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Original Articles

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Efficacy of virtual reality for pain management during central venous access device procedures in pediatric oncology: A systematic review and meta-analysis of randomized controlled trials

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MAIN POINTS

- Central venous access devices (CVADs) are commonly used in paediatric oncology.
- Virtual reality (VR) is a technology-based distraction intervention that has been increasingly used to support symptom management in children.
- This systematic review and meta-analysis evaluated randomised controlled trials investigating the effects of VR during CVAD access in paediatric oncology patients.
- VR interventions were associated with reduced pain, fear, and anxiety during CVAD access compared with control conditions.
- Findings suggest that VR may be a useful non-pharmacological strategy to support nurses and other healthcare professionals in improving procedural experiences for children with cancer.

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ABSTRACT

Aim: Procedures involving central venous access devices (CVADs) are painful and distressing for pediatric oncology patients. This systematic review and meta-analysis evaluated the efficacy of virtual reality (VR) interventions in reducing pain, fear, and anxiety during CVAD puncture among pediatric oncology patients.

Materials and Methods: PubMed, EBSCO, Web of Science, and the Cochrane Library were systematically searched from inception to September 2025. Randomized controlled trials (RCTs) evaluating the use of VR for pain management during CVAD procedures in children and adolescents under 18 years of age were included. Two independent reviewers performed study selection and data extraction, and assessed risk of bias using RoB 2.0. Effect sizes were pooled as standardized mean differences using a DerSimonian–Laird random-effects model. Heterogeneity was quantified using the τ^2 and I^2 statistics.

Results: Five RCTs were included (participants aged 6-18 years). VR reduced child-reported pain (SMD=-1.11; 95% CI: -1.82 to -0.40; I^2 =82.4%). Parent-reported pain likewise favored VR (SMD = -1.18; 95% CI: -1.89 to -0.47; I^2 =82.0%). VR reduced child-reported fear (SMD = -1.29; 95% CI: -2.33 to -0.25; I^2 = 87.2%) and parent-reported fear (SMD = -1.46; 95% CI: -2.00 to -0.91; I^2 = 36.1%). VR also lowered child-reported anxiety/distress (SMD =-1.18; 95% CI: -1.73 to -0.62; I^2 =61.4%). Parent-reported anxiety showed a statistically significant reduction (SMD = -1.12; 95% CI: -2.08 to -0.16; I^2 =85%). Risk of bias was generally low for randomization and outcome completeness, whereas some concerns were identified in the risk of bias assessment, mainly related to the lack of blinding.

Conclusion: VR interventions during CVADS access appear to be effective in reducing pain and fear based on both child- and parent-reported outcomes, and they significantly reduce child-reported anxiety; however, they also reduce parent-reported anxiety. However, the certainty of the evidence is limited due to small sample sizes, heterogeneity, and methodological variability across the included studies.

Keywords: Anxiety, Cancer, Central venous catheter, Port catheter, Pain management, Virtual reality therapy

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INTRODUCTION

Childhood cancer constitutes a major source of stress for both children and adolescents due to prolonged treatment processes, frequent hospital visits, and the physical and psychosocial challenges associated with the disease [1]. Despite improvements in survival rates, the side effects accompanying cancer treatment continue to markedly impair the quality of life of affected children [1,2]. In pediatric oncology, the use of central venous access devices (CVADs) has become an integral component of clinical practice to ensure the safe and con-

tinuous delivery of treatment [3,4]. These devices are commonly used for blood sampling, parenteral fluid and nutritional support, chemotherapy administration, transfusion of blood products, and stem-cell transplantation [3,5,6]. Depending on duration of use, insertion site, and the number of lumens, various types of CVADs including non-tunneled catheters, tunneled catheters (Broviac, Hickman, Groshong), subcutaneous implanted ports, totally implanted venous access systems, and peripherally inserted central catheters-are utilized in clinical settings [3-5].

Cancer treatment inherently involves frequent and invasive procedures, children often experience substantial pain, fear, and psychological distress [7-11]. CVADs access, even when supported with pharmacological approaches such as topical anesthetics, is consistently reported by children as a painful and frightening experience [12]. Given that access to CVADs is a frequently repeated, invasive procedure in pediatric oncology, focusing on this context permits a more clinically relevant evaluation of interventions to reduce procedure-related distress.This is largely due to the needle insertion required to access the CVADs; the insertion induces physiological nociceptive responses. Compared with peripheral venous access, CVADs-particularly implanted ports-are associated with higher levels of pain during both insertion and access procedures [13]. Furthermore, a child undergoing a six-month chemotherapy regimen may be exposed to approximately 25-50 CVADs punctures, contributing to cumulative procedural burden [12]. Consequently, pain, fear, and anxiety experienced during CVADs access represent some of the most distressing aspects of cancer care for pediatric patients [14-16].

Psychological support and distraction-based interventions are widely recommended as part of standard care to reduce procedural distress in children and adolescents receiving cancer treatment. VR, a distraction technique offering immersive and interactive sensory experiences, has emerged as an effective tool for modulating attention, emotional states, and neurobiological responses, thereby providing therapeutic benefits during painful procedures [1,17]. Numerous studies have shown that VR applications used during needle-related procedures, including CVADs access, successfully reduce acute pain, fear, and anxiety in pediatric patients [18-21].

However, existing studies differ in sample characteristics, control conditions, and outcome measures, creating heterogeneity that limits the generalizability of findings. Furthermore, current meta-analyses examining VR use in pediatric oncology have focused primarily on needle-related procedures, and no meta-analysis to date has specifically evaluated VR effectiveness during puncture of CVADs. Thus, synthesizing RCTs that investigate VR during CVAD access is essential to address this gap and to provide robust evidence to inform clinical practice. The aim of this systematic review and meta-analysis was to evaluate the effects of VR on pain, fear, and anxiety during access to CVADs in pediatric oncology patients.

MATERIALS AND METHODS

This study is a systematic review and meta-analysis of RCTs that evaluated the effect of VR applications on pain during access via CVADs in pediatric oncology patients. Outcomes related to anxiety and fear that were reported alongside pain were included. The study selection process is reported in accordance with the PRISMA guidelines, and all search updates are reflected in the PRISMA flow diagram [22] (Figure 1). A detailed PRISMA 2020 checklist was completed and submitted as required (Supplementary File 1).

Search strategy

RCTs published up to September 2025 were identified by searching PubMed, EBSCO, Web of Science, and the Cochrane Library. No time restrictions were applied during the literature search. The search strategy was implemented using the following keywords and their combinations, as listed in Supplementary File 2 (Table S1).

Inclusion criteria

- Conducted with pediatric oncology patients,
- Designed as RCTs,
- Use of VR during CVADs access or procedure-related pain,
- Quantitative assessment of pain including mean and standard deviation,
- Availability of full-text and published in English.

Exclusion criteria

- Reported combined data for pediatric and adult populations without separation,
- Presented outcomes of CVC puncture together with other invasive procedures.

The primary outcome was pain, while secondary outcomes, as prespecified, included fear and anxiety/distress. The meta-analysis was designed and implemented according to the PICOS framework (Population, Intervention, Comparator, Outcomes, Study Design), with all inclusion and exclusion criteria presented in Table 1.

Selection process

The literature search, study selection, data extraction, and quality assessment for this review were carried out independently by two researchers in three consecutive stages. In the first stage, all records retrieved from the databases were screened for duplicates, and duplicate entries were removed. In the second stage, the titles and abstracts of the studies were reviewed according to the inclusion criteria; the extracted information was compared for accuracy and consistency, and

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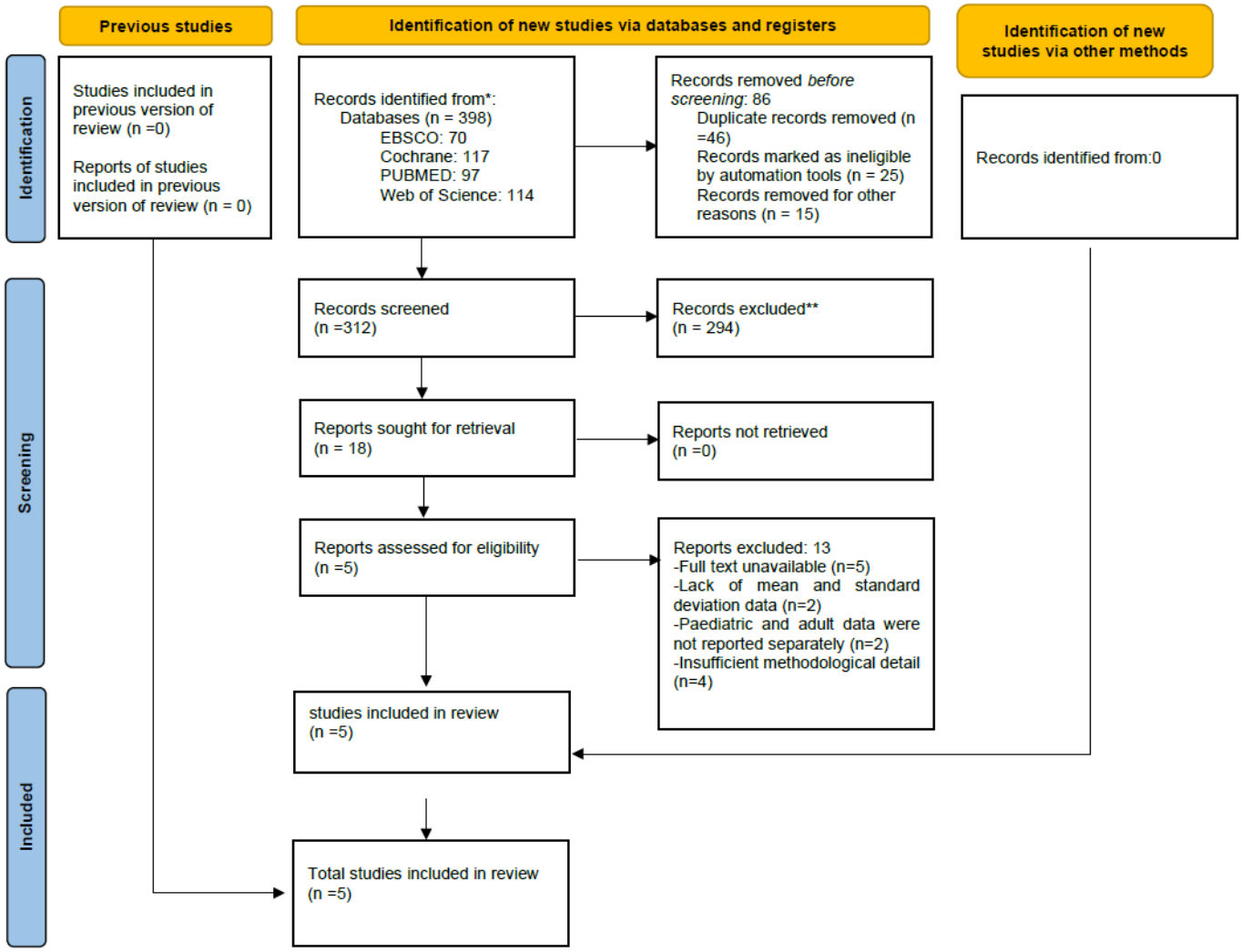

Figure 1. PRISMA flow diagram.

Table 1. PICOS framework.

Population (P)	Children and adolescents undergoing CVC access during cancer treatment.
Intervention (I)	VR applications used during CVC/port access.
Comparison (C)	Standard care, no intervention, or other distraction techniques.
Outcomes (O)	Primary: Pain Secondary: Fear or anxiety
Study Design (S)	RCTs

any disagreements were resolved through consultation with a third researcher. In the final stage, the full texts of potentially eligible studies were examined against the inclusion and exclusion criteria, and the final set of studies included in the review was determined. The number of reviewers, their independent operations, and the conflict-resolution procedure were aligned with PRISMA guidelines. No automated screening or selection tools were used.

Data extraction process

The inclusion criteria were operationalized in a data extraction form developed by the researchers, and the extraction process was performed independently by two reviewers. This form ensured systematic data collection from each included study, capturing information such as the first author's name, publication year, sample size (intervention and control), characteristics of the VR intervention, assessment tools, and primary outcomes (Table 2). Data extraction included participant demographics, intervention components, VR device specifications, control conditions, timing of assessments, and statistical variables (means, standard deviations, and sample sizes). When data were missing or unclear, study authors were not contacted, and analyses were conducted using only the reported values. Full electronic search strategies for each database, including exact search strings, were documented in Supplementary File 2 (Table S1). Additionally, reference

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Table 2. Characteristics of included studies (n=5).

