited number of reported healthcare-associated infection outbreaks associated with contaminated textiles[15-19], their involvement may be undermined, and not sought out during incidences or as part of the general infection control process.

Taken cumulatively, the findings of our study add insight to the literature and clearly suggest that staff attire, including uniforms and scrubs, when contaminated, can transmit pathogenic micro-organisms onto patients' skin, thus acting as bacterial vectors. It can also be assumed that contaminated hands can in turn contaminate uniforms[10]. Hence, strict and targeted preventive measures targeting textile and attire are needed to break the chain of transmission. Current evidence from various studies suggests against the use of white coats and ties in healthcare settings, as they are more

frequently contaminated compared to other HCP attire[4]. On the other hand, short-sleeved uniforms can be more beneficial as regards to lower transmission of pathogenic micro-organisms[20]. However, these measures alone seem insufficient to control MDRB spread and support the need for additional control measures, such as ensuring application of appropriate home laundering practices, use of a hospital laundry service, wearing single-use protective aprons or gowns wherever applicable, promoting hand hygiene before and after patient interaction, daily uniform change, and application of contact precautions particularly in high-prevalence settings[21,22]. Our experimental study was performed under the same environmental conditions for all 3 studied MDRB. Therefore, the different observations in regard to time intervals and concentrations among the 3 studied MDRB in our study, probably reflect the properties of these bacterial strains.

The strength of the current study lies in its design, which aimed to resemble as much as possible a real-life situation. Specifically, in addition to evaluating the possibility of textile inoculation and transfer onto pig skin, we confirmed the viability of clinically important MDRB over time, in an attempt to mimic the environmental conditions in healthcare settings during HCP shifts. Our findings demonstrate that 6 h after MDRB inoculation of textiles, transfer from a dry textile surface on pig skin was possible. Simulation of real-life conditions showed that MDRB can survive, grow and transmit from textiles of two different compositions onto pig skin, which is the closest parallel to human skin.

Limitations of our study include the small spectrum of micro-organisms used and the fact that we used only two types of fabrics. We also acknowledge the fact that during our experiment, we didn't quantify the bacterial colony concentrations after inoculation. However, we performed the experiment under the hypothesis that bacterial persistence through time and transmission is evidence that textiles can be infectious, regardless of the growth amount. In addressing the current research gaps in the literature and the limitations of our study, future studies can include inoculation of textiles not solely with pure bacterial cultures, but also mixed with organic matter such as bodily fluids, to better emulate real-life conditions. Furthermore, the role of skin flora could be investigated in the survival of microorganisms on textiles and to evaluate their effect on MDRB binding capacity and replication.

Of note, only few similar studies have attempted to associate textiles with the transmission and survival of microorganisms. In line with the general perception that the risk of transmission from textiles is low, there is not enough emphasis given to the importance of cleaning and decontamination of textiles, compared to other inanimate surfaces[22,23]. However, our findings are supportive that textiles can be responsible for the transmission of pathogens in healthcare settings and thus, they should be managed accordingly. Moreover, our study strongly suggests that textiles should be included in the transmission triad "patient - healthcare professional - environment". To this end, healthcare settings should opt to analyse all possible steps in the chain of transmission and introduce appropriate action plans that include reduction of transmission risk through textiles and HCP attire. In the absence of protocols for testing uniform cleanliness and compliance, our findings provide evidence towards enforcing appropriate measures to reduce bacterial reservoirs in healthcare settings.

CONCLUSION

In conclusion, the current experimental study using 3 types of MDRB, provides evidence of their sustainability and transmission from textiles to skin. MRSA exhibited maximum sustainability for the whole duration of the 6-h study in all concentrations, VRE also remained for the whole duration of the study but only in the highest concentration, whereas CRKP remained for less time overall. Cumulatively, our data adds support to increasing evidence that textiles should be considered as vehicles of transmission in the healthcare setting.

ARTICLE HIGHLIGHTS

Research background

The isolation of microorganisms from textiles, including healthcare professionals' (HCP) attire, has been previously demonstrated in several studies.

Research motivation

The ability of microorganisms to adhere and survive on textiles, raises questions as to their possible role in transmission from textile to skin in healthcare environments.

Research objectives

The present experimental study aimed to evaluate the presence, survival and transmission of different multidrug-resistant bacteria (MDRB) from HCP attire onto skin.

Research methods

Inoculation of 3 MDRB [methicillin-resistant *Staphylococcus aureus* (MRSA); vancomycin-resistant *Enterococcus faecium* (VRE); carbapenem-resistant *Klebsiella pneumoniae*, CRKP)] on textiles from two types of textiles (60% cotton-40% polyester and 100% cotton) at 3 different concentrations (10^8 CFU, 10^5 CFU and 10^3 CFU per mL) and at different time intervals ranging from 1 min to 6 h. At the end of each time period, textiles were imprinted onto pig skins and each skin

square was inverted onto selective chromogenic media. Growth from the pig skins was recorded for the 3 MDRB at the three above concentrations, for the whole length of the 6-h experiment.

Research results

Recovery of MDRB from pig skins differed for each strain, with MRSA recording the longest and most sustained recovery at all concentrations and for up to 6 h. VRE showed no growth from 10^3 CFU/mL and was recovered from 10^8 CFU/mL for 6 h and from at 10^5 CFU/mL for up to 3 h. CRKP was recovered from 10^8 CFU/mL for 6 h, up to 30 min from 10^5 CFU/mL and for only 1 min from 10^3 CFU/mL.

Research conclusions

Evidence from the current study shows that all 3 studied MDRB can be sustained and transferred onto skin, with MRSA showing the highest level of persistence on textiles and transmission to skin even at low concentrations.

Research perspectives

Our findings support that textiles can be implicated as vectors of bacterial spread.

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FOOTNOTES

Author contributions: Lena P contributed to the conceptualization of this study; Lena P, Liatsou M, Lamnisos D, Papageorgis P, and Tsioutis C involved in the methodology of the manuscript; Lena P, Karageorgos S, Agouridis AP, Spernovasilis N, Lamnisos D, Papageorgis P, and Tsioutis C participated in the formal analysis and investigation of this manuscript; Lena P, Karageorgos S, and Tsioutis C drafted the manuscript; Lena P, Karageorgos S, Agouridis AP, Spernovasilis N, and Tsioutis C contributed to the review and editing of this manuscript; Lena P, Papageorgis P, and Tsioutis C involved in the supervision of this study; and all authors approved final article version published.

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ORIGINAL ARTICLE

Basic Study

Exploring the mechanism of action bitter melon in the treatment of breast cancer by network pharmacology

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Abstract

BACKGROUND

Bitter melon has been used to stop the growth of breast cancer (BRCA) cells. However, the underlying mechanism is still unclear.

AIM

To predict the therapeutic effect of bitter melon against BRCA using network pharmacology and to explore the underlying pharmacological mechanisms.

METHODS

The active ingredients of bitter melon and the related protein targets were taken from the Indian Medicinal Plants, Phytochemistry and Therapeutics and SuperPred databases, respectively. The GeneCards database has been searched for BRCA-related targets. Through an intersection of the drug's targets and the disease's objectives, prospective bitter melon anti-BRCA targets were discovered. Gene ontology and kyoto encyclopedia of genes and genomes enrichment analyses were carried out to comprehend the biological roles of the target proteins. The binding relationship between bitter melon's active ingredients and the suggested target proteins was verified using molecular docking techniques.

RESULTS

Three key substances, momordicoside K, kaempferol, and quercetin, were identified as being important in mediating the putative anti-BRCA effects of bitter melon through the active ingredient-anti-BRCA target network study. Heat shock protein 90 AA, proto-oncogene tyrosine-protein kinase, and signal transducer and activator of transcription 3 were found to be the top three proteins in the protein-protein interaction network study. The several pathways implicated in the anti-BRCA strategy for an active component include phosphatidylinositol 3-kinase/protein kinase B signaling, transcriptional dysregulation, axon guidance, calcium signaling, focal adhesion, janus kinase-signal transducer and activator of transcription signaling, cyclic adenosine monophosphate signaling, mammalian

target of rapamycin signaling, and phospholipase D signaling.

CONCLUSION
Overall, the integration of network pharmacology, molecular docking, and functional enrichment analyses shed light on potential mechanisms underlying bitter melon's ability to fight BRCA, implicating active ingredients and protein targets, as well as highlighting the major signaling pathways that may be altered by this natural product for therapeutic benefit.

Key Words: Bitter melon; Momordica charantia; Network pharmacology; Molecular docking; Breast cancer; Indian Medicinal Plants, Phytochemistry and Therapeutics

Core Tip: The phytochemicals and molecular processes in bitter melon that are thought to be involved in the therapy of breast cancer (BRCA) were investigated using network Pharmacology. Our research demonstrated that the anti-BRCA benefits of bitter melon are likely caused by negative regulation of transcription, cell differentiation, apoptosis, proteolysis, negative control of neuron apoptosis, and cell migration. Further discovered nine important pathways like phosphatidylinositol 3-kinase/protein kinase B signaling, transcriptional dysregulation, axon guidance, calcium signaling, focal adhesion, janus kinase-signal transducer and activator of transcription signaling, cyclic adenosine monophosphate signaling, mammalian target of rapamycin signaling, and phospholipase D signaling, are likely to be involved in the mechanism of action of bitter melon for the treatment of BRCA.