Researcher, Year	Age, Intervention (Participants, n)	Outcome Measure/Reporter	Outcome
Gerçeker et al., 2021	6-17 years	Pain: Wong-Baker FACES Pain Rating Scale (WB-FACES)-child and parent report	Pain: In the VR group, both child- and parent-reported mean pain scores during port needle insertion were significantly lower compared with the control group (p<0.001).
	Intervention group: VR application (n = 21)	Fear: Child Fear Scale (CFS)-child and parent report	Anxiety and Fear: Post-procedure fear and anxiety scores were significantly lower in the VR group based on both child and parent reports (p<0.001; p<0.001, respectively).
	Control group: Standard care (n = 21)	Anxiety: Children's Anxiety Meter-State (CAM-S)-child and parent report	
Hundert et al., 2022	8-18 years	Pain: Numeric Rating Scale (NRS)-child/adolescent self-report, parent report, nurse report, and research staff report	Pain: Although the likelihood of experiencing pain was three times higher in the iPad group compared with the VR group, this difference was not statistically significant (p=0.123).
	Intervention group: VR application (n = 20)	Fear: CFS-child/adolescent self-report, parent report, nurse report, and research staff report	Distress: Participants in the iPad group had a 4.1-fold higher likelihood of experiencing distress compared with the VR group; however, this finding was not statistically significant (p=0.086). There was no significant difference between groups in overall distress scores (p=0.860).
	Control group: Ipad watching (n = 19)	Distress: Numeric Rating Scale (NRS)-child/adolescent self-report, parent report, nurse report, and research staff report Parent Distress Questionnaire-parent report Immersion: Child/adolescent self-report	Immersion: Immersion levels were significantly higher in the VR group (p=0.031).
Savas et al., 2024	6-12 years	Pain: WB-FACES-child self-report and parent report	Pain: Post-procedure pain scores reported by both children and mothers were significantly lower in the intervention group (p<0.001; p<0.001, respectively).
	Intervention group: VR application (n=31)	Fear: CFS-child self-report and parent report	Fear: Post-procedure fear scores reported by children and mothers were significantly lower in the intervention group (p<0.001; p<0.001, respectively).
	Control group: Standard care (n=31)	Anxiety: Child Anxiety Scale--State-child self-report and parent report	Anxiety: Post-procedure anxiety scores reported by both children and mothers were significantly lower in the intervention group (p<0.001; p<0.001, respectively).
		Physiological Parameter: Respiratory rate/minutes)	Respiratory Rate: Respiratory rate was significantly lower in the intervention group (p<0.001).
		Satisfaction: Visual Analog Scale (VAS) -child self report and parent report	Satisfaction: Satisfaction scores reported by children and mothers were significantly higher in the intervention group (p<0.001; p<0.001, respectively).
Semerci et al., 2021	7-18 years	Pain: WB-FACES-child and parent report	Pain: Mean procedural pain scores reported by children and parents were significantly lower in the intervention group (p=0.001; p<0.001, respectively).
	Intervention group: VR application (n=36) Control group: Standard care (n=35)		
Wolitzky et al., 2005	7-14 years	Pain: VAS-child, parent, and nurse report	Pain: Based on researcher-reported assessments, mean pain scores in the VR group were significantly lower than those in the control group (p<0.01).
	Intervention group: VR application (n=10)	Children's Hospital of Eastern Ontario Pain Scale (CHEOPS)-researcher report	Heart Rate: Both intra-procedure and post-procedure heart rate values were lower in the VR group compared with the control group; the difference was statistically significant during the procedure (p<0.05), but not post-procedure (p=0.12).
	Control group: Standard care (n=10)	Anxiety:How-I-Feel Questionnaire-child self-report	
		Distress:VAS-child, parent, and nurse report Pulse: Heart rate/minutes	

Abbreviations: VR; Virtual Reality, n; Number of Participants, WBFPS; Wong–Baker FACES Pain Rating Scale, CFS; Children's Fear Scale, VAS; Visual Analog Scale, NRS; Numerical Rating Scale, p; Probability, CHEOPS; Children's Hospital of Eastern Ontario Pain Scale.

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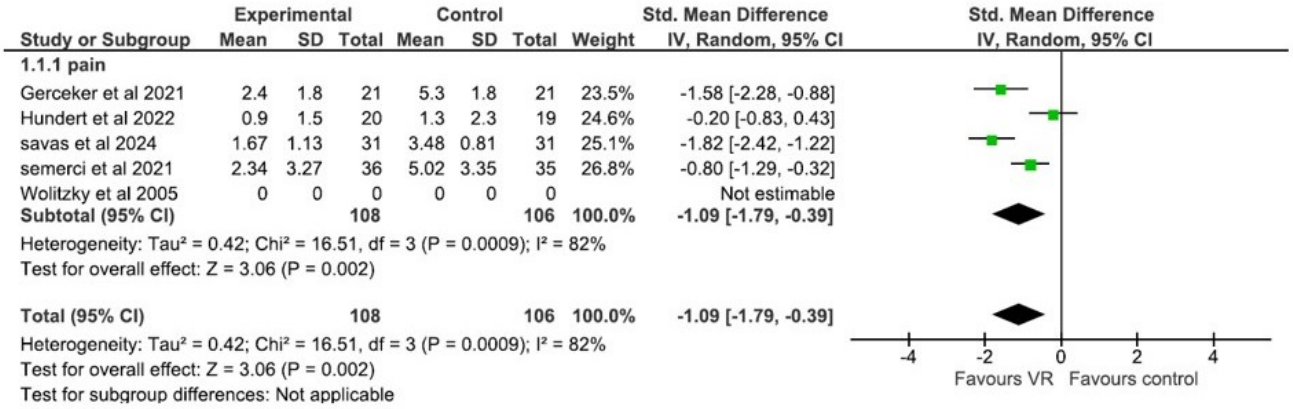
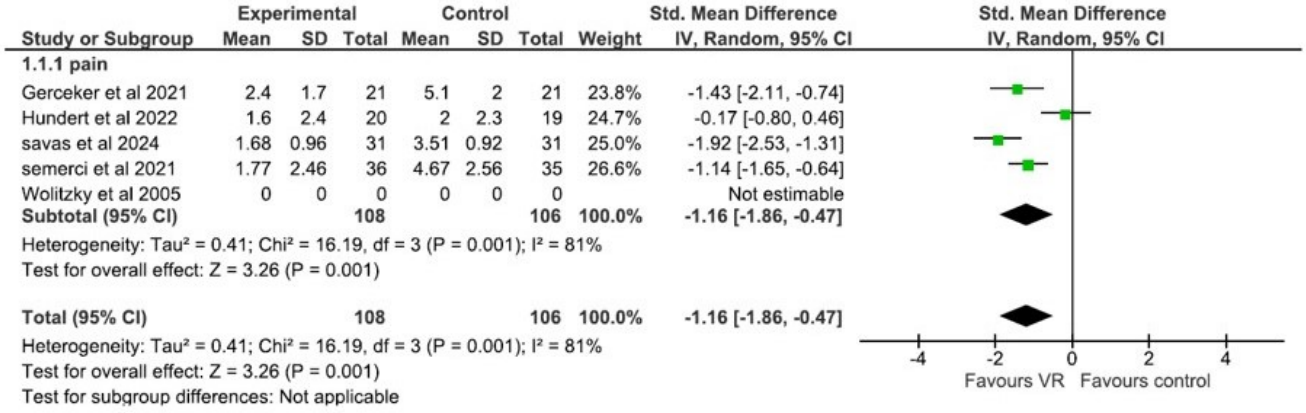


(a) Children's self-reported pain scores.



(b) Parents' reported pain scores.

Figure 2. Forest plot illustrating the effect of VR on pain scores.

lists of all included articles were screened manually to identify any potentially eligible studies not captured through database searching. No automation tools were used during the search or selection processes.

Data items

Outcomes included pain intensity, fear, and anxiety/distress, each assessed using validated measurement scales reported in the original studies; when multiple measurements were available for the same outcome, the most clinically relevant time point (needle insertion) was selected. Additional extracted variables included age, sex distribution, diagnosis, types of CVADs, characteristics of the VR content (active vs. passive VR), type of control condition (routine care vs. distraction), and study funding status.

Risk of bias assessment

The methodological quality of the included studies was evaluated independently by two reviewers using the Cochrane Risk of Bias (RoB)2 tool, as recommended by Cochrane for randomized controlled trials. The RoB2 assessment was conducted at the outcome level and covered five domains: (1) bias arising from the randomization process, (2) bias due to deviations from intended interventions, (3) bias due to missing outcome data, (4) bias in measurement of the outcome, and (5) bias in selection of the reported result. Any disagreements between reviewers were resolved through discussion, with consultation from a third researcher when necessary. All domain-level judgments and final RoB2 decisions for each outcome and each study are presented in Supplementary File 3 (Table S2a, S2b, and S2c), providing a transparent evaluation of potential sources of bias.

Data synthesis

Continuous outcomes were pooled using Standardized Mean Differences (SMD/Cohen's d), as the use of different validated scales across studies (e.g., Wong Baker FACES, Numeric Rating Scale, Visual Analog Scale) made raw mean differences inappropriate for cross-study comparison. Statistical analyses were performed using Review Manager 5.4.1. Given the anticipated clinical and methodological variability across trials, a random-effects model (DerSimonian-Laird) was prespecified as the primary analytical approach [23]. Statistical signif-

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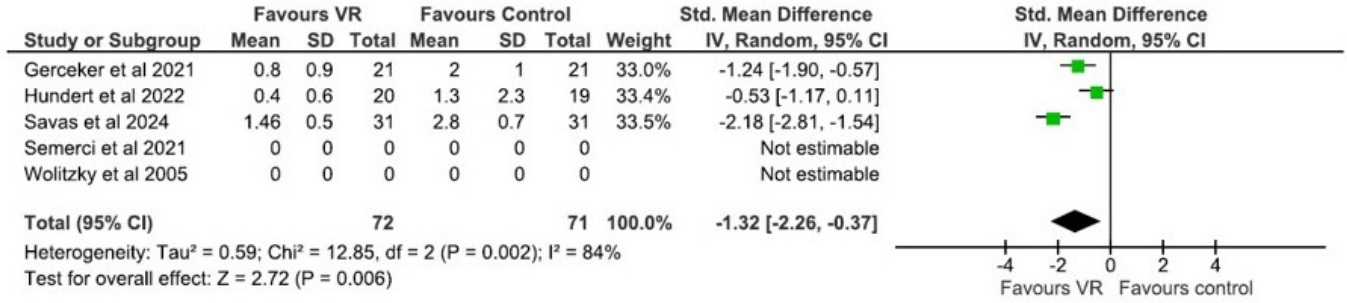
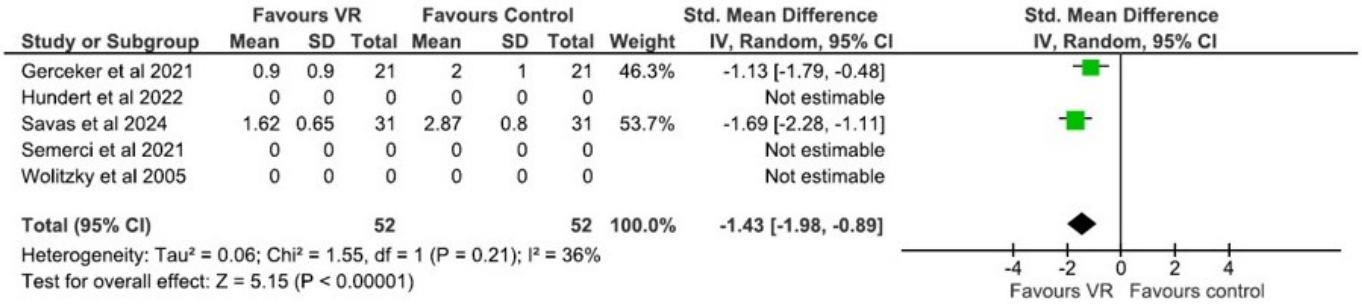


(a) Children's self-reported fear scores.



(b) Parents' reported fear scores.

Figure 3. Forest plot illustrating the effect of VR on fear scores.

icance was set at $p<0.05$.

Heterogeneity was quantified using Cochrane's Q ($Chi^2(3)$, $p< 0.10$), Tau^2, and I^2 statistics, with I^2 used solely to describe heterogeneity rather than to determine model selection [24]. Due to the limited number of trials, no subgroup, sensitivity, or meta-regression analyses were performed. Because fewer than ten studies were included, publication bias could not be reliably assessed [23]. The certainty of evidence for each outcome was evaluated using the GRADE approach. Overall confidence in the estimates was rated low because of concerns about risk of bias, inconsistency, imprecision, and small sample sizes.

RESULTS

Study selection

A total of 398 records were identified through searches conducted in PubMed, EBSCO, Web of Science, and the Cochrane Library. After removing duplicates and conducting the title-abstract screening, 18 full-text articles were assessed for eligibility. Studies were excluded if they were not published in English, if pediatric and adult data were not analyzed separately, if outcomes from CVC access were combined with other invasive procedures, if statistical data were incomplete, or if the full text was unavailable. Ultimately, five studies met all eligibility criteria and were included in this systematic review and meta-analysis (Figure 1).

Characteristics of the studies

A total of five RCTs evaluating the effects of VR during CVADs access in children undergoing cancer treatment were included in this systematic review and meta-analysis [8-10,25,26]. The studies, conducted between 2005 and 2024, included participants aged 6-18 years. VR was used as the intervention in all experimental groups, while the control groups received either standard care [8-10,26] or a passive distraction method such as an iPad-based activity [25] (Table 2).