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INTRODUCTION

Breast cancer (BRCA) is the highest-escalation cancer in women with incidence rates rising at a rate of 3% annually in recent years around the world[1]. BRCA is the most prevalent cancer in women, with more than 2.3 million cases diagnosed each year[2]. BRCA is the top or second cause of death from cancer in women in 95% of the world's nations[3]. The age at which BRCA symptoms first appear is also decreasing, which has a severe psychological impact on patients, puts a large amount of financial and life burden on patients and their families, and lowers happiness levels dramatically. BRCA is currently treated with a combination of chemotherapy, radiotherapy, and surgery which have several shortcomings. The psychological and physical side effects include slow wound healing, changes in body shape, liver and kidney damage, changes in metabolism, and severe alopecia[4,5]. As a result, it is important to create medicines that have a wide range of applications and few negative effects. Herbal medicine has a lot of potential because it has few adverse effects and a powerful healing effect. Emergence, expansion, invasion, and metastasis of BRCA are complex processes. Herbal medicine is a useful technique for treating BRCA regardless of stage[6,7].

Bitter melon, also known as momordica charantia, is a member of the Cucurbitaceae family that exhibits anti-inflammatory, potential antibacterial, and antiviral activities[8-10]. bitter melon extracts are also effective against tumors[11] and were also found to be effective for the treatment of ulcers, malaria, pain, psoriasis, hyperlipidemia, and hypertension[12-17]. It includes several different active ingredients, including polysaccharides, anthrone, peptides, proteins, terpenoids, phenolics, and many more. bitter melon anticancer potentials have become more widely known in recent years. According to research, bitter melon suppresses the growth of several malignant tumors, including gastric, liver, and BRCA[18].

According to Ray *et al*[19], the bitter melon extract reduces cell proliferation and induces apoptotic cell death. The fact that bitter melon extract administration increased p53, p21, and pChk1/2 while inhibiting cyclin B1 and cyclin D1 production suggests yet another mechanism involved in controlling the cell cycle. Muhammad *et al*[20] also noted that bitter melon extract treatment enhances phospho-adenosine monophosphate activated protein kinase expression in BRCA cells and suppresses the mammalian target of rapamycin (mTOR)/protein kinase B (Akt) signaling pathway. Additionally, they showed that bitter melon extract feeding effectively slowed the development of BRCA in syngeneic and xenograft animal models. Additionally, they noticed that tumors from animals fed bitter melon extract showed enhanced p62 accumulation, autophagy activation, and apoptotic cell death[20]. Shim *et al*[21] have demonstrated a therapy with bitter melon extract inhibiting the growth of triple-negative BRCA tumors in mouse models more effectively than treatment with estrogen receptor-positive breast tumors. However, the anti-cancer mechanism of bitter melon extract connecting lipid metabolism in BRCA growth is still unknown. They also showed that bitter melon extract feeding reduces acyl-CoA: Cholesterol acyltransferase-1 expression and slows tumor growth in non-obese diabetic scid gamma mice implanted with triple-negative BRCA mammospheres. Their presented study was the first study indicting bitter

melon extract's role in the inhibition of Acyl-CoA: Cholesterol acyltransferase-1 and the proliferation of triple-negative BRCA cells, suggesting it to be beneficial in combination with other therapeutic agents to treat human BRCA[21].

A recently developed analytical technique is network pharmacology. It performs "multitarget and multi-pathway" analysis of pharmaceuticals and diseases through the integration of various databases, and then creates a "drug active ingredient disease" model to visually depict the investigated action mechanism and precisely forecast the targets[22,23]. Even though a significant number of studies have demonstrated bitter melon's clinical effectiveness in the treatment of BRCA, the exact mechanism by which it works is still unknown[24]. This study aimed to identify potential bitter melon components, targets, and biological pathways against BRCA using network pharmacology. Furthermore, we validated and provided a theoretical foundation for the possible mechanism using molecular docking studies (Figure 1).

MATERIALS AND METHODS

Collection and screening of active constituents of bitter melon

Chemical constituents were collected from the Indian Medicinal Plants, Phytochemistry and Therapeutics (IMPPAT) 2.0 database (https://cb.imsc.res.in/imppat/)[25]. The bioactive obtained were analyzed and only the chemical constituents of the fruit part were taken by applying the filter. By typing the names of the constituents into the search box on PubChem (https://pubchem.ncbi.nlm.nih.gov/), the canonical smiles of the active constituents were acquired[26]. Moreover, the Molsoft database was used to obtain information regarding the molecular formula, molecular weight, number of hydrogen bond acceptors, number of hydrogen bond donors, MolLogP, and drug-likeness (DL) (https://www.molsoft.com/)[27] to get an idea of whether the compound is following the Lipinski rule. The bioactive violating more than two rules of Lipinski were eliminated. Additionally, the DL and oral bioavailability (OB) thresholds of 0.18 and 30%, respectively, were used to identify the potential active phytochemicals in bitter melon. These two crucial components greatly influence a substance's potential to have pharmacological effects, and from that the final bioactives were selected, and data were prepared and integrated.

Predicting the targets of the compounds

For identification of the potential targets of the bio-actives, the online platform SuperPred was used (https://prediction.charite.de/subpages/target_prediction.php). The targets of the compound were obtained after entering the canonical smiles[28]. The genes of strong binders and predicted targets were taken, and, on the latter, predicted targets with 75% probability were chosen[29]. The gene's names corresponding to the proteins were found in the string by putting all the protein's names, and obtaining the gene UniProt, or official symbol.

Screening for gene targets

The genes related to BRCA were sourced from GeneCards (http://www.genecards.org), using the term "BRCA" as the search query[30]. A Venn diagram was created using the online tool Venny (https://bioinfogp.cnb.csic.es/tools/venny/) to compare the targets of bitter melon and BRCA[31]. The overlap diagram represents the anti-BRCA targets of bitter melon.

Construction and analyses of the protein-protein interactions (PPI) network

The PPI network was constructed by uploading the anti-BRCA targets of bitter melon to the STRING platform (https://string-db.org) with "Homo sapiens" chosen in the organism box[32]. After selecting "Medium Confidence (0.400)" in the basic setting column and "Hide disconnected nodes in the network" in the advanced setting column, the tab-separated values (TSV) format file was exported. The TSV format file was imported into the Cytoscape software to produce the visual analysis of the PPI network[33].

Network construction of active phytoconstituents and anti-BRCA targets

The network of active ingredients in bitter melon and the corresponding targets was constructed and analyzed using the software Cytoscape 3.7.2[34,35]. The nodes in the network represented molecules or genes, which were distinguished by different shapes, and the line between one molecule and one gene meant they were related[36].

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis

The biological role of bitter melon's anti-BRCA targets was hypothesized using GO and KEGG pathway enrichment analysis. The GO analysis included molecular function, biological processes, and cellular components[37]. The anti-BRCA targets of bitter melon were uploaded to the DAVID platform (https://david.ncifcrf.gov/) to access the database of GO functions and KEGG pathways[38]. Applying a p value of 0.01 and organising the gene count in descending order allowed the top 10 data from the GO analysis to be sorted. The top 10 pathways were then displayed in the form of an enrichment dot bubble by uploading the data to the Bioinformatics Platform (http://www.bioinformatics.com.cn/)[39].

Verification by molecular docking stimulation

The network pharmacology identified possible bitter melon active components as well as the main BRCA-fighting targets and pathways. The bitter melon active compounds with the highest edge counts and which interact the most with targets were chosen as the ligands. Three bioactives were chosen as the ligands, and the Chem 3D program was used to create their 3D structures after searches in the PubChem database (https://pubchem.ncbi.nlm.nih.gov). After adding

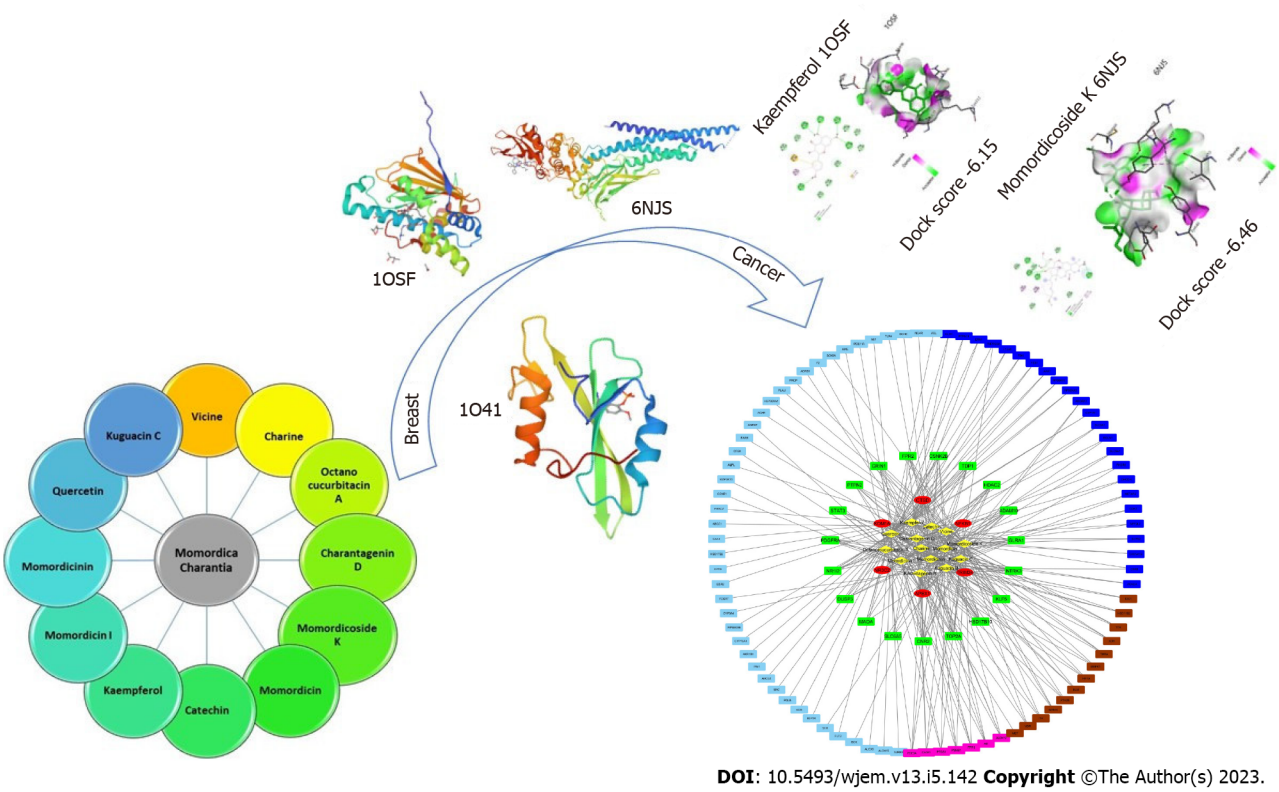

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Figure 1 Graphical abstract.

hydrogens, calculating Gasteiger charges, identifying the root, and selecting rotatable bonds with the help of AutoDock Tools, the ligands were stored in the format of PDBQT. The targets chosen as the protein receptors were those with the top three values in the PPI network, and the 3D structures were taken from the protein data bank (PDB) database (http://www.rcsb.org/)[40]. The specifications of the grid box created around the ligand are shown below.

Hydrogen atoms were added to the receptor structure after the water molecules had been removed, and charges were determined after assigning AD4-type atoms. Thus, prepared receptors were stored in the PDBQT format using the AutoDock tool. All residues remained rigid for the duration of the molecular docking process. The outcomes were shown, and the generation of the binding energy (docking score) was done[40].

RESULTS

Organizing the database of bitter melon

By using the keyword Momordica Charantia in the IMPPAT 2.0 database and reviewing the literature, 304 active ingredients were obtained. By applying a filter from the aerial part of the plant to the fruit part, 43 chemical constituents were obtained, and their canonical smiles were taken to the Molsoft database to demonstrate Lipinski properties and DL. After that, the phytoconstituents that contain DL greater than 0.18, oral OB thresholds of more than 30%, and which do not violate more than 2 Lipinski rules were chosen. So, at last, 14 phytoconstituents' remains were further analyzed for results.

Active phytochemicals targets

The SuperPred database was used to identify possible protein targets for the bitter melon's active phytochemicals. Targeted genes for corresponding compounds were determined by taking strong binders, and 75% filtered probability predicted targets were taken. After redundant information was removed, 133 putative protein targets of bitter melon's active phytochemicals were further examined.

Establishing a database of BRCA

In the GeneCards database, "BRCA" was used as the keyword, and around 15970 genes were obtained. So, to reduce the no. of genes, GeneCards Inferred Functionality Scores filter of > 50 was applied, which resulted in BRCA genes to about 3601.

Acquisition of the venn diagram and construction of the component-target network

A Venn diagram was produced by submitting the bitter melon and BRCA targets to the Venny platform, respectively.

The results conclusively showed that 112 instances of 133 genes connected to bitter melon were likewise linked to BRCA (Figure 2).

Construction and analyses of the PPI network

By inputting 112 anti-BRCA targets into the STRING database and using the Cytoscape program to show them, the PPI network of possible targets was created. There were 428 edges and 112 nodes in the PPI network. The size of map nodes increases as the node degrees rise, and their color shifts from orange to blue. The map edge size increased and the color changed from orange to blue as the total score rose (Figure 3A). On a bar graph, the top 10 genes were displayed in order of their degree (Figure 3B). Each bar was plotted using the degree value associated with each gene. The top 3 potential anti-BRCA targets, *i.e.*, heat shock protein 90 (HSP90) AA1, proto-oncogene tyrosine-protein kinase (SRC), and signal transducer and activator of transcription 3 (STAT3), were chosen for molecular docking studies, with key active phytochemicals described in molecular docking section under result.

Network of active phytoconstituents and anti-BRCA targets

Using the network analysis program Cytoscape, the active ingredient and BRCA target network was created (Figure 4). There are 369 edges and 126 nodes in the network. The 112 circular nodes surrounding the 14 active components of bitter melon, which were represented by the network's yellow diamond nodes in the centre, were anticancer targets. Red was used to indicate 6 target nodes linked to more than 10 active compounds, green for 19 target nodes linked to in the range of 5 to 10 corresponding chemicals, pink for 7 target nodes linked to 4 compounds, brown for 13 target nodes linked to 3 active molecules, dark blue for 24 target nodes linked to 2 compounds, and blue for 43 target nodes linked to only 1 active compound. Amongst all, three phytoconstituents, quercetin, kaempferol, and momordicoside K are considered the most important and key active phytochemicals found in bitter melon for BRCA treatment. The findings show that the majority of bitter melon's active compounds had several targets, and many of these targets related to different active ingredients, demonstrating that bitter melon is a multicomponent, multitarget medication. These outcomes highlight the complex nature of the interactions between many targets and active phytochemicals in bitter melon.

Enrichment analysis

To further analyze the BRCA targets of bitter melon, GO enrichment was performed on 112 intersecting targets using the web-based DAVID program. The top 10 representative enrichment terms for cellular components, molecular processes, and biological processes, sorted by count in descending order and P value < 0.01, are displayed in Figure 5. Orange bars represent a biological process that mainly consisted of G-protein-coupled receptor activity (10/45) and protein kinase binding (9/45). Bars with the green color represented the cellular component, which mainly included the nucleus (42/45), membrane (28/45), and extracellular region (20/45); and lastly, blue colored bars signify the molecular function. Of all 45 potential genes, 9 genes were involved in negative regulation of transcription, DNA template, and cell differentiation.

KEGG pathway enrichment analysis

Using the DAVID web-based tool, KEGG pathway analysis was carried out on 112 overlapping targets to clarify the molecular mechanism by which bitter melon treats BRCA. By uploading 112 anti-BRCA targets to the DAVID platform, around 90 KEGG pathways were obtained, which were further filtered and sorted by P value < 0.01 and gene count in descending order. The top 20 representative enrichment terms were plotted by an enrichment dot bubble in Figure 6. Phosphatidylinositol 3-kinase/protein kinase B (PIK3-Akt) signaling, transcriptional misregulation, axon guidance, calcium signaling, focal adhesion, janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling, cyclic adenosine monophosphate (cAMP) signaling, mTOR signaling, and phospholipase D signaling pathways may play important roles in the mechanism of bitter melon against BRCA. These signaling pathways might work in concert to provide the therapeutic benefits of bitter melon against BRCA.

Molecular docking

HSP90AA, SRC, and STAT3 were employed as the receptors because they have the top three greatest values in the PPI network and the highest level of resolution, while quercetin, kaempferol, and momordicoside k were chosen as the ligands since they each have more than ten gene targets. The PubChem, PDB, Open Babel, and AutoDock tools were used to prepare the ligands and receptors. The three compounds were docked through AutoDock to three different targets to validate the prediction. The result has been displayed as an affinity value (Table 1). Whereas 1O41 had the weakest capacity to attach to the ligands of the three, 1OSF had the easiest time doing so. At a value of -4.47 kcal/mol, quercetin's affinity for 1O41 was the lowest. Figures 7A and B display the docking interactions of kaempferol with 1OSF and 6NJS, respectively, while Figures 8A and B display the docking interactions o momordicoside K with 1OSF and 6NJS, respectively.

Kaempferol showed many interactions with 1OSF. The hydroxyl group present on the chromen-4-one ring showed hydrogen bonding with Asn106, Lys112, Gly135, and Phe138, while the hydroxyl group present on the phenyl ring showed hydrogen bonding with Asp93. Phenyl ring is also involved in pi-sulfur and pi-alkyl interactions with Met98 and Ala55, respectively. Similarly, the hydroxyl group present on chromen-4-one ring showed hydrogen bonding with Glu638, and Lys658 on 6NJS. The hydroxyl group attached to the phenyl ring showed interactions with Pro639, and Gln644 through hydrogen bonding. Pi-alkyl interactions are observed between chromen-4-one ring and Val637.