VR in pain management

All five studies included in the meta-analysis assessed pain outcomes. Four studies reported pain ratings obtained from both children and their mothers [8,9,25,26], while one study measured pain based on nurse assessments [10]. Pain evaluation tools used across the studies included the Wong–Baker FACES Pain Rating Scale [8,9,26], the Numeric Rating Scale [25], and the Visual Analog Scale [25]. In the studies comparing VR with standard care, reductions in children's self-reported pain levels were consistently observed [8,9,26]. However, in the study comparing VR with an iPad-based distraction method, no significant difference in pain outcomes was identified between the groups [25].

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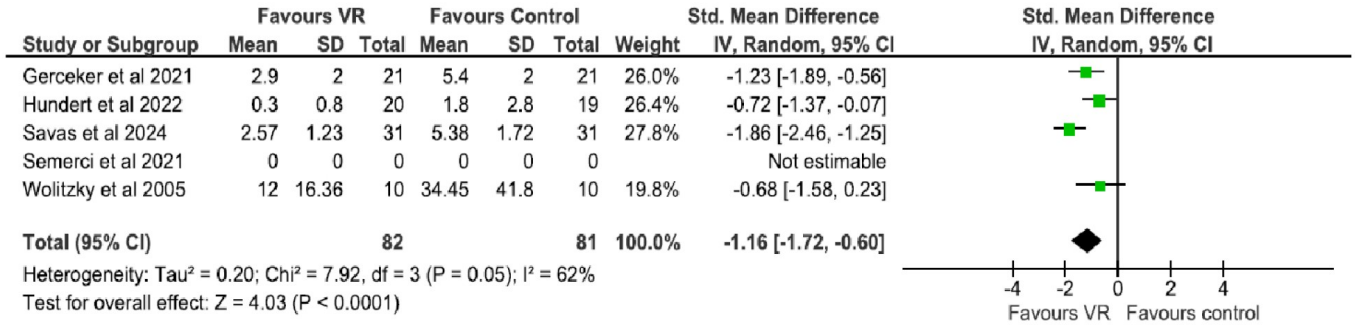
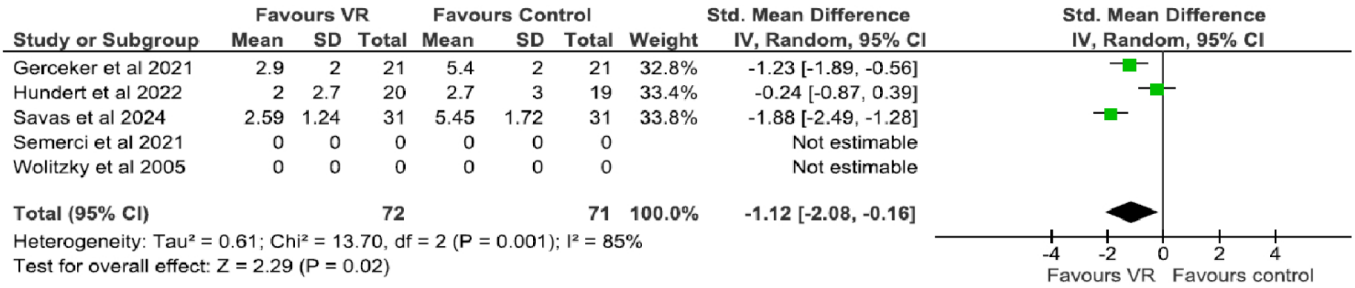


(a) Children's self-reported anxiety scores.



(b) Parents' reported anxiety scores.

Figure 4. Forest plot illustrating the effect of VR on anxiety scores.

Under the random-effects model, the pooled effect size was calculated as SMD=-1.09 (95% CI:-1.79 to -0.39). Between-study heterogeneity was substantial (Tau^2=0.42; I^2=82%). The overall effect test was statistically significant (Z=3.06, p=0.002). Individual study effects ranged from -0.20 to -1.82, with study weights varying between 23.5% and 26.8%. The heterogeneity test yielded Chi^2(3)=16.51, p= 0.0009 (Figure 2a).

Under the random-effects model, the pooled effect size was calculated as SMD =-1.16 (95% CI: -1.86 to -0.47). Between-study heterogeneity was high (Tau^2= 0.41; I2= 81%). The overall effect was statistically significant (Z= 3.26, p=0.001). The heterogeneity test yielded Chi^2(3)=16.19, p=0.001. Individual study effect sizes ranged from -0.17 to -1.92, with study weights varying between 23.8% and 26.6% (Figure 2b).

VR in fear management

All three studies included in this systematic review and meta-analysis assessed fear outcomes based on ratings provided by both children and their mothers [8,25,26]. These studies utilized the Child Fear Scale to measure fear levels [8,25,26].

In comparisons between VR and standard care, reductions in fear levels were consistently observed according to both child- and parent-reported assessments [8,26]. However, in the study comparing VR with an iPad-based distraction approach, no significant difference in fear scores was detected between the groups [25].

Under the random-effects model, the pooled effect size was calculated as SMD=-1.32 (95% CI: -2.26 to -0.37). A high level of heterogeneity was observed among the studies (Tau^2=0.59; I^2 = 84%). The heterogeneity test was significant, Chi^2(3)=12.85, p=0.002. The overall effect test also indicated statistical significance (Z=2.72, p=0.006). Individual study effect sizes ranged from -0.53 to -2.18, and study weights varied between 33.0% and 33.5% (Figure 3a).

Under the random-effects model, the pooled effect size for fear was calculated as SMD=-1.43 (95% CI:-1.98 to -0.89). Between-study heterogeneity was low (Tau^2= 0.06; I^2= 36%), and the heterogeneity test was not significant, Chi^2(3)= 1.55, p=0.21. The overall effect was statistically significant (Z= 5.15, p<0.00001). Individual study effect sizes ranged from -1.69 to -1.13, and study weights ranged from 46.3% to 53.7% (Figure 3b).

VR in anxiety/distress management

All three studies included in this systematic review and meta-analysis assessed anxiety or distress using both child- and parent-reported measures [8,10,25, 26], while one study additionally included nurse-reported assessments [25]. The assessment tools used across the studies included the Children's

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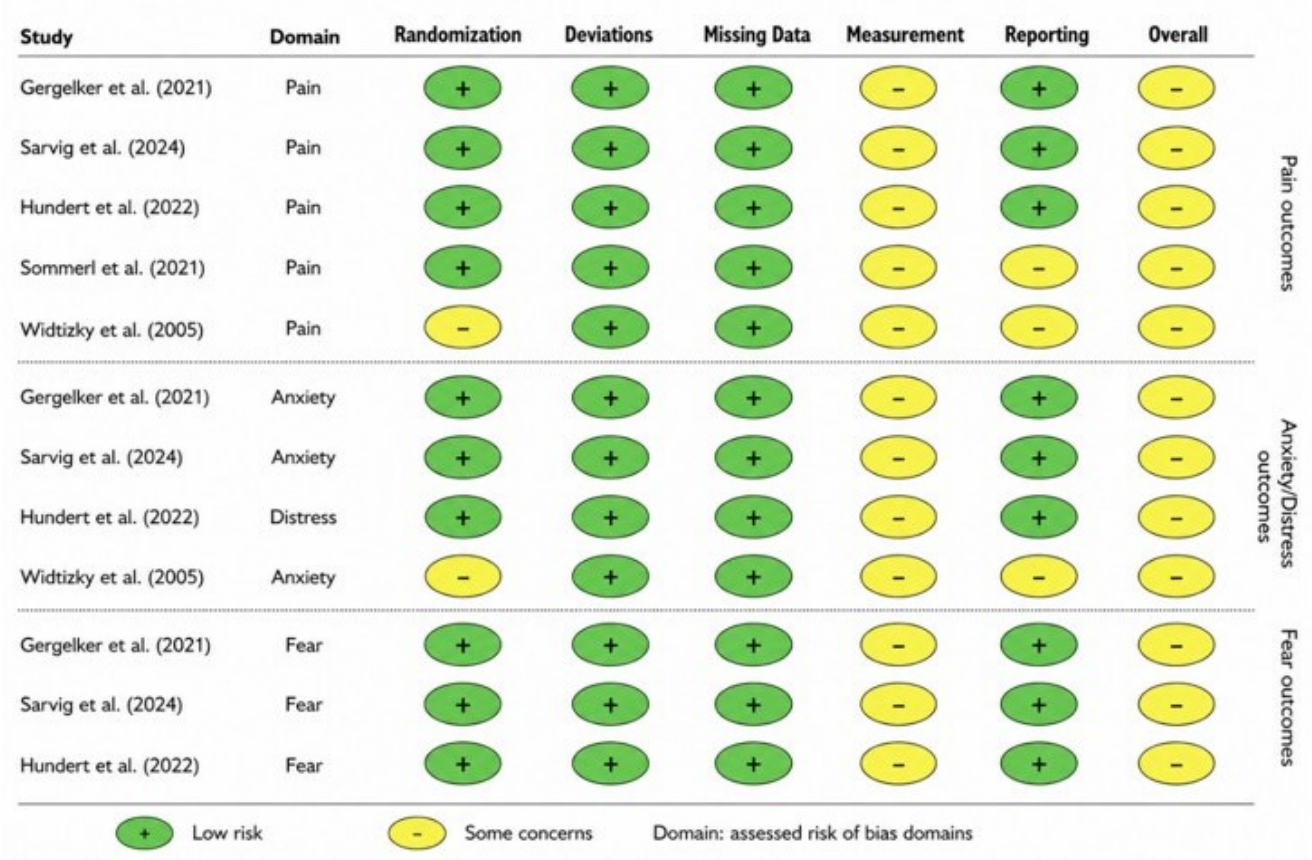

Figure 5. Risk of bias in the studies for Pain, Anxiety/Distress, and Fear.

Anxiety Meter State [8,26], the Numeric Rating Scale [25], and the Visual Analog Scale [10].

In the comparison of VR with standard care, reductions in anxiety and distress were reported. However, no significant difference was observed in anxiety/distress scores in the study comparing VR with an iPad-based distraction method [25].

Under the random-effects model, the pooled effect size was calculated as SMD = -1.32 (95% CI: -1.96 to -0.67). Between-study heterogeneity was moderate (Tau^2= 0.19; I^2= 59%), and the heterogeneity test indicated no significance, $Chi^2(3)$= 4.90, p=0.09. The overall effect test was statistically significant (Z= 4.01, p<0.00001). Individual study effect sizes ranged from -0.68 to -1.86, with study weights varying between 26.7% and 37.9% (Figure 4a).

Under the random-effects model, the pooled effect size was calculated as SMD = -1.12 (95% CI: -2.08 to -0.16). Between-study heterogeneity was high (Tau^2= 0.61; I2= 85%), and the heterogeneity test was significant, $Chi^2(3)$= 13.70, p=0.001. The overall effect was statistically significant (Z= 2.29, p= 0.02). Individual study effect sizes ranged from -0.24 to -1.88, and study weights varied between 32.8% and 33.8% (Figure 4b).

Risk of bias traffic-light plot summarizing outcome-level RoB2 assessments for all included randomized controlled trials. Each domain reflects potential sources of bias as defined by the Cochrane Risk of Bias 2.0 tool, including bias arising from the randomization process, deviations from intended interventions, missing outcome data, measurement of the outcome, and selection of the reported result. Green indicates low risk of bias, yellow indicates some concern, and red indicates high risk of bias. Risk-of-bias assessments were classified separately for pain, anxiety/distress, and fear outcomes in all included randomized controlled trials. However, due to the inherent nature of VR interventions in which participant blinding is not feasible, the measurement of subjective outcomes (pain, fear, and anxiety) was judged to have 'some concerns' in all trials. Furthermore, 'some concerns' were noted in the selective reporting domain for Semerci et al. [9] and Wolitzky et al. [10] due to the absence of preregistered protocols. Overall, the included studies were predominantly categorized as having 'some concerns,' consistent with the nature of subjective, non-blinded interventions in pediatric procedural pain research. (Figure 5). Detailed justifications for the judgments regarding each outcome and study are provided in Supplementary File 3 (Table S2a, S2b, and S2c).

DISCUSSION

The results of this systematic review and meta-analysis, which aimed to evaluate, based on existing RCT findings, the effects of VR on pain, fear, and anxiety during CVAD access in pediatric oncology patients, are valuable because this is the first study of its kind in the literature. Five RCTs, conducted with children who used VR during CVADs access and pub-

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lished up to September 2025, were included in this review. Among the five studies included in the meta-analysis, four assessed pain based on child self-report, and four assessed pain based on parent report. In addition, three studies evaluated fear, and four evaluated anxiety and pain. VR used during access to CVADs was effective in reducing pain, fear, and anxiety in children compared with children in the control group, according to both child and parent reports. Although these findings suggest a beneficial effect, the magnitude of the observed effects should be interpreted with respect to their clinical applicability, particularly given the procedural context and variability across studies. However, considering the risk of bias (RoB) and the sample sizes of the included studies, the certainty of the evidence must be interpreted cautiously. In addition, the limited number of included studies and their relatively small sample sizes may have reduced the statistical power of the pooled analyses and may have limited the generalizability of the findings.