Momordicoside K also showed many interactions with 1OSF. Three hydroxyl groups present on the oxane ring form hydrogen bonds with Asn106, Lys112, Gly135, Val136, and Phe138. While the sidechain oxygen atom is involved in hydrogen bond interaction with Gly135. An alkyl interaction is observed between the phenanthrene ring and the alkyl

Table 1 Affinities of compound to the targets (dock score)

Compound	HSP90AA (1OSF)	SRC (1O41)	STAT3 (6NJS)
Quercetin	-5.57	-4.47	-5.61
Kaempferol	-6.15	-4.74	-5.78
Momordicoside K	-6.07	-5.39	-6.46

HSP90: Heat shock protein 90; SRC: Proto-oncogene tyrosine-protein kinase; STAT3: Signal transducer and activator of transcription 3.

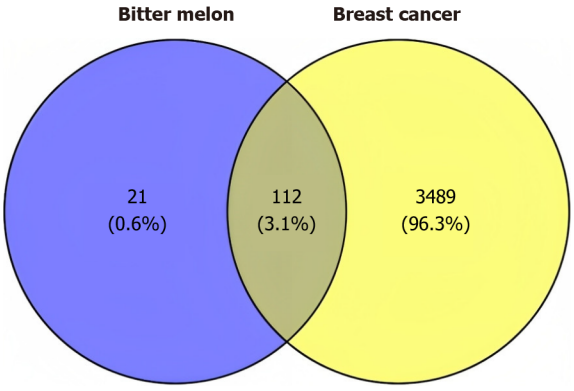

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Figure 2 Venn diagram of targets of bitter melon and breast cancer where blue color indicates targets of bitter melon while yellow color indicates targets of breast cancer.

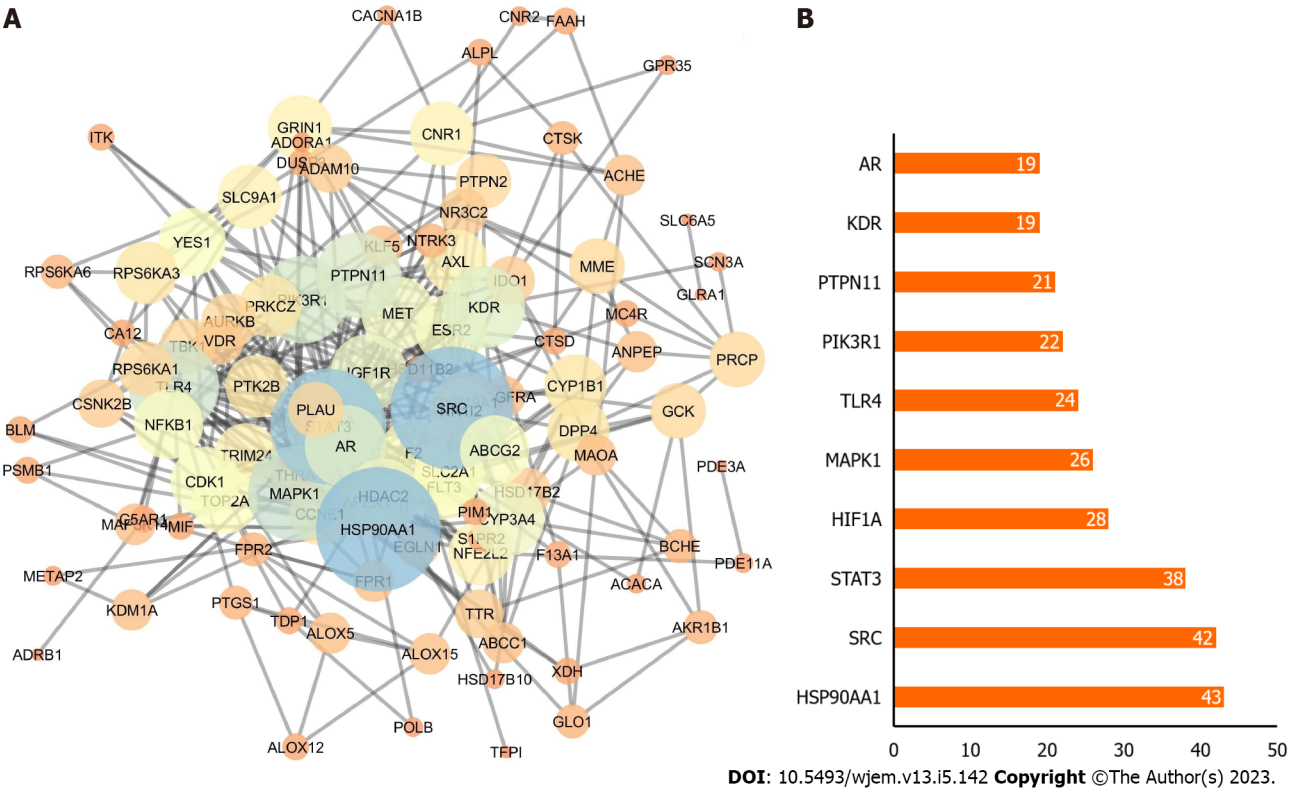

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Figure 3 Potential targets. A: Protein-protein interaction network; B: Bar chart of the top 10 genes according to degree where X-axis is the degree value, and the Y-axis is the gene name.

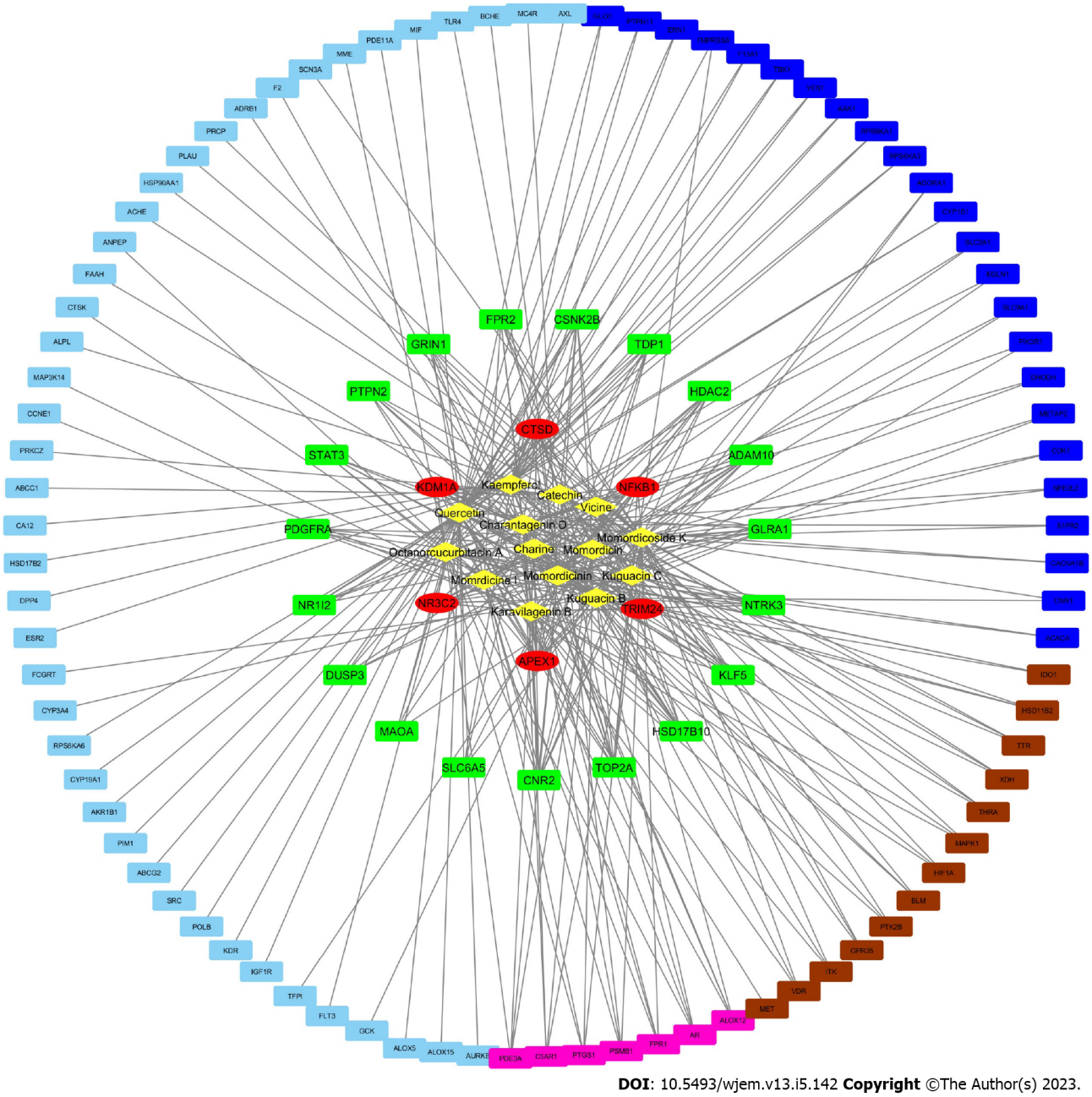

Figure 4 Active ingredient-anti-breast cancer target network of bitter melon.

substituent present on the phenanthrene ring with Met98. As with 1OSF, momordicoside K showed interactions with 6NJS. A hydrogen bond interaction is observed between the phenanthrene ring containing the hydroxyl group and Glu638. Three hydroxyl groups present on the oxane ring form hydrogen bonds with Tyr657, Met660, and Lys658. Also, Van der Waal's interaction and pi-alkyl interaction are observed between the sidechain oxygen atom and methyl substituent, respectively, with Tyr657. Similarly, pi-alkyl interactions between the sidechain and the methyl substituent are present on the sidechain with Ile653 and Tyr640, respectively.