Cancer diagnosis and treatment are physiologically and psychologically challenging for children. Children are exposed to long treatment protocols, painful procedures, and extended hospitalizations [27,11]. CVADs, which are used to ensure continuity and facilitate the application of treatment, may contribute to improving the child's comfort and quality of life [28]. However, considering the duration and intensity of treatment, an increase in the number of CVADs access procedures may elevate the child's pain, fear, and anxiety related to the procedure [29]. Children may attempt to avoid the procedure and demonstrate non-compliance with treatment due to the pain they experience [29]. Additionally, pain experienced during CVADs access may lead to changes in the sympathetic or parasympathetic system, increasing heart rate; this may cause vasoconstriction and make vascular access more difficult [28].

VR, which is one of the distraction techniques, may be used to reduce the pain, fear, anxiety, and related physiological changes that children experience during CVADs access [11]. In children who used VR during CVADs access, reductions in heart rate [10], pain, fear, and anxiety were reported compared with the control group [8,9,26]. Although these physiological and emotional effects are promising, the strength of supporting evidence remains limited, particularly because the included studies varied substantially in intervention content, measurement tools, and procedural conditions. This variability contributes to heterogeneity across studies, which lowers confidence in the pooled results despite consistent directional findings; this heterogeneity is partly attributable to the use of different outcome measurement tools (e.g., Wong-Baker FACES, Numeric Rating Scale, and Visual Analog Scale), and it also limits the extent to which pooled effect sizes can be directly compared or generalized to different clinical settings.

The studies included in this systematic review and meta-analysis revealed that children who received VR reported significantly lower pain levels compared with the control group. When the pain scores, which were the primary outcome of the included RCTs, were examined, most studies with a high level of evidence [8,9,26] reported a statistically significant and clinically pronounced reduction in the VR group compared to the control group. In contrast, Hundert et al. [25] did not find a significant difference between the groups, despite showing higher rates of pain-free procedures in the VR group. In clinical practice, although topical anesthetic cream is applied to the needle insertion site before CVADs access, procedural pain often continues at a considerable level, and invasive interventions such as port needle insertion may exceed children's pain threshold [13]. The pain-reducing effect of VR may be related not only to psychosocial distraction mechanisms but also to potential neurophysiological processes associated with multisensory immersion. The synchronized presentation of visual and auditory stimuli may facilitate the activation of the gate-control mechanism at the spinal level, reducing the transmission of pain signals to the central nervous system [30,31]. This mechanism may contribute to decreased allocation of cognitive resources to pain-related neural networks and reduce behavioral manifestations of pain intensity. These findings are consistent with, but do not directly confirm, the proposed neurophysiological mechanisms. The findings indicate that VR is safe and applicable as a complementary method to pharmacological analgesia in pediatric procedures. Although the pooled results demonstrate statistically significant reductions in pain, the extent to which these reductions translate into clinically meaningful improvements (e.g., improved procedural cooperation or reduced need for additional interventions) may vary depending on the clinical setting and implementation conditions. Nevertheless, the certainty of evidence for pain reduction was rated as moderate rather than high, reflecting the presence of methodological concerns and variability among studies. Thus, while VR appears clinically beneficial, further large-scale, high-quality RCTs are required to confirm its analgesic effect with greater certainty.

The studies included in this systematic review and meta-analysis also showed that children who used VR reported significantly lower levels of fear and anxiety than children in the control group. Gerçeker et al. [8] and Savaş et al. [26] demonstrated that the VR intervention significantly reduced procedural fear and anxiety in both child and parent reports; while Wolitzky et al. [10] showed reductions in parent reports. However, Hundert et al. [25] found no difference between the groups. This result may be explained by a mechanism in which psychological distraction processes that reduce threat perception, together with potential neurophysiological modulation may operate. Immersive VR experience diverts the child's cognitive resources away from the procedure by separating attention from threatening stimuli; this may strengthen cognitive control executed in the prefrontal cortex and anterior cingulate cortex and may contribute to the suppression of limbic system activity [32,33]. Such neural redirection may be associated with lower emotional responses

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during the procedure. Overall, the findings suggest that VR may be an effective complementary intervention during pediatric procedures to support emotional regulation and possible neurobiological modulation of threat perception. However, the certainty of the evidence for fear and anxiety outcomes is limited because of small sample sizes, high heterogeneity, and 'some concerns' identified in the Risk of Bias assessment, particularly the lack of blinding and reporting bias. Therefore, while these emotional benefits are consistently observed, the findings should be interpreted with caution and be confirmed through larger, rigorously designed future trials.

The studies included in this systematic review and meta-analysis also demonstrated that parent-reported levels of pain, fear, and anxiety in children who used VR were significantly lower than those of children in the control group. In general, parents' reports were consistent with children's self-reports, revealing that children in the VR group experienced significantly less pain, fear, and anxiety than those in the control group. Parental reports across the included studies were consistent with children's self-reports, reinforcing the efficacy of the VR intervention. In the studies by Gerceker et al. [8], Semerci et al. [9], and Savas et al. [26], parents rated post-procedural pain significantly lower in the VR group compared to controls. Similarly, parents reported significantly reduced fear and anxiety levels in studies assessing these outcomes [8,26]. While the pilot study by Hundert et al. [25] observed a non-significant trend favoring VR in parental scores due to limited sample size, Wolitzky et al. [10] reported a strong correlation between parent and child assessments, underscoring the reliability of parental observations in validating the benefits of VR. The consistent reduction in parental reports suggests that VR may not only influence children's subjective experience but also positively affect parents' observations of the procedure. The immersive nature of VR may help redirect the child's attention away from the intervention, leading to a noticeable reduction in emotional reactions; this may enable parents to perceive behavioral and physiological cues as calmer and more manageable [34,35]. The strong tendency in parent reports favoring VR is consistent with VR functioning as a holistic intervention that may reduce emotional burden for both children and parents. However, the certainty of the evidence for parent-reported anxiety was rated very low because of extreme heterogeneity and inconsistent effect sizes. For this reason, conclusions regarding parental perceptions should be interpreted with caution.

The strength of the findings should be considered in the context of the methodological quality and RoB of the included RCTs. Our RoB 2 analysis demonstrated that the majority of studies were at low risk across the domains of the randomization process, deviations from intended interventions, and missing outcome data. However, the VR intervention was not amenable to blinding of participants and practitioners, which consequently resulted in 'some concerns' regarding the measurement of subjective outcomes (pain, fear, anxiety) across all included studies. The potential for subjective reports to be biased by the lack of blinding is a primary factor limiting the certainty of the evidence and represents an inherent methodological constraint that should be considered when interpreting the magnitude of the observed effects. In addition, the absence of preregistered protocols in the studies by Semerci et al. [9] and Wolitzky et al. [10] raises concerns regarding selective reporting. The studies were generally classified as 'some concerns', consistent with the nature of behavioral interventions in pediatric procedural pain research. In addition, the restriction to English-language studies may have introduced language bias. In addition, the study authors were not contacted to obtain missing data, which may have limited the completeness of the information available. These methodological limitations indicate that the statistically significant positive effects of VR should be interpreted with cautious optimism, taking into account potential risks of bias, rather than as definitive conclusions.

CONCLUSION

The results of this systematic review and meta-analysis indicate that VR reduces pain, fear, and anxiety during CVAD access in pediatric oncology patients, based on both child- and parent-reported outcomes. While these findings are clinically meaningful, their direct translation into routine clinical practice should be approached with caution, as effect sizes may vary depending on procedural context, VR content, and patient characteristics, and the overall certainty of evidence-particularly for fear and anxiety-remains limited due to small sample sizes, methodological variability, and heterogeneity among the included trials.Therefore, VR should be considered a promising complementary intervention rather than a definitive standard of care.

In clinical practice, incorporating VR into routine access procedures for CVADs may help improve children's procedural tolerance, reduce parental distress, and support healthcare professionals, such as nurses and physicians, in managing invasive interventions. However, broader implementation should be guided by further high-quality research to strengthen the evidence base and determine optimal VR protocols for different pediatric populations.

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Effects of remote ischemic postconditioning treatment on ferroptosis and hexokinase II levels in a rat model of cerebral ischemia-reperfusion injury

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MAIN POINTS

- Following I/R, Increased levels of the ferroptosis marker ALOX12 and GPX4 suggest that ferroptotic cell death contributes to the injury.
- The neuroprotective mechanism of RePostC may be associated with the reduction of ALOX12 levels and the elevation of GPX4 levels.
- GPX4, ALOX12, LCN2, and HKII have been identified as novel biomarkers and therapeutic targets for cerebral I/R injury.
- RePostC's protective effect is linked to the activation of internal tolerance mechanisms and the strengthening of antioxidant defenses.

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ABSTRACT

Aim: Cerebral ischemia-reperfusion (I/R) injury is an unavoidable outcome of reperfusion therapy following a stroke, initiating intricate death mechanisms at both cellular and molecular levels. Comprehending the molecular pathways that result in neuronal death post-stroke is crucial for the development of effective neuroprotective strategies. Ferroptosis is a mechanism of cell death characterized by iron-dependent lipid peroxidation and is believed to contribute to the damage associated with cerebral I/R injury.

Materials and Methods: Thirty male Sprague-Dawley rats were randomly assigned to three experimental groups: Control, IR, and IR+REPOSTC (n=10). Cerebral ischemia was induced using the intraluminal filament technique for 60 minutes, and the animals were euthanized after a 24-hour reperfusion period. The femoral artery of the right hind limb of the rat underwent RePostC consisting of three cycles of 5 minutes of ischemia followed by 5 minutes of reperfusion. Triphenyl tetrazolium chloride (TTC) staining was used to measure infarct size.

Results: Compared to the control group, the I/R group showed a significant increase in glutathione peroxidase 4 (GPX4) levels, while RePostC treatment significantly decreased GPX4 levels. Compared to the control group, the I/R group showed a significant decrease in hexokinase II (HKII) levels. The RePostC group, however, exhibited a substantial increase in these levels compared with the I/R group. Levels of arachidonic acid 12-lipoxygenase (ALOX12) and lipocalin 2 (LCN2) were significantly increased in the I/R group compared with the control group. No statistically significant difference was observed between the RePostC treatment group and the I/R group.

Conclusion: Ferroptotic cell death plays a role in the damage that occurs after cerebral I/R. GPX4, ALOX12, LCN2, and HKII levels may serve as potential targets for the management of cerebral ischemia-reperfusion injury. The RePostC application may help protect against I/R injury by activating the body's own tolerance mechanisms.

Keywords: Cerebral I/R, Ferroptosis, GPX4, ALOX-12

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INTRODUCTION

Stroke is one of the leading causes of death and illness worldwide, and its incidence is rising [1]. Ischemic stroke is the most common type of stroke, accounting for approximately 87% of cases. It occurs when blood flow to the brain suddenly stops because thrombosis or embolism blocks the blood vessels that supply it. Statistics show that more than 14 million people are affected by this condition each year [2]. Thrombolysis with tissue plasminogen activator (tPA), which targets reperfusion, and mechanical thrombectomy are the two most common treatments for ischemic stroke. However, these methods don't work well enough to treat strokes [3]. Reperfusion can exacerbate injury in ischemic tissue, causing ischemia/reperfusion (I/R) injury [4]. I/R injury induces cell

death, thereby exacerbating damage to ischemic tissue. This is followed by the infiltration of neutrophils, the accumulation of reactive oxygen species (ROS), the disruption of cellular ion homeostasis, and the activation of inflammatory responses [5].

The mechanisms responsible for cerebral I/R injury are not fully elucidated; nevertheless, emerging evidence suggests that ferroptosis significantly contributes to this phenomenon [6]. Ferroptosis is a regulated mechanism of cell death characterized by iron-dependent lipid peroxidation and is genetically, morphologically, and biochemically distinct from other forms of cell death [7].

Extensive research in recent years has examined mechanisms regulating ferroptosis, including the roles of iron, lipids, and amino acids [8]. GPX4 plays a role in ferroptosis and in amino acid metabolism by reducing lipid hydroperoxides to lipid alcohols. This inhibits the formation of toxic lipid ROS that are dependent on iron (Fe^{2+}) [9]. In addition to blocking hydroperoxide-dependent processes, GPX4 inhibits the ALOX (arachidonic acid lipoxygenase) family. This family is thought to be involved in the iron metabolism pathway in ferroptosis [10,11].

ALOX12 (arachidonic acid 12-lipoxygenase), a member of the lipoxygenase family, is involved in pro-oxidant and pro-inflammatory processes [12]. Nonetheless, the function of ALOX in lipid peroxidation resulting from GPX4 deficiency is yet to be elucidated.

Lipocalin-2 (LCN2) is an acute-phase protein that binds siderophores released by microorganisms, thereby sequestering iron. Its levels increase during infections or after various traumas [13,14]. Research indicates that LCN2 diminishes ferroptotic cell death [15]. Patients who have experienced ischemic stroke exhibit increased plasma levels of LCN2 [16]. Nonetheless, the function of LCN2 in stroke and the impact of RePostC on LCN2 remain incompletely understood.

The enzyme that phosphorylates glucose to form glucose-6-phosphate [17] is hexokinase II (HK-II). This is the first step in glycolysis. HK-II attaches to the outer mitochondrial membrane via the Voltage-Dependent Anion Channel 1 (VDAC1). This causes HK-II to dissociate from the mitochondria, leading to cell death [18]. Research indicates that elevated HK-II expression confers neuroprotective effects in a model of cerebral ischemia, reduces oxidative stress, and preserves mitochondrial stability [19].