DISCUSSION

In Ayurveda medicine, bitter melon has numerous elements that have therapeutic effects against a variety of ailments. All parts of the plant, including the fruit, are frequently eaten and prepared in many ways, such as stir-frying, stuffing, or using a small amount to add a mildly bitter flavor to soups or beans. Antioxidant, anti-inflammatory, cancer-fighting, anti-diabetic, anti-bacterial, anti-obesity, and immunomodulatory properties are said to be present in the plant. A fascinating discovery showed that bitter melon extract appeared to be beneficial in halting the growth and spread of cancer tumors in studies employing mice models[41]. By causing apoptosis, cell cycle arrest, autophagy, and blocking cancer stem cells, the plant extract reduces the proliferation of cancer cells. The anticancer effects of bitter melon and its

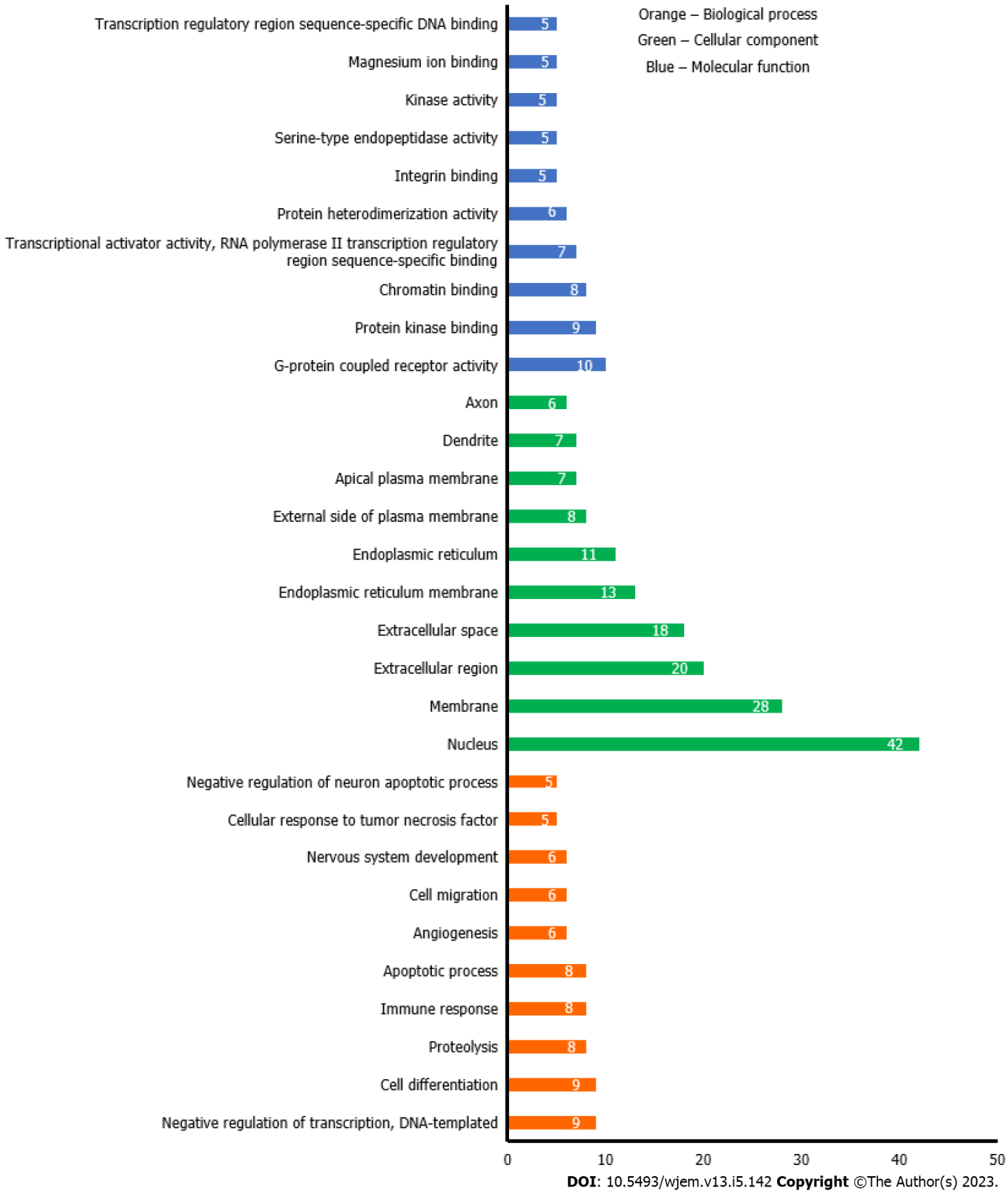

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Figure 5 Gene ontology analysis of anti-breast cancer targets of bitter melon where Y-axis is the number of the targets involved in the gene ontology analysis, and the X-axis is the molecular function, biological process, or cellular component.

active components have been studied; however, the underlying processes have not been comprehensively uncovered. In this study, network pharmacology-an efficient and cost-effective method for conducting systematic therapeutic research-was used to examine the mechanism by which bitter melon treats cancer.

The 133 relevant targets were found by gathering and screening the 14 active components from bitter melon that were screened out from the IMPPAT 2.0 database. The Venn diagram revealed that 112 of the 133 targets connected to bitter melon were also connected to targets for BRCA, indicating that the majority of the pertinent targets in bitter melon were connected to effects that were anti-BRCA. The interactions between different bitter melon targets were visible in the PPI network. The three targets with the top three greatest degree values, HSP90AA1 (43), SRC (42), and STAT3 (38) may be the primary anti-BRCA targets. To further confirm the potential interaction between the important targets and the active compounds, molecular docking was done.

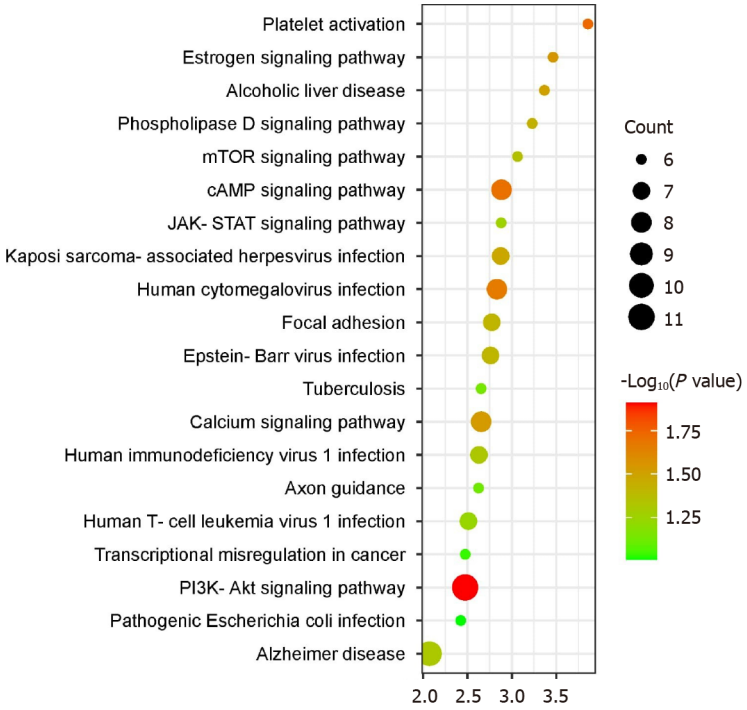

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Figure 6 Kyoto encyclopedia of genes and genomes enrichment analysis (top 20) where X-axis is the enrichment gene count, and the Y-axis is the kyoto encyclopedia of genes and genomes pathway. Bubble size represents the number of genes involved in the kyoto encyclopedia of genes and genomes enrichment while color represents -log 10 (*P* value). PIK3-Akt: Phosphatidylinositol 3-kinase/protein kinase B; JAK-STAT: Janus kinase-signal transducer and activator of transcription; mTOR: Mammalian target of rapamycin.

One of the most prevalent proteins in mammalian cells, HSP90, together with the oestrogen receptor, progesterone receptor, key HER2 signaling molecules (HER2, AKT, c-SRC, RAF, and HIF-1), and epidermal growth factor receptor, are crucial for the growth and survival of cancer. Moreover, HSP90 has an N-domain ATP binding site, and all its cellular functions depend on its ATPase activity[11]. HSP90 activation may sustain the malignant phenotype, enhance metastasis, and foster treatment resistance in a variety of BRCA subtypes because it functions as a key integrator of numerous pathways. According to research findings, treating BRCA with up-regulated HSP90 may lessen the chance of fatal recurrence and distant metastases. The transcriptional program that are triggered by various cytokines, growth factors, and carcinogenic stimuli are mediated by the STAT3 protein, which is a signal transducer and transcription activator. Its expression and activity are frequently associated with cellular transformation, tumor development, and tumor initiation. The interleukin 6 (IL-6)/JAK/STAT3 signaling pathway is an actionable target with preclinical and clinical studies demonstrating therapeutic potential in both primary and metastatic BRCA, according to recent findings elucidating the role of IL-6 in BRCA progression, metastasis, and anti-cancer immunity. Importantly, direct targeting of IL-6, IL-6R, gp130 receptor, JAKs, or STAT3 has been examined as a means of blocking the IL-6/JAK/STAT3 signaling axis. Numerous cancers have dysregulated SRC, a non-receptor tyrosine kinase. SRC plays a significant role in a variety of features of tumor formation, including growth, survival, adhesion, migration, invasion, and, most significantly, metastasis in a variety of tumor forms. SRC boosts proliferation, diminishes apoptosis, and promotes metastasis.

The molecular docking results showed that the binding energies of kaempferol to STAT3 and HSP90AA are the highest. Furthermore, momordicoside K and quercetin have good STAT3 and HSP90AA binding properties. The most significant anti-BRCA targets of bitter melon may be STAT3 and HSP90AA. SRC, a member of the family of nonreceptor protein tyrosine kinases, is important for many signaling pathways that are involved in cell growth, survival, motility, and angiogenesis. Compared to STAT3 and HAP90AA, SRC had poor binding activity to kaempferol, momordicoside K, and quercetin, which suggested that SRC may not be the direct target of bitter melon in BRCA treatment.

The findings of the KEGG pathway study identified bitter melon's anti-BRCA pathways. Ten out of the top 20 pathways were directly linked to cancer. These pathways, which include PIK3-Akt signaling, transcriptional dysregulation, axon guidance, calcium signaling, focal adhesion, JAK-STAT signaling, cAMP signaling, mTOR signaling, and phospholipase D signaling, may be crucial in the bitter melon's defense against BRCA. According to the findings, bitter melon's targets are crucial in the management of BRCA.

In the PIK3-Akt signaling pathway, which is involved in the control of numerous cellular physiological processes by activating downstream matching effector molecules and is critical for the cell cycle, growth, and proliferation, bitter melon has 11 anti-BRCA targets. Eight anti-BRCA targets in bitter melon are connected to the calcium and cAMP signaling pathways. By the activation of cAMP-dependent protein kinase, the cAMP signaling pathway controls a wide range of intracellular events that are connected to the regulation of cellular proliferation, differentiation, and apoptosis. BRCA development and chemotherapy resistance are both influenced by essential processes regulated by Ca^{2+} signaling pathways, ranging from inflammation to apoptosis. There are seven anti-BRCA targets in the focal adhesion pathway.

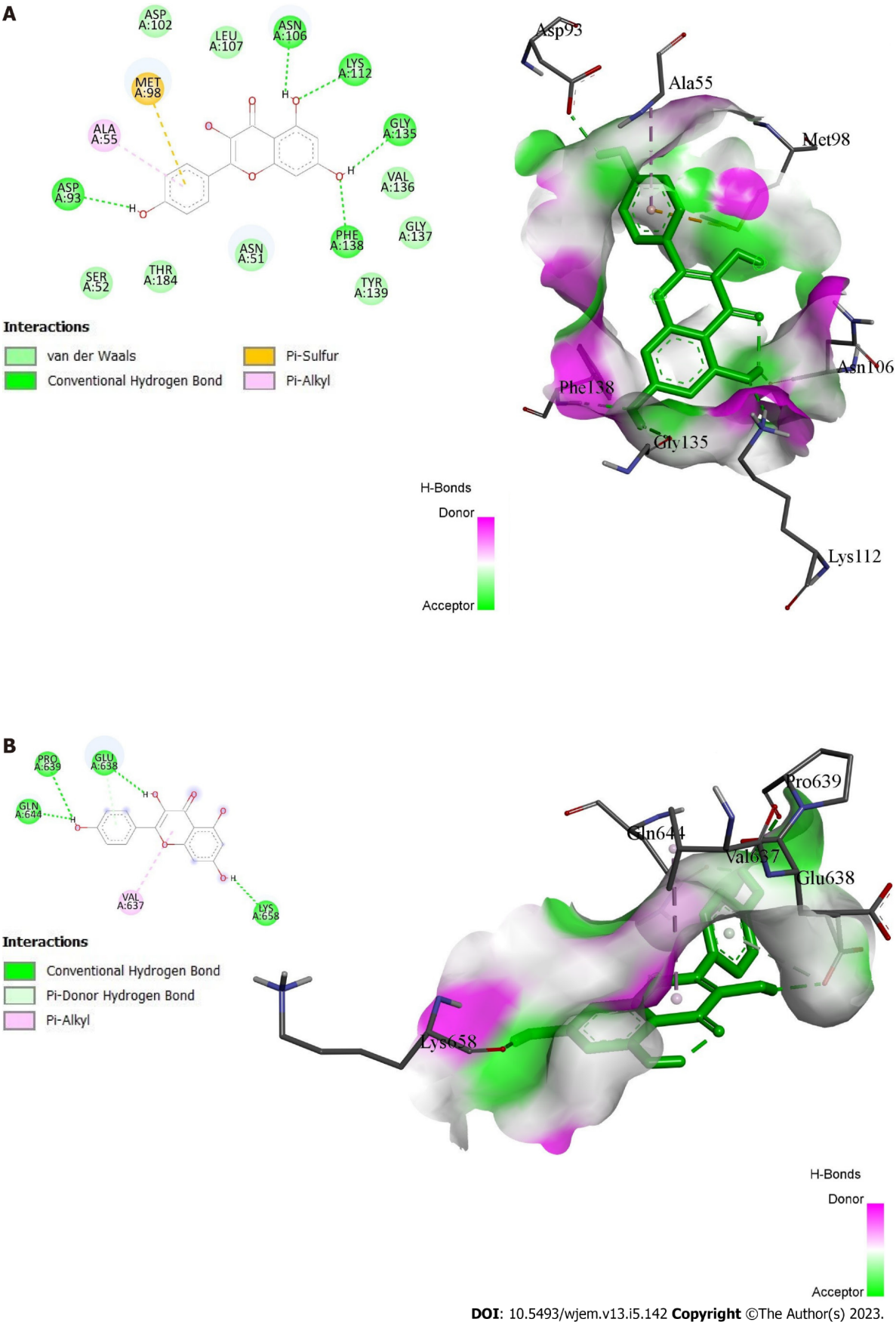

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Figure 7 Docking interactions of kaempferol. A: With protein 1OSF; B: With protein 6NJS.