Remote ischemic postconditioning (RePostC) is a noninvasive technique designed to safeguard vital organs compromised by ischemia via interventions administered to remote body regions [20]. Its accessibility and noninvasive nature have demonstrated its therapeutic potential across numerous conditions, encompassing ischemic neurological disorders in the acute, subacute, and chronic phases, including acute ischemic stroke [21].

This study investigated the role of ferroptosis in cerebral I/R injury, highlighting interactions of GPX4, ALOX12, and LCN2 with mitochondrial HKII. We also sought to clarify the regulatory impacts of RePostC on GPX4, ALOX12, LCN2, ferroptosis, and HKII at the molecular level to inform treatment strategies for cerebral I/R injury. Our study is the first to show how RePostC affects the levels of ALOX12, LCN2, and HKII. The RePostC application inhibited ferroptotic cell death by elevating GPX4 and HKII levels and exhibited a substantial neuroprotective effect against cerebral I/R injury.

MATERIALS AND METHODS

Statement of ethics and animal care

The Fırat University Faculty of Medicine Animal Experimentation Ethics Committee (Date: 21.06.2023; Protocol No: 2023/12-07) established and approved the ethical rules governing all animal experiments. The sample size analysis, conducted prior to the study using the "Web-based sample size and power analysis software" [22], indicated that at least 18 rats (6 per group) should be included to evaluate the parameters (power = 80%, $\alpha = 0.05$, $\beta = 0.2$). To increase the power of the study and minimize mortality from surgical procedures, 30 male Sprague-Dawley rats aged 10 to 12 weeks were used. The rats lived in a room with a temperature range of 22 ± 1°C and a 12-hour light/dark cycle. Animals had ad libitum access to food and water. The study used the minimum number of animals required, and measures were implemented to mitigate pain and discomfort.

Groups and experimental design

The study involved weighing all rats and randomly assigning them to three groups of 10 animals each: the Control Group (C), the Ischemia-Reperfusion Group (IR), and the Ischemia-Reperfusion and Remote Ischemic Postconditioning Treatment Group (IR+RePostC). In the IR+RePostC group, three cycles of 5-minute ischemia followed by 5-minute reperfusion were applied to the femoral artery in the right hind leg at the onset of reperfusion.

Initiation of the experimental cerebral ischemia-reperfusion model

Control, IR, and IR+RePostC rats underwent occlusion of the right middle cerebral artery (MCA) via the Koizumi intraluminal filament technique [23], thereby initiating a 60-minute period of ischemia. The animals were given ketamine-xylazine [24] to induce anesthesia, and their body temperature was maintained between 36.5°C and 37°C throughout the protocol. The right common carotid artery (CCA), the carotid bifurcation, and the external and internal carotid arteries (ECA and ICA) were carefully exposed by cutting away the surrounding tissues. The ECA was tied off distally, the CCA was tied off proximally, and a temporary microvascular clamp was placed on the ICA. A 4/0 nylon filament with

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a silicone-coated tip was inserted into the ICA through a incision in the right CCA. After the clamp was removed, the filament was gently advanced until it met resistance (approximately 18–20 mm), indicating occlusion of the middle cerebral artery (MCA). The filament stayed in place for

It was applied for an hour to induce ischemia, and then it was taken out to allow reperfusion. The same surgical steps were performed on the control animals, but no filament was inserted. After the procedure, the incision was closed with sutures, and the animals were returned to their cages to recover.

Analysis of Western blots

We used Western blotting to assess HKII levels. Western blot analysis of tissue HKII levels was performed using the appropriate antibodies. Before use, primary antibodies were mixed with a buffer containing 0.05% Tween-20 and then diluted 1:1000. We incubated nitrocellulose membranes with primary antibodies at +4°C overnight. Subsequently, the nitrocellulose membranes were washed five times, each wash lasting five minutes, with a buffer. After washing, the nitrocellulose membranes were incubated at 37°C for 90 minutes with goat anti-rabbit immunoglobulin conjugated to peroxidase, diluted 1:1000 in the same buffer. Lastly, the washing process was performed five times, each lasting five minutes. We used beta-actin for normalization.

Analyzing ELISA

The ELISA method was used to measure the levels of GPX4, ALOX12, and LCN2. Blood samples from the experimental groups were allowed to coagulate at room temperature for 30 minutes and then centrifuged at 5000 rpm for 15 minutes to separate the serum. The collected serum samples were stored at -80°C until testing. Using ELISA kits from BTLab (Shanghai Korain Biotech Co., Ltd., China), we measured the serum levels of the proteins GPX4, ALOX12, and LCN2 according to the manufacturer's instructions.

The kits were purchased with the following catalog numbers: GPX4 (Cat. No. YLA1783RA), ALOX12 (Cat. No. E3428Ra), and LCN2 (Cat. No. E1434Ra).

Statistical analysis

The data were analyzed using the IBM SPSS Statistics version 21.0 (Armonk, NY: IBM Corp.) , and the results are presented as mean ± standard deviation. The Shapiro-Wilk test was used to assess normality, and one-way ANOVA followed by Tukey's post hoc test was used to assess differences between groups. The correlation analysis employed Pearson's correlation test and two-tailed significance tests, with a significance threshold set at $p<0.05$.

RESULTS

GPX4

The IR group (11.57 ± 0.67) had significantly higher GPX4 protein levels than the control group (7.35 ± 0.13) ($p = 0.0003$). The IR+RePostC group (7.85 ± 0.13) had significantly lower GPX4 levels compared with the IR group ($p = 0.006$), but there was no significant difference between the control group and the IR+RePostC group ($p = 0.30$) (Figure 1).

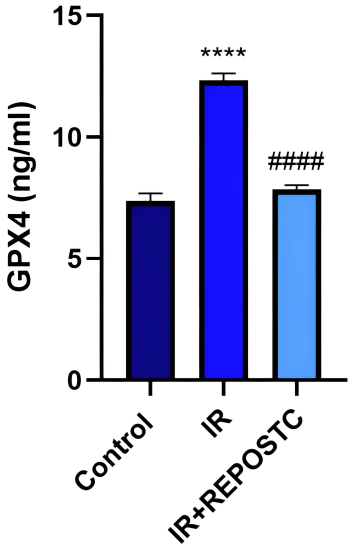

Figure 1. Impact of groups on GPX4 concentrations. ****p<0.0001 when compared to control, and ####p<0.0001 when compared to the IR group.

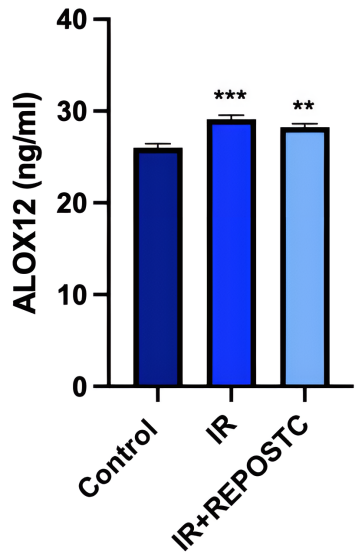

Figure 2. The impact of groups on ALOX12 levels. **p<0.01 in relation to control.

ALOX12

The levels of ALOX12 protein were statistically significantly higher in the IR (29.10 ± 0.45) and IR+RePostC (28.21 ± 0.37) groups than in the control group (26.01 ± 0.43) ($p = 0.0003$). No statistically significant changes were observed in the IR+RePostC group compared with the IR group ($p = 0.30$) (Figure 2).

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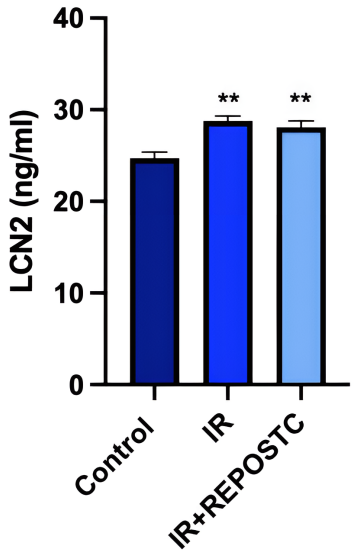

Figure 3. The influence of groups on LCN2 levels. **p<0.01 compared to control.

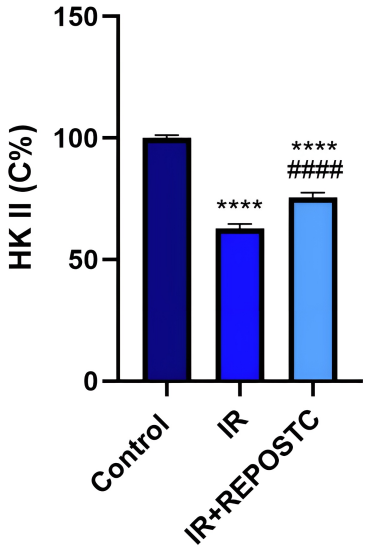

Figure 4. Impact of groups on HKII levels. ****p<0.0001 in comparison to control, ####p<0.0001 in comparison to the IR group.

LCN2

The IR (28.79 ± 0.52) and IR+RePostC (28.09 ± 0.68) groups had significantly higher levels of LCN2 protein than the control group (24.70 ± 0.67) (p = 0.0018). No statistically significant difference was observed between the IR+RePostC and IR groups (p = 0.72) (Figure 3).

HKII

The IR group (62.82 ± 1.92) had significantly lower levels of HKII protein than the control group (100.0 ± 1.13) (p<0.0001). In the IR+RePostC group (75.71 ± 1.79), HKII levels were statistically significantly elevated in comparison to the IR group (p<0.0001) (Figure 4).

DISCUSSION

This study investigated the role of ferroptosis in cellular death pathways related to cerebral I/R injury, the protective effects of remote postconditioning as a mechanical intervention, and its influence on the levels of GPX4, ALOX12, LCN2, and HKII. In the I/R group, HKII were significantly lower compared to the control group, while GPX4 levels were significantly higher. After RePostC administration, the increase in HKII levels and the decrease in GPX4 levels were significant compared to the I/R group. The I/R group exhibited substantially higher levels of ALOX12 and LCN2 than the control group. There was no statistically significant difference between the RePostC treatment group and the I/R group.

Ferroptosis is a regulated form of cell death characterized by iron-dependent lipid peroxidation [25]. Ferroptosis involves the iron-dependent production of lipid reactive oxygen species (L-ROS), the depletion of glutathione (GSH), the inactivation of glutathione peroxidase 4 (GPX4), the disturbance of cellular antioxidant equilibrium, and the consequent accumulation of lipid peroxides, leading to cell death [26].

GPX4, an important antioxidant enzyme, reduces lipid peroxides by converting membrane lipid hydroperoxides (L-OOH) to lipid alcohols (L-OH) with the help of GSH, thereby maintaining membrane stability [27]. GPX4 levels decrease during post-stroke ferroptosis, and upregulation of GPX4 may protect neuronal function from ferroptosis-induced damage [28]. However, Wenyuan et al. reported that GPX4 levels increased following I/R injury [29]. Similar to this finding, our study showed an increase in GPX4 levels after I/R injury; however, a significant decrease was observed in the RePostC-treated group compared to the I/R group. The increase in GPX4 levels may result from an acute adaptive stress response initiated by the cell to protect itself from ferroptosis caused by lipid peroxidation. In contrast, the decrease in GPX4 levels in RePostC-treated groups is thought to be a secondary effect of the treatment's successful reduction of early-stage ROS production and lipid peroxidation.

RePostC. This suggests that RePostC may provide protection against ferroptosis by modulating GPX4 levels.

Ferroptosis is a process of lipid peroxidation caused by iron accumulation via non-enzymatic Fenton reactions. ALOX enzymes and related enzymatic pathways are primarily responsible for this.

ALOX12 induces ferroptosis by initiating lipid peroxidation via the Fenton reaction when Fe^{2+} levels are elevated. In our investigation, serum ALOX12 levels increased following I/R injury, whereas RePostC administration did not produce a statistically significant change. The literature indicates that ALOX12 is activated locally, especially in neuronal and glial cells, suggesting that systemic levels may not accurately represent these intracellular alterations. Future investigations of tissue-specific ALOX12 expression levels will enhance understanding of the mechanisms underlying RePostC.

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Impaired expression of LCN2 may lead to neuronal degeneration and cognitive dysfunction. LCN2 can exacerbate neuronal injury and apoptosis through mechanisms that increase oxidative stress and induce mitochondrial dysfunction [30].

Research indicates that LCN2 increases intracellular Fe2+ levels in neurons, thereby augmenting oxidative damage and promoting ferroptosis [31]. Previous studies have demonstrated that LCN2 facilitates astrocyte activation [32] and exacerbates inflammatory damage in cerebral ischemia via pro-inflammatory cytokines [33]. In our study, serum LCN2 levels increased after IR injury; however, the treatment did not produce a statistically significant change in those levels. RePostC's protective mechanism may not directly affect LCN2 expression. Serum LCN2 may not completely represent tissue alterations; localized neuronal LCN2 variations may occur independently of serum concentrations.

In a model of ischemic stroke, the elimination of HK-II has been shown to exacerbate inflammatory responses and augment cerebral damage. The pro-inflammatory effects observed following deletion of HK-II are thought to be associated with impaired mitochondrial function and accumulation of reactive oxygen species (ROS) [17]. Li et al. established an HK-II-dependent connection between hyperglycolysis and neuroinflammation, proposing that selective inhibition of HK-II induction may represent an innovative therapeutic approach for ischemic stroke [34]. In our study, mitochondrial HK-II levels diminished following I/R injury. This finding corroborates that mitochondrial dysfunction and cell death mechanisms linked to I/R injury are activated, aligning with existing literature on HK-II's role in safeguarding mitochondrial membrane integrity and in energy metabolism. HK-II levels were markedly increased in the groups receiving RePostC compared with the I/R group. According to these results, PostC and RePostC applications may exert neuroprotective effects by increasing HK-II levels.

CONCLUSION

The rise in ferroptosis markers GPX4 and ALOX12 levels post-I/R, suggests that ferroptotic cell death contributes to the injury. Blocking ALOX12 and LCN2 and increasing GPX4 levels may help prevent ferroptosis. The levels of GPX4, ALOX12, LCN2, and HKII may represent viable targets for therapeutic intervention in cerebral I/R injury. The neuroprotective effect of RePostC may be attributed to elevated levels of GPX4 and HKII. The administration of RePostC may induce endogenous tolerance mechanisms that offer protection against I/R injury.

Ethics Committee Approval: Experimental Animal Ethics Committee of Fırat University, Faculty of Medicine (Date: 21.06.2023; Protocol No: 2023/12-07).

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The role of cognitive flexibility in the relationship between pregnancy risk perceptions and vaccine hesitancy in expectant mothers

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MAIN POINTS

- The study included 204 volunteer pregnant women living in Samsun; the average age was 29.5 years, and most were university graduates and middle-income.
- Cognitive flexibility scores were significantly higher among university and postgraduate graduates than among primary school graduates.
- A trend toward higher cognitive flexibility scores with increasing income was observed.
- Vaccine hesitancy varied by employment status; self-employed individuals, in particular, were more hesitant. Pregnant women employed in the public sector have the highest perceived risk.
- Cognitive flexibility did not significantly moderate the relationship between risk perception and vaccine hesitancy.

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ABSTRACT

Aim: Vaccine hesitancy has emerged as a significant public health problem in recent years. The aim of this study was to examine the moderating role of cognitive flexibility in the relationship between pregnant women's risk perceptions and vaccine hesitancy.

Materials and Methods: The study employed a relational screening model. The study population consisted of pregnant women. The sample included 204 participants who were randomly selected and consented to participate. Data were collected using a Personal Information Form, the Vaccine Hesitancy Scale, the Pregnancy Risk Perception Scale, and the Cognitive Flexibility Scale. Data analysis was conducted using SPSS v26 and Hayes' PROCESS Macro Model 1.

Results: The sample's mean age was 29.5 years; most participants had at least a university degree and reported middle-income status, while a majority lived in nuclear families. Participants with university or postgraduate education had higher cognitive flexibility scores than those with primary education. A marginal trend suggested greater cognitive flexibility among participants with higher incomes. Employment status significantly influenced vaccine hesitancy: self-employed women demonstrated higher hesitancy than those who were unemployed, and public-sector employees exhibited the highest levels of risk perception. A moderate positive correlation was observed between cognitive flexibility and vaccine hesitancy. A moderation analysis confirmed that cognitive flexibility did not significantly moderate the relationship between pregnancy risk perception and vaccine hesitancy. The overall model explained 7.8% of the variance in vaccine hesitancy, but was not statistically significant.

Conclusion: The findings underscore the complexity of vaccine hesitancy, indicating that cognitive processes alone may be insufficient to predict vaccination attitudes during pregnancy. Expanding such research to a national scale and integrating qualitative insights could enrich interpretations of how personal and contextual variables jointly shape vaccine hesitancy during pregnancy, ultimately supporting targeted interventions to promote maternal and fetal health.

Keywords: Vaccine hesitancy, Risk perception in pregnancy, Cognitive flexibility, Expectant mothers

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INTRODUCTION

The World Health Organization (WHO) defines vaccine hesitancy as a behavioral pattern resulting from multiple, interacting factors, such as distrust, complacency toward vaccines, and access problems, and emphasizes that these drivers rarely act alone [1]. The SAGE Vaccine Hesitancy Working Group concluded that vaccine hesitancy refers to delay in or refusal to accept vaccination despite the availability of immunization services [2]. Recently, reluctance and refusal to be vaccinated have increased significantly, both globally and in Türkiye. Social opposition to vaccination in Türkiye has increased markedly. According to a study, only 183 families refused vaccination in 2011, whereas this number reached 23,600 as of 2017 [3]. This trajectory underscores the urgency of addressing the interwoven social, cognitive, and structural determinants that shape vaccination decisions.

In her research titled COVID-19 vaccine hesitancy in pregnant women: a systematic review and meta-analysis, conducted by Oashe Bhattacharya [4], she examined studies that reported quantitative data on COVID-19 vaccine acceptance across countries and regions and found that acceptance levels were low among pregnant women. This situation threatens efforts to maintain and improve the effectiveness of immunity policies. As Hobson-West notes, "vaccination is just one of many decisions faced by parents." Other daily concerns related to child health and nutrition may take precedence at certain times or influence willingness to be vaccinated [5]. There is an urgent need for future research to address priority-setting for health behavior change before, during, and after pregnancy [6].

Across all pregnancies, a complication that could threaten the health of the mother or the fetus may occur in approximately 5 to 20 per 100 pregnancies. When pregnancies in Turkey were examined, it was determined that 31.1% and 60.5% of births were risky [7]. Accurately understanding the perception of risk during pregnancy is essential determinant of how women utilize health services, their motivation to seek care, and their decision-making processes regarding childbirth [8,9]. In a study using self-reports from Australian individuals on whether they had received the 2019 influenza vaccine, researchers examined the impact of perceived disease severity on vaccination behavior. Participants were asked, on a scale from 0 to 10, "If you had the flu, how severe would you expect it to be?". 31% anticipated a mild flu, 44% moderate, and 25% severe. Those who perceived influenza as more serious were significantly more likely to be vaccinated than those perceiving moderate severity, while no significant difference emerged between mild and moderate groups [10]. These results reveal that risk perception can be a determinant of vaccination behavior and that higher perceived risk encourages preventive action.

Individual risk perception is a multilayered structure that describes how individuals perceive environmental risks and is directly related to the individual's past experiences, cultural background, and cognitive-perceptual style. Societal norms and value systems can influence which risks are prioritized, while cognitive characteristics, such as cognitive flexibility, attentional processes, and information-processing capacity, play an important role in determining how risk-related information is interpreted and which behavioral responses it elicits. Cognitive flexibility is the capacity to adapt logical strategies and develop alternative solutions to unexpected or complex situations [11]. This ability facilitates coping with uncertainty, supports evaluation of alternative information sources, and fosters development of flexible strategies. Therefore, The relationship between vaccine hesitancy and risk perception depends on how an individual interprets health risks and the attitude that individual develops toward those risks. In this theoretical framework, cognitive flexibility may play a moderating role in the relationship between risk perception and vaccine hesitancy. However, empirical research addressing cognitive flexibility as a mediator or moderator in this context is quite limited, and in Türkiye, studies that jointly examine vaccine hesitancy, risk perception and cognitive flexibility, especially among pregnant women, are not reported in the literature review. This gap reveals the need for comprehensive, model-based research to clarify not only direct relationships but also how cognitive flexibility shapes ambivalence in vaccination decisions.

The main purpose of this study is to evaluate the effect of perceived risk situations experienced by expectant mothers during pregnancy on vaccine hesitancy and to empirically examine the moderating role of cognitive flexibility in this relationship. To this end, answers were sought to the following subquestions:

1. What are the vaccine hesitancy levels of expectant mothers?
2. What are the risk perception levels of expectant mothers during pregnancy?
3. What are the cognitive flexibility levels of expectant mothers?
4. Is there a significant relationship between risk perception and vaccine hesitancy during pregnancy?
5. Does cognitive flexibility moderate the relationship between risk perception and vaccine hesitancy during pregnancy?

MATERIALS AND METHODS

Research model

The relational screening model, one of the screening research models designed to reveal the current status of a particular subject or population, was used in the study. The relational screening model is used to examine the existing or assumed levels of relationship among two or more variables [12].

Sample

This research was approved by the Ondokuz Mayıs University Social and Human Sciences Research Ethics Committee (decision number 2025-424, dated March 28, 2025). The study population comprises pregnant individuals residing in Samsun. The sample group was composed of 204 participants who applied to the Obstetrics and Gynecology Outpatient Clinic of Ondokuz Mayıs University Hospital and voluntarily agreed to take part in the study. These participants were selected through convenience sampling. Convenience sampling was preferred because it allowed practical and timely access to pregnant individuals who regularly visited the outpatient clinic during the data collection period. The sample size for this study was determined in accordance with established

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Table 1. Demographic information of the study group.

	N	Min.	Max	Mean	SD
Age	204	19.00	41.00	29.50	4.78
Marriage Age	204	14.00	38.00	24.23	3.91
				N	%
Educational Status		Primary education		39	19.1
		High school		65	31.9
		University		93	45.6
		Postgraduate		7	3.4
		Total		204	100.0
Financial Income		Low		25	12.3
		Middle		169	82.8
		High		10	4.9
		Total		204	100.0
Employment Status		Not working		136	66.7
		Public personnel		30	14.7
		Private sector employee		34	16.7
		Private sector own business		4	2.0
		Total		204	100.0
Family Structure		Nuclear family		186	91.2
		Extended family		18	8.8
		Total		204	100.0

Table 2. Statistical information regarding the data collection tool.

	Number of Items	Item Load Ranges	Explained Variance	Cronbach Alpha	KMO	p-value	Skewness		Kurtosis	
							Statistics	SE	Statistics	SE
Vaccine Hesitancy Scale	10	0.36-0.86	67.75%	0.84	0.88	<0.001	-0.56	0.17	-0.2	0.34
Cognitive Flexibility Scale	12	0.25-0.61	51.12%	0.70	0.74	<0.001	0.22	0.17	-0.28	0.34
Risk Perception Scale in Pregnancy	9	0.31-0.68	55.71%	0.82	0.77	<0.001	0.18	0.17	-0.82	0.34

methodological recommendations in the literature. According to Tavşancıl, the minimum sample size in scale-based research should be at least five times the total number of items [13]. Given that the measurement tools used in this study included 31 items, the minimum sample size was calculated to be 155, with an additional allowance of approximately 10% to account for potential data loss or exclusions. Furthermore, Aiken and West emphasize that moderation analyses often require larger samples, typically 200 or more participants, because of the generally low statistical power associated with interaction effects [14]. Therefore, conducting the analysis with 204 participants meets both the item-based sample-size criterion and the recommended methodological standard for moderation analysis.

Table 1 below shows descriptive statistics for the demographic characteristics of the study group.

Data collection tools and processes

The study used a researcher-prepared personal information form and three different scales as data collection tools.

Personal information form

It consists of questions aimed at obtaining the participants' descriptive information, such as age, age at marriage, education level, income, employment status, and family structure.

Cognitive flexibility scale

The Cognitive Flexibility Scale, developed by Martin and Rubin, consists of 12 items and a single dimension [15]. BEÖ is a 6-point Likert-type measurement tool, with responses ranging from 1 "strongly disagree" to 6 "strongly agree." In this study, the measurement tool had an internal consistency coefficient (α) of 0.80 and a test-retest reliability coefficient of 0.83. In the reliability study conducted by Martin and Anderson [16], the internal consistency of the BES was reported as 0.72, 0.73, and 0.81. The positive and significant correlation between CFS scores and self-efficacy scores for communication skills and related behaviors, together with findings that individuals form positive friendships as their cognitive flexibility increases, also support the criterion-related validity of the CFS [15]. The scores obtainable from the measurement tool, in which items 2, 3, 6, and 10 are reverse-scored, range from 10 to 60. Increased scores indicate a high level of cognitive flexibility. In another study, Maltby et al. [17] reported

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an internal consistency coefficient for the BÖ of 0.92 [18].

Risk perception scale in pregnancy

The scale was developed by Heaman and Gupton [19] to assess perceived risk during pregnancy, and its Turkish reliability and validity were evaluated by Evcili and Dağlar [20]. The scale comprises nine items: five relate to pregnant women's risk perception towards their babies, and four relate to pregnant women's risk perception towards themselves. The scale includes the response options "no risk" and "extremely high risk" for each item. These statements are marked on a 0-10 cm linear scale according to pregnant women's risk perceptions. The total scale score is calculated by dividing the sum of all item scores by nine. Scores on the GRA scale range from 0 to 10. An increase in the score on the scale is interpreted as an increased risk perception among pregnant women regarding themselves and their babies. The Cronbach's Alpha coefficient of the scale was found to be 0.84. In this study, the coefficient was found to be highly reliable (0.90).