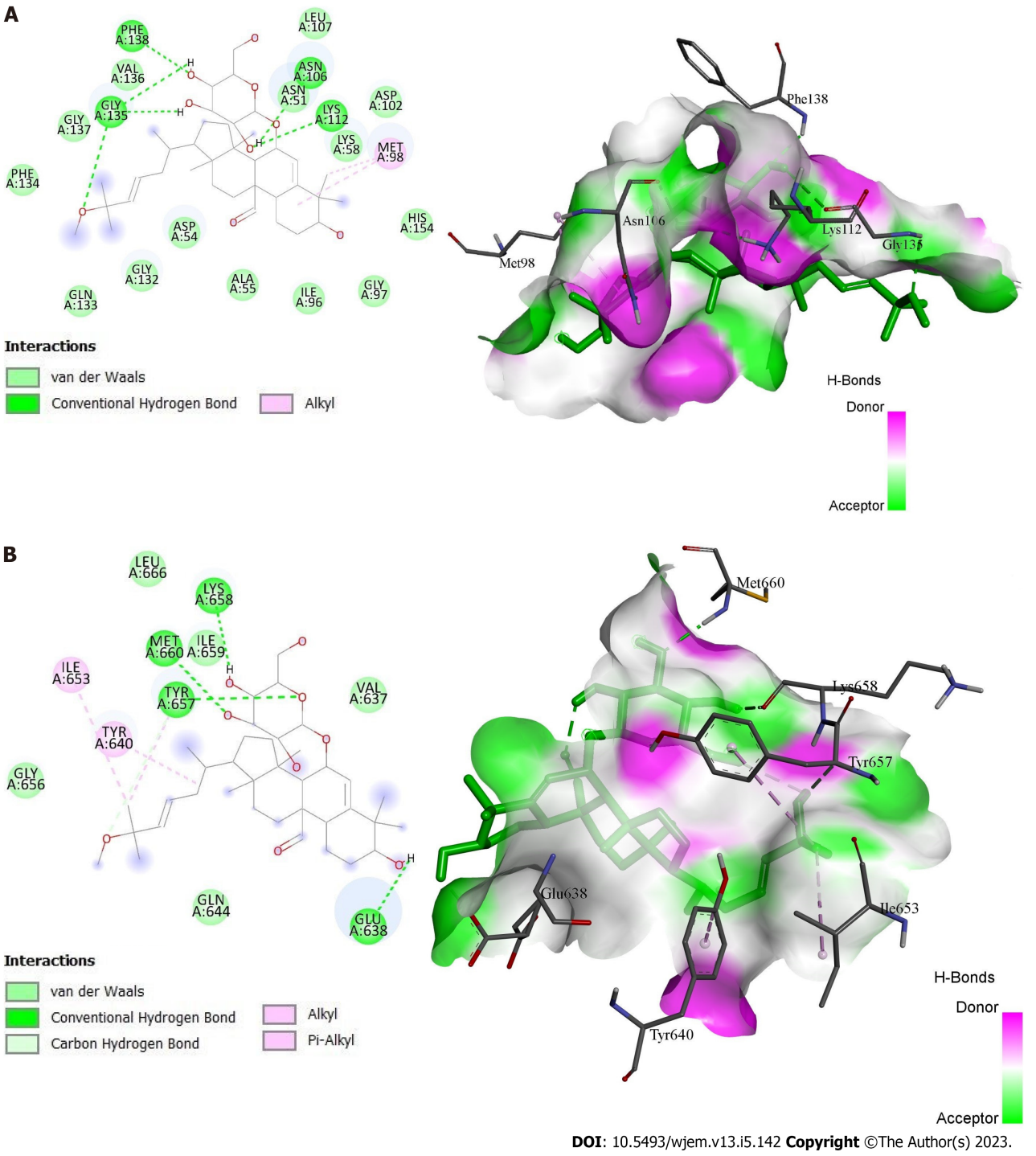

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Figure 8 Docking interactions of momordicoside K. A: With protein 1OSF; B: With protein 6NJS.

bitter melon has six anti-BRCA targets that are involved in the mTOR, JAK-STAT, and phospholipase D signaling pathways. A range of biological processes, including cellular proliferation, survival, metabolism, autophagy, and immunity, are aided by the mTOR signaling pathway. While the phospholipase D signaling system has been linked to survival signaling and the control of cell cycle progression, the JAK-STAT pathway plays critical roles in the carcinogenesis, maintenance, and metastasis of BRCA. As a result, bitter melon has significant therapeutic potential in the treatment of BRCA and functions in several ways.

CONCLUSION

We have successfully investigated the main phytochemicals and molecular processes in bitter melon that are thought to be involved in the therapy of BRCA. This study found 14 major active phytoconstituents as well as 112 anti-BRCA targets in bitter melon. Our research demonstrated that the anti-BRCA benefits of bitter melon are likely caused by negative

regulation of transcription, cell differentiation, apoptosis, proteolysis, negative control of neuron apoptosis, and cell migration. Further, we discovered that nine important pathways, including PIK3-Akt signaling, transcriptional dysregulation, axon guidance, calcium signaling, focal adhesion, JAK-STAT signaling, cAMP signaling, mTOR signaling, and phospholipase D signaling, are likely to be involved in the mechanism of action of bitter melon in the treatment of BRCA. Our results support the hypothesis that bitter melon's potential anti-BRCA properties may be a result of the direct or indirect synergistic effects of multitarget and multi-pathway activities. According to molecular docking research, the principal active phytochemicals in bitter melon may be able to bind to HAP90AA1, STAT3, and other BRCA-related targets. The results of this study offer suggestions for additional investigation into the phytochemicals and mechanisms of bitter melon's anti-BRCA activity. This serves as a foundation for creating cutting-edge anti-BRCA medications based on phytochemicals found in bitter melon but experimental validation is necessary to confirm the predicted therapeutic effects of bitter melon on BRCA. Without experimental evidence, the clinical relevance of these findings remains uncertain.

ARTICLE HIGHLIGHTS

Research background

Bitter melon has been used to stop the growth of breast cancer (BRCA) cells. However, the underlying mechanism is still unclear.

Research motivation

The motivation of this research is to explore the underlying pharmacological mechanisms.

Research objectives

The goal of this study was to predict the therapeutic effect of bitter melon against BRCA using network pharmacology.

Research methods

The active ingredients of bitter melon and the related protein targets were taken from the Indian Medicinal Plants, Phytochemistry and Therapeutics and SuperPred databases, respectively. The GeneCards database has been searched for BRCA-related targets. Through an intersection of the drug's targets and the disease's objectives, prospective bitter melon anti-BRCA targets were discovered. Gene ontology and kyoto encyclopedia of genes and genomes enrichment analyses were carried out to comprehend the biological roles of the target proteins. The binding relationship between bitter melon's active ingredients and the suggested target proteins was verified using molecular docking techniques.

Research results

Through the active ingredient-anti-BRCA target network analysis, three major components were found to be important in mediating the putative anti-BRCA actions of bitter melon: momordicoside K, kaempferol, and quercetin. In the protein-protein interaction network analysis, the top three proteins were determined to be heat shock protein 90 AA, proto-oncogene tyrosine-protein kinase, and signal transducer and activator of transcription 3 (STAT3). According to molecular docking research, the principal active phytochemicals in bitter melon are able to bind to HAP90AA1, STAT3, and other breast cancer-related targets.

Research conclusions

Overall, the integration of network pharmacology, molecular docking, and functional enrichment analyses shed light on potential mechanisms underlying bitter melon's ability to fight BRCA, implicating active ingredients and protein targets, as well as highlighting the major signaling pathways that may be altered by this natural product for therapeutic benefit.

Research perspectives

Database resources were used to examine active compounds found in bitter melon. Network pharmacology and molecular docking techniques were used to explore the mechanism through which bitter melon was used to treat BRCA. Momordicoside K, kaempferol, and quercetin were identified as potentially important active ingredients of bitter melon showing anti-BRCA actions. The study identified several possible molecular targets as well as signaling pathways involved in bitter melon's anti-BRCA actions, like phosphatidylinositol 3-kinase/protein kinase B signaling and janus kinase-signal transducer and activator of transcription signaling. The study suggests further experimental verification to confirm the potential findings of bitter melon in the treatment of BRCA.

FOOTNOTES

Author contributions: Panchal K designed, performed and wrote the paper; Nihalani B designed, performed and wrote the paper; Oza U edited the paper; Panchal A edited the paper; Shah B designed, supervised and edited the paper.

Conflict-of-interest statement: The authors declare no conflicts of interest in this paper.

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LETTER TO THE EDITOR

Research on nanosciences involvement in pharmaceutical education should be reinforced

Zheng-Wei Huang, Ye-Qi Huang

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Abstract

Inclusion of nanoscience in pharmaceutical education should be reinforced, in order to match the demand of current pharmaceutical talent cultivation.

Key Words: Nanosciences; Pharmaceutical education; Talent cultivation; Reformation

Core Tip: Nanosciences have currently boosted the development of various disciplines, including pharmaceutical sciences. Theoretically, nanosciences should be involved in pharmaceutical education, in order to cultivate pharmaceutical talents familiar with nanosciences. However, the current courses on nanoscience are insufficient in pharmaceutical curricula, and only a few studies on this topic are documented in databases. It seems that this field is rarely exploited. We therefore urge investigators and educators to perform more studies, and include nanosciences in pharmaceutical education.

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TO THE EDITOR

Since the 1990s, nanoscience has gained rapid momentum in research and development. Although nanotoxicology is a rising concern worldwide[1], and the ethical issues (so-called "nanoethics")[2] and the ecological risks[3] of nanotechnology have been identified, the application of nanomaterials and nanotechnologies is still a focus.

Consequently, various nanomaterials and nanotechnologies have been developed and introduced into different fields, including the chemical, biological, environmental, medical, and pharmaceutical sciences. In the chemistry field, it is possible to apply nanotechnologies to various chemical compounds, including polymers, to modify their structure and function. In a recent study, metal-organic framework based nano-adsorbents have made a number of noteworthy advances in anti-chemical warfare reagents[4]. In the biology field, one of the most acclaimed achievements in nanotechnology in molecular biology is identification of the vaccination mechanism for coronavirus disease 2019 (COVID-19) using nanoscale vector systems[5]. In the environment field, nanotechnology is utilized not only to enhance the environment but also to produce renewable sources of energy. A paradigmatic example is the employment of nanofluids in solar cells which can produce electricity at a competitive cost[6]. In the medical field, the successful application of nanomedicine has helped to develop enhanced versions of diagnostics, treatment, prevention, and proactive healthcare measures[7]. Recent research has shown that the controllability of nanorobots has advanced, allowing for efficient remodeling of dense tumor stromal microenvironments to enable deep tumor penetration[8].

As pharmaceutical researchers, the authors of this paper consider that nanoscience is deeply involved in the field of pharmaceutical sciences. The following are several key examples: (1) Pharmaceutical chemistry: One-component new chemical entity nanomedicines are synthesized to enhance therapeutic efficacy[9]; (2) Pharmacology: Nanovesicles can be used as analytical tools to investigate cellular signaling pathways[10]; (3) Pharmaceutics: Nanoparticles are effective carriers for drug loading and delivery[11]; and (4) Pharmaceutical analysis: Nanotechnologies can facilitate the separation, identification, and quantification of drug molecules[12]. This information is schematically illustrated in Figure 1.