Vaccine hesitancy scale

The Turkish validity and reliability analysis of the vaccine hesitancy scale developed by Larson et al. [21] was conducted by Aslan et al. [22]. In this study, the WHO Vaccine Hesitancy Scale, adapted to Turkish culture by Aslan et al. [22] and shown to be usable by Turkish parents, was administered in Turkish. Responses to this 10-item, 5-point Likert-type scale range from "Strongly agree" to "Strongly disagree". While questions 5, 9, and 10 on the scale are negatively worded, all other questions are positively worded. Higher scores for positive statements are associated with lower vaccine hesitancy, whereas higher scores for negative statements are associated with higher vaccine hesitancy. Therefore, responses 5, 9, and 10 were reverse-coded. Therefore, as the total score on the scale increases, the level of vaccine hesitancy decreases. There is no cut-off point that distinguishes those who are hesitant from those who are not.

Statistical analysis

Data from study participants collected using "Vaccine Hesitancy", "Cognitive Flexibility", and "Risk Perception in Pregnancy" instruments were analyzed using IBM SPSS Statistics version 26.0 [23] and Hayes PROCESS model macros. A total of 220 participant responses were transferred to the analysis program. Before analysis, outlier and extreme-value analyses were conducted on the dataset, and participants who negatively impacted the dataset's reliability were excluded from the study. In the statistical evaluation of the data, the assumption of normality was first examined using the Kolmogorov-Smirnov and Shapiro-Wilk tests ($P > 0.05$) and by calculating skewness and kurtosis coefficients: skewness = 0.39 ± 0.17 for age and −0.44 ± 0.34 for marriage age; kurtosis = 0.52 ± 0.17 for age and 1.19 ± 0.34 for marriage age. After removing 11 outlier responses and 5 extreme responses from the data set, the analysis proceeded the responses of 204 participants. Table 2. below provides statistical information regarding the validity and reliability of the data collection tools in the current study.

Table 2 indicates that the Vaccine Hesitancy Scale (10 items; loadings. 0.36–0.86) explains 67.75% of variance with high internal consistency (α =0.84; KMO = 0.88; $p<0.001$), the Cognitive Flexibility Scale (12 items; loadings. 0.25–0.61) explains 51.12% (α = 0.70; KMO=0.74; $p<0.001$), and the Pregnancy Risk Perception Scale (9 items; loadings. 0.31–0.68) explains 55.71% (α=0.82; KMO=0.77; $p<0.001$). Skewness/kurtosis values (Vaccine Hesitancy: −0.56/−0.20; Cognitive Flexibility: 0.22/−0.28; Risk Perception: 0.18/−0.82) fall well within Kline's thresholds (±3, ±10), supporting approximate normality [24]. Following George and Mallery, α values between 0.70–0.90 evidence acceptable–good reliability [25]. Independent-samples t tests were applied to dichotomous groupings, one-way ANOVA to multigroup comparisons, and Pearson correlations to examine associations among instruments. Following significant ANOVA results, Bonferroni post hoc tests were conducted to identify pairwise group differences. Effect sizes were interpreted via η^2 with [26]. 0.01, 0.06, and $\geq$0.14 denoting small, medium, and large effects, respectively. Moderation was tested using Hayes PROCESS Model 1 to evaluate whether the association between X (pregnancy risk perception) and Y (vaccine hesitancy) varies by W (cognitive flexibility) [27].

This study tests the effect of pregnancy risk perception (X) on vaccine hesitancy (Y) and examines the moderating role of cognitive flexibility (W) within the framework of Hayes PROCESS Model 1, with X, W, and X×W terms included in the model. Statistical significance of the effects was tested using multiple linear regression.

RESULTS

This section presents findings from statistical analyses examining the moderating role of cognitive flexibility in the relationship between vaccine hesitancy and risk perception during pregnancy.

Examination of Table 3 indicates that education level did not yield significant group differences in vaccine hesitancy ($F=0.765$, $p=0.515$, $\eta^2=0.011$) or pregnancy risk perception ($F=0.492$, $p=0.688$, $\eta^2=0.007$; $F=2.777$, $p=0.052$, $\eta^2=0.040$, with university (M=55.36 ±7.78) and postgraduate (M=57.57±5.85) participants scoring higher than primary-school graduates (M=51.94±7.48). Financial income showed no significant effects on hesitancy ($F=1.365$, $p=0.258$, $\eta^2=0.013$) or risk perception ($F=0.700$, $p=0.498$, $\eta^2=0.006$), whereas flexibility in the high-income group yielded ($F=2.384$, $p=0.095$, $\eta^2=0.023$; high M=58.10±4.01 vs. low M=52.04±7.88). Employment status mattered: vaccine hesitancy varied significantly ($F=2.776$, $p=0.042$, $\eta^2=0.040$), with self-employed individuals reporting higher hesitancy (M=45.25±2.21) than unemployed peers

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Table 3. Comparison of participants' vaccine hesitancy, cognitive flexibility, and risk perception levels during pregnancy according to their socio-demographic characteristics.

	Vaccine Hesitancy					Cognitive Flexibility					Risk Perception in Pregnancy				
Variables	N	Mean±SD	Test (F/t)	η2	p-value	N	Mean±SD	Test (F/t)	η2	p-value	N	Mean±SD	Test (F/t)	η2	p-value
Educational Status															
Primary Education	39	36.35±7.12				39	51.94±7.48				39	3.41±1.87			
High School	65	35.06±6.89	0.765	0.011	0.515	65	53.24±7.03	2.777	0.040	0.052	65	3.33±1.80	0.492	0.007	0.688
University	93	36.84±7.71				93	55.36±7.78				93	3.68±1.94			
Postgraduate	7	36.14±7.90				7	57.57±5.85				7	3.58±1.68			
Material Income															
Low	25	33.92±7.46				25	52.04±7.88				25	3.44±1.63			
Medium	169	36.50±7.30	1.365	0.013	0.258	169	54.18±7.57	2.384	0.023	0.095	169	3.49±1.91	0.700	0.006	0.498
High	10	35.90±7.47				10	58.10±4.01				10	4.20±1.72			
Working Status															
Unemployed	136	36.04±6.98				136	53.59±7.39				136	3.56±1.84			
Public Employee	30	37.16±7.51	2.776	0.040	0.042	30	56.43±8.84	1.184	0.017	0.317	30	4.26±2.00	4.159	0.059	0.007
Private Sector	34	34.67±8.30				34	54.02±7.09				34	2.66±1.65			
Self-Employed	4	45.25±2.21				4	55.00±2.30				4	3.61±1.19			
Family Structure															
Nuclear	186	36.44±7.33	1.754	0.015	0.081	186	54.33±7.70	1.38	0.009	0.169	186	3.56±1.89	1.004	0.005	0.317
Extended	18	33.27±7.01				18	51.77±5.02				18	3.09±1.57			

Table 4. Pearson correlation analysis results.

Variables		2	3	4	5
1. Age	r p	0.398** p<0.01	0.046 p>0.05	0.009 p>0.05	0.117 p>0.05
2. Age of Marriage	r p		0.188** p<0.01	0.142* p<0.05	0.147* p<0.05
3. Vaccine Hesitancy	r p			0.271** p<0.01	-0.004 p>0.05
4. Cognitive Flexibility	r p				0.069 p>0.05
5. Pregnancy Risk Perception	r p	- -	- -	- -	- -

N=204, p<0.05=*, p<0.01**

Table 5. Moderation analysis results (Hayes PROCESS Model 1).

Dependent Variable: Vaccine Hesitancy	B (β)	SE	t-value	p-value	95% Lower Limit	95% Upper Limit
Constant	28.623	7.180	3.99	<0.001	14.465	42.781
Risk Perception in Pregnancy (X)	-1.824	1.755	-1.04	0.300	-5.284	1.636
Cognitive Flexibility (W)	0.145	0.130	1.12	0.266	-0.112	0.402
Interaction (X × W)	0.032	0.032	1.00	0.317	-0.031	0.094

(M=36.04±6.98), and pregnancy risk perception differed across sectors (F=4.159, p=0.007, η^2=0.059), peaking among public personnel (M=4.26±2.00) and lowest in private employees (M=2.66±1.65), Cognitive flexibility is not affected by employment status (F=1.184, p=0.317, η^2=0.017). Finally, family structure produced no statistically significant differences in vaccine hesitancy (F=1.754, p=0.081, η^2=0.015), cognitive flexibility (F=1.380, p=0.169, η^2=0.009), or pregnancy risk perception (F=1.004, p=0.317, η^2=0.005).

Examination of Table 4 revealed a significant positive relationship between age and age of marriage (r=0.398, p<0.01). Age at marriage was found to be slightly positively correlated with vaccine hesitancy (r=0.188, p<0.01), cognitive flexibility

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(r=0.142, p<0.05), and pregnancy risk perception (r=0.147, p<0.05). A moderate, positive correlation was observed between vaccine hesitancy and cognitive flexibility (r=0.271, p<0.01). The relationships between other variables are not statistically significant (p>0.05).

Table 5 shows the results of the moderation analysis. In the Hayes PROCESS Model 1 moderation test, we examined pregnancy risk perception (X), cognitive flexibility (W), and their interaction (X×W) as predictors of vaccine hesitancy (Y). The model was statistically significant overall (F(3, 200) = 6.31, p=0.004), explaining 7.8% of the variance (R^2 = 0.078). Risk perception during pregnancy negatively, but non-significantly, predicted hesitancy (B=−1.824, SE=1.755, t=−1.04, p=0.300, 95% CI [−5.284, 1.636]); cognitive flexibility showed a small, non-significant positive association (B=0.145, SE=0.130, t=1.12, p=0.266, 95% CI [−0.112, 0.402]); the interaction term did not indicate moderation (B=0.032, SE=0.032, t=1.00, p=0.317, 95% CI [−0.031, 0.094]).Despite the importance of the model, risk perception and cognitive flexibility do not explain the variations in vaccine hesitancy. This indicates that other factors may come into play in determining hesitancy, and that cognitive flexibility does not moderate the relationship between risk perception and hesitancy under current circumstances.

DISCUSSION

This study aimed to analyze the relationship between vaccine hesitancy and risk perception among pregnant individuals and to examine the moderating effect of cognitive flexibility on this relationship. Using Hayes' PROCESS Model 1, we tested the effects of pregnancy risk perception (X), cognitive flexibility (W), and their interaction (X×W) on vaccine hesitancy. Although the overall model was statistically significant, its explanatory power was low (R^2 = 0.078), suggesting that additional determinants of vaccine hesitancy were not included, such as health literacy, trust, and religious-spiritual orientations. The independent variable, pregnancy risk perception, negatively predicted vaccine hesitancy, but not significantly, consistent with findings that women's total risk perception scores during pregnancy tend to be low [28]. Cognitive flexibility did not significantly predict hesitancy, and the interaction term (Risk Perception × Cognitive Flexibility) did not yield a significant moderating effect. These results indicate a multifactorial structure in which cognitive flexibility may be related to hesitancy but does not alter the risk perception–hesitancy link in this sample.

By educational level, no significant differences emerged in vaccine hesitancy or pregnancy risk perception, indicating that attitudes cannot be explained solely by academic attainment and may instead be shaped by cognitive and sociocultural factors. This aligns with Sevinç et al. [29], who reported no relationship between education and vaccine hesitancy. Yet other evidence is mixed: Çetin and Söğüt [30], identified education as a factor, finding higher vaccine opposition among primary school graduates; Çınar et al. [31] observed that high school or higher education was associated with increased tetanus vaccination among pregnant women; and Afolabi et al. [32] concluded that lower education was associated with greater hesitancy toward hepatitis B vaccination. Regarding pregnancy risk perception, Gözüyeşil and Özertürk [7] determined that overall levels were low, with risk perception for self and baby peaking in the second trimester, a pattern congruent with our findings.

A significant effect of education on cognitive flexibility was observed: Participants with university and postgraduate education scored higher than primary school graduates, consistent with Oral and Kolburan [33]. No significant differences in vaccine hesitancy or pregnancy risk perception were observed across levels of financial income. Prior work links lower education and income to higher anxiety, which may complicate information-seeking and communication with health services [34]. In our data, cognitive flexibility exhibited a marginal income gradient, with higher scores in the upper-income group, whereas Saadi [35] reported no statistically significant association; this discrepancy may reflect our study population or its relatively high socioeconomic setting.

Employment status was important. Self-employed individuals exhibited significantly higher vaccine hesitancy than the unemployed; we found no directly comparable results in the literature, and this pattern may stem from broader interpersonal networks and greater exposure to heterogeneous opinions among the self-employed. Risk perception differed by sector: it was highest among public-sector employees and lower among private-sector employees, plausibly linked to occupational risk profiles such as stress, chemical exposures, sharps injuries, hepatitis B, and violence-related injuries [36]. Cognitive flexibility did not differ by employment status. Family structure (nuclear vs. extended) was not associated with significant differences in vaccine hesitancy, cognitive flexibility, or risk perception, and we found no studies assessing these variables jointly.

Age and age at marriage were positively correlated, and age at marriage was slightly positively correlated with vaccine hesitancy, cognitive flexibility, and risk perception. Prior findings are nuanced: Oral and Kolburan [33] noted high cognitive flexibility at ages 26–35, whereas Altunkol [37] and Dağgeçcen Başsu [38] reported no significant age–flexibility link, and Xia et al. [39] found that cognitive flexibility decreases in older adults. We observed a moderately positive, statistically significant relationship between vaccine hesitancy and cognitive flexibility. Sevinç et al. [29] reported a negative correlation between age and vaccine hesitancy. Importantly, participants with lower cognitive flexibility have been shown to endorse more personal, but not external, barriers to vaccination [40], emphasizing that vaccine hesitancy is multidimensional and that different mechanisms may underlie personal versus structural influences. Overall, our results highlight limited moderation by cognitive flexibility and underscore the need

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for holistic models integrating cognitive, sociocultural, and structural determinants.

CONCLUSION

Findings indicate a moderately positive and statistically significant association between vaccine hesitancy and cognitive flexibility, whereas relationships among the remaining constructs were not significant. Within the Hayes PROCESS Model 1 framework, pregnancy risk perception (X), cognitive flexibility (W), and their interaction (X×W) were entered as predictors of vaccine hesitancy. The model was statistically significant overall (R^2 = 0.078), explaining 7.8% of the variance; however, neither X, W, nor X×W reached significance. These results imply that pregnancy risk perception does not directly predict vaccine hesitancy, that cognitive flexibility alone is not a sufficient determinant of vaccine hesitancy, and that cognitive flexibility does not moderate the link between pregnancy risk perception and vaccine hesitancy. The study is limited to Samsun Province, which constrains generalizability; therefore, multi-site sampling across Türkiye is warranted. Future research should complement these quantitative findings with qualitative inquiry and develop integrative models that test additional psychosocial determinants, such as information literacy, health anxiety, trust, and conspiracy beliefs, as potential mediators or moderators. Including pregnant women, their spouses/partners, healthcare professionals, and recent mothers could yield a more comprehensive map of societal perspectives and vaccination decision-making dynamics. Pregnant women participating in the study were not asked whether they had been vaccinated , which constituted a limitation. Another important limitation of this study is that no questions were asked regarding the differential diagnosis of vaccine rejection in expectant mothers.

Ethics Committee Approval: The research was approved by the Ondokuz Mayıs University Social and Human Sciences Research Ethics Committee (decision number 2025-424, dated March 28, 2025).

Informed Consent: Participants were informed about the purpose of the study, their voluntary consent was obtained after explaining the study's potential contributions to women's health and the scientific literature. The study was completed face-to-face, with the understanding that the data would be used only for scientific purposes.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that there are no conflicts of interest.

Author Contributions: Conception: HT; Design: ED; Supervision: YB; Fundings: SÇ; Materials: ED; Data Collection and/or Processing: HT; Analysis and/or Interpretation: SÇ; Literature Review: ED; Writing: HT; Critical Review: YB.

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Evaluating awareness of lysosomal storage diseases: A step toward early diagnosis

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MAIN POINTS

- Most physicians demonstrated limited awareness of lysosomal storage diseases (LSD).
- Only a minority of physicians recognized that LSD can manifest at any age, indicating that adult-onset cases may be overlooked.
- Despite the availability of non-invasive diagnostic methods, many physicians still include invasive procedures, such as bone marrow aspiration and liver biopsy, in the diagnostic process.
- The findings highlight the urgent need for targeted educational programs to enhance awareness and promote early diagnosis of LSD.

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ABSTRACT

Aim: Lysosomal storage diseases (LSD) are a diverse group of rare inherited metabolic disorders that frequently go undiagnosed for extended periods because of their non-specific, overlapping clinical features. Raising awareness among healthcare professionals is crucial for facilitating early diagnosis and improving outcomes. This study aimed to evaluate physicians' awareness of and knowledge about five LSDs: Gaucher disease, Fabry disease, mucopolysaccharidoses, Pompe disease, and Niemann--Pick types A/B.

Materials and Methods: A descriptive cross-sectional survey was conducted among 106 physicians, including 56 pediatricians and 50 internal medicine specialists. A 17-item online questionnaire assessed participants' knowledge of LSD symptoms, diagnosis, and treatment.

Results: 81.9% of the physicians participating in the study reported limited knowledge of LSD. When the pediatric and internal medicine groups were compared, pediatric specialists generally exhibited higher levels of awareness than their internal medicine counterparts. This difference was statistically significant for MPS and Gaucher disease (p=0.016 and p=0.012, respectively). Among the participants, only 25.7% recognized that LSD can occur at any age. 86.7% of respondents identified genetic testing as accurate, while 38% and 58.1% indicated roles for bone marrow aspiration and liver biopsy, respectively, in diagnosis. The vast majority of physicians (99%) emphasized the need for increased training in this field.

Conclusion: Our study demonstrates that physicians working at a tertiary training hospital lack sufficient awareness of LSD. Therefore, strengthening educational programs for physicians is essential to enhance awareness and improve early diagnosis of the disease.

Keywords: Lysosomal storage diseases, Awareness, Early diagnosis

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INTRODUCTION

Lysosomal storage diseases (LSD) are a heterogeneous group of genetically inherited disorders; they result from deficiencies or functional impairments of lysosomal enzymes, this leads to widespread damage across organs and systems [1]. Although rare, these conditions can be life-threatening. The diagnostic and therapeutic processes are often challenging and prolonged, as symptoms are usually recognised late, and clinical manifestations may overlap with those of more common disorders. Awareness of the symptoms of LSD and recognition of the importance of early diagnosis and treatment play a crucial role in improving diagnostic timelines and patient outcomes. Furthermore, novel therapeutic approaches, such as enzyme replacement therapy (ERT), substrate reduction therapy (SRT) and gene therapy, are being developed at an increasing rate, which further emphasises the importance of early detection [2]. Accordingly, raising awareness among healthcare professionals of LSD is critical to ensuring early diagnosis and access to effective treatment.

This study aimed to evaluate the knowledge of physicians from various specialties regarding five LSDs: Gaucher disease, Fabry disease, mucopolysaccharidoses (MPS), Pompe disease, and Niemann–Pick types A/B, which are considered more prevalent in our region than in other regions.

The findings are expected to contribute to the development of strategies aimed at improving awareness and facilitating earlier recognition of these disorders.

MATERIALS AND METHODS

Study design

This was a cross-sectional descriptive survey. The questionnaire was prepared using the online platform Google Forms and distributed by email and through social media to 226 physicians working in the Departments of Pediatrics and Internal Medicine at the Adana City Training and Research Hospital. Participants were asked to respond voluntarily. No names or personal identifiers were collected from the physicians included in the study. A total of 106 physicians completed the survey, including 56 pediatricians and 50 internal medicine specialists (response rate: 46.9%). Data collection was carried out between April and May 2025. The study protocol was designed in accordance with the 1964 Helsinki Declaration and approved by the Ethics Committee of Adana City Training and Research Hospital (Decision No: 478, Date: 10.04.2025).

Questionnaire design

The survey comprised 17 questions designed to evaluate physicians' professional experience and their knowledge of LSD, as well as their awareness of potential clinical manifestations, diagnostic approaches, treatment methods, and educational requirements. Participants' specific knowledge of and ability to recognize symptoms related to Gaucher, Fabry, MPS, Pompe, and Niemann-Pick A/B diseases were also evaluated. Most of the questions were multiple-choice or allowed multiple responses, with open-ended responses limited to the 'Other, please specify' option. The survey was developed based on input from a pediatric metabolism specialist and was not adapted from previously published instruments; however, pilot testing was not conducted.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics for Windows version 23.0 (IBM Corp., Armonk, NY, USA). The distributions of numerical variables were assessed for Kolmogorov-Smirnov test. Continuous variables were presented as means ± standard deviation or medians (minimum [min]-maximum [max]) according to distribution of data and categorical variables as numbers and percentages (%). Statistical analyses were conducted using tests appropriate for the type and distribution of the variables. Categorical variables were compared using the Chi-square test. A p-value <0.05 was considered statistically significant.

RESULTS

81.9% of the participants considered their knowledge of LSD inadequate. When awareness of LSD was analyzed separately among pediatric specialists (N = 56) and internal medicine specialists (N = 50), the majority of participants in both groups reported having "no knowledge" or "limited knowledge" of these diseases (pediatric specialists: 3.4% "no knowledge", 77.6% "limited knowledge"; internal medicine specialists: 6.7% "no knowledge", 78% "limited knowledge"). A smaller proportion of participants in both groups reported a moderate level of knowledge (pediatric specialists, 19%; internal medicine specialists, 15.6%). There was no statistically significant correlation between years of experience and knowledge level among physicians (p=0.479). However, when the pediatric and internal medicine groups were compared, pediatric specialists generally exhibited higher awareness than their internal medicine counterparts (Figure 1). This difference was found to be statistically significant for MPS and Gaucher disease (p=0.016 and p=0.012, respectively). Of the participants, 66.7% indicated that LSDs manifest during infancy and childhood, while 25.7% believed that they could occur at any age. When assessing knowledge levels for five different LSD, Gaucher disease received the highest proportion of participants reporting a 'moderate level of knowledge'; (76.3%), while Niemann-Pick A/B disease received the lowest (31.1%). The highest rates of correct responses to questions about commonly observed symptoms were obtained for MPS (91.4%) and Gaucher disease (86.7%) (Table 1). In terms of diagnostic methods, enzyme analysis (98.1%) and genetic testing (86.7%) were the options most frequently indicated by the participants. Thirty-eight per cent of participants reported that bone marrow aspiration plays a role in diagnosis, while 58.1 per cent reported a role for liver biopsy. In terms of treatment, 85.7% of the participants were aware that ERT was used. When responses regarding treatment for the five lysosomal storage diseases (Gaucher, Fabry, MPS, Pompe, and Niemann-Pick A/B) were evaluated, most participants (95.2%) reported that treatment improved outcomes. Only 2.9% indicated that the treatment had no effect on improvement. The majority of participants (85.7%) acknowledged the availability of ERT as a treatment option, and 44.8% were aware of SRT. Additionally, 95.2% of participants reported that early diagnosis and treatment facilitate improvement; 99% indicated that educational programs for physicians should be improved.

DISCUSSION

Individuals with rare diseases, such as LSD, often initially present to the departments of pediatrics and internal medicine. It is crucial that these physicians are aware of these diseases to enable early diagnosis and treatment, and to improve the patients' quality of life. However, these conditions may not exhibit obvious clinical signs initially, and metabolic conditions in adults can be easily overlooked. According to the National Commission on Orphan Diseases, 30% of individuals with rare diseases wait up to five years to receive an accurate diagnosis, whereas 15% require six years or longer [3]. Taking a detailed family history, accurately documenting symptoms, and ensuring timely referral to the relevant specialist can reduce diagnostic delays and enable earlier intervention. Therefore, it is crucial for physicians to consider LSD in their clinical practice and in differential diagnoses.

In our study, although most clinicians were aware of LSD,

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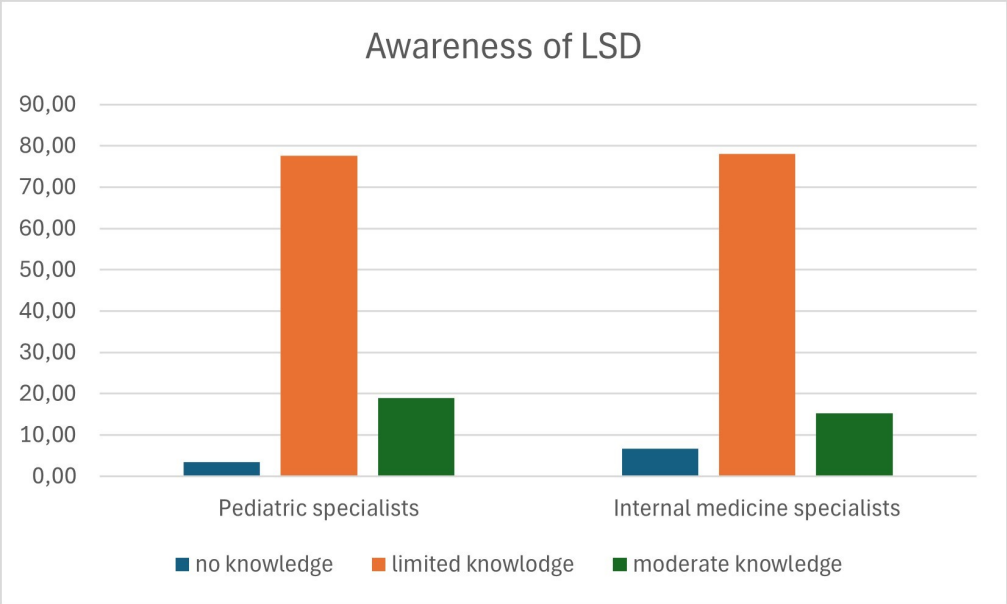

Figure 1. Awareness levels regarding lysosomal storage diseases among pediatric and internal medicine specialists.

Table 1. Distribution of clinical and laboratory findings among patients with different lysosomal storage disorders(LSDs)as reported by participating physicians.

Disease	Findings	Occurs n (%)	Does not occur n (%)
Gaucher Disease	Splenomegaly	91 (86.7)	13 (12.4)
	Hepatomegaly