As nanoscience has become an ever more integral part in pharmaceutical sciences, individuals with pharmaceutical interests (*i.e.*, pharmaceutical scientists, pharmacists, staff in the pharmaceutical industry, and governors in drug administration) should master the relevant knowledge and skills regarding nanoscience. Pharmaceutical education is the fundamental and vital approach for cultivating pharmaceutical talent; hence, pharmaceutical curricula should involve imparting knowledge and skills on nanoscience. However, for several pharmaceutical education systems worldwide, the core courses in pharmaceutical curricula remain focused on the subdisciplines of pharmaceutical sciences (such as pharmaceutical chemistry, pharmacology, pharmaceutics, and pharmaceutical analysis[13] and fundamental chemistry (such as inorganic, organic, and physical chemistry and biochemistry[14], and courses focused on nanoscience are insufficient or even absent. The reasons for this may include the following: Nanotechnology is a cutting-edge research field; its novelty may present some challenges to the faculty. Even in a nanoscience or nanotechnology training program, some teachers show a preference for teaching more familiar courses so that they can apply the knowledge into the classroom in a timely manner[15]. It is documented that low self-confidence, associated with a lack of knowledge on the new content, sometimes hinders the acceptance and the willingness to use it in the classroom[16]. Another aspect is that the study of basic theoretical subjects is still generally considered necessary in pharmacy education. Some people therefore reject the educational importance of nanoscience, believing that there is no room for a new science curriculum like that[17].

Moreover, not only are current courses lacking in coverage of nanoscience, retrospective/progressive/prospective studies on the inclusion of nanoscience in pharmaceutical education are also in their infancy. The authors consulted six databases associated with Web of Science (Web of Science Core Collection, Chinese Science Citation Database, KCI-Korean Journal Database, Medline, ProQuest Dissertations & Theses Citation Index, and SciELO Citation Index) using various search sets (Table 1) on July 13, 2023. Documents published between 1965 and 2023 were searched, and all document types and languages were included. Duplicate and irrelevant publications were manually excluded. However, according to the results of the literature survey, only eight documents were retrieved, which was surprisingly low. For comparison, the number of documents on pharmaceutical education and nanoscience were 50537 and 2985487, respectively. These results are shown in a Venn diagram in Figure 2. From the authors' perspective, characterized by interdisciplinarity, nanoscience is a deeply interconnected discipline, encompassing diverse areas of modern science and technology. When taking nanoscience into the classroom, it has been confronted with the dilemma of whether it should be taught as a new or a subsidiary discipline. Accordingly, the intricate nature of nanoscience may be the possible reason that has resulted in a scarcity of research exploring its intersection with a specialized field such as pharmacy education.

Overall, the current scenario is that insufficient attention has been paid to the inclusion of nanoscience into pharmaceutical education. Consequently, the cultivation of pharmaceutical talents mastering the required knowledge and skills of nanoscience cannot be guaranteed. With the continuous development of nanoscience, its inclusion in pharmaceutical science will become more comprehensive in the future. It is worth noting that the market for nanomedicines for disease management has great potential. The global market for nanomedicine was estimated at $53 billion in 2009. It is expected to grow by 13.5% to reach $100 billion in recent years[18]. These data demonstrate global interest in the nanoscience field. Therapeutic formulations utilizing nanotechnology hold potential for improving clinical outcome. Engineered nanomaterials are rapidly evolving in drug development, and offer promise in overcoming biological barriers and achieving precise drug delivery for precision medicine[19]. In addition, the potential of nanotechnology in pharmacy will be further expanded with efforts to combine nanomaterials with some established formulations. Recent studies have pointed out that the combined properties of hydrogels and nanoparticles in smart nanogels can improve drug loading capacity, drug stability, target delivery and therapeutic efficacy[20]. Considering these factors, it is foreseeable that nanotechnology will become more widely and tightly integrated into pharmaceuticals in the future. Thus, correspondingly, continued efforts are required to promote the inclusion of nanoscience in pharmaceutical education.

To achieve this aim, the authors propose three suggestions:

Firstly, colleges and universities should establish scientific foundations for the educational reformation of nanoscience courses; the role of foundations in support of the education process is essential. A positive example to be studied is the National Science Foundation (NSF). The NSF is charged with funding basic research programs to maximize the

Table 1 Search sets for literature retrieval

Step	Search set	Interpretation
#1	TS = pharmacy educat[1], TS = pharmaceutical educat[1], TS = pharmacy train[1], TS = pharmaceutical train[1], TS = pharmacy curricul[1], TS = pharmaceutical curricul[1]	Pharmaceutical education as the topic
#2	TS = nano[1]	Nano-related terms as the topic
#3	#1 AND #2	Intersection of #1 and #2

[1]Is an infinite truncator, which allows the term to be infinitely expanded.

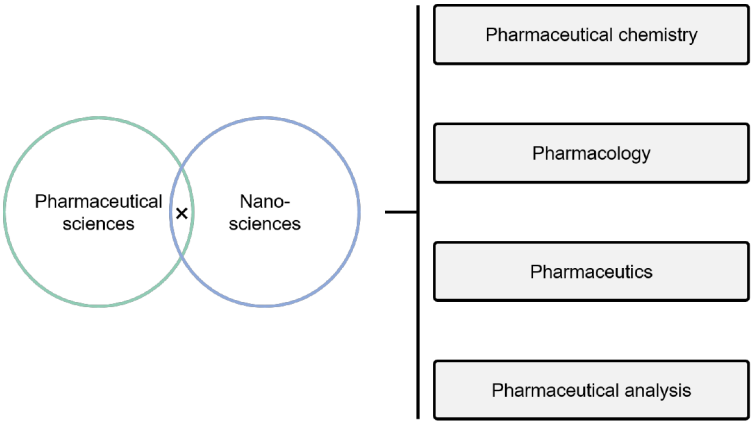

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Figure 1 Main applications of nanoscience and nanotechnology in pharmaceutical sciences.

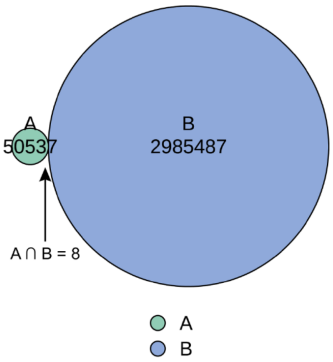

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Figure 2 Euler Venn diagram showing the intersection of the publications on pharmaceutical education (A) and nanoscience (B).

advancement of science in the United States through the development of scientific information. In the National Nanotechnology Initiative, NSF assumed responsibility for funding basic research and education in nanoscience and nanotechnology, leading to a healthy growth of nanotechnology in the United States[21]. Referring to the operation and supporting model of NSF, sufficient attention from colleges and universities should be given to the investment of adequate funding to meet the development of nanoscience in pharmacy education.

Secondly, faculties must initiate changes to their curricular systems and add nanoscience courses. In the post-pandemic era, the rate of change in healthcare has rapidly accelerated. Consequently, healthcare professionals must dedicate themselves to lifelong learning through continuing education and professional development programs, including those associated with nanoscience[22]. For instance, teachers should learn about the applications of nanotechnology in COVID-19 treatment, and pass this knowledge to the students in the classroom. Through these efforts, students can gain scientific and technological literacy, which has a significant impact on curriculum design[15]. It can also serve as an effective means of bridging the gap between workforce needs and cutting-edge fields[23].

Lastly, journals, such as the *World Journal of Experimental Medicine*, should encourage the submission of relevant studies as a publishing platform. Currently, the evaluation of papers by impact factor is still the dominant approach used by journals. However, this single-factor approach has led to much discussion about its update or revolution[24], and the

actual implication of papers for the real world should be reconsidered. As a publishing platform, considering and encouraging papers based on multiple factors may be a positive guide for the conduct of research focusing on a rare field (the very scenario of nanoscience education in pharmacy). The authors envision that the degree of inclusion of nanoscience in pharmaceutical education can be increased in the near future.

In summary, nanoscience is rapidly evolving in a number of disciplines and fields. It has been widely used in the fields of chemistry, biology, environment, medicine, and pharmacy, and has attracted much attention. Especially in the field of pharmacy, nanoscience and nanotechnology have played a significant role. However, at present, the coverage of nanoscience in pharmaceutical courses and educational studies is lacking, which is detrimental to the cultivation of talents in this field. The gaps in this area should be further addressed by all groups. We propose three suggestions to boost the inclusion of nanoscience in pharmacy education: (1) Colleges and universities should establish scientific foundations for the educational reformation of nanoscience courses; (2) faculties must initiate changes to their curricular systems and add nanoscience courses; and (3) journals should encourage the submission of relevant studies as a publishing platform. Similar to the situation in pharmacy education, clinical medicine training should also include more courses on nanotechnology, and we will be conducting in-depth research on this topic in the future.

FOOTNOTES

Author contributions: Huang Z designed the study and wrote the letter; Huang Y polished the language and prepared the artwork.

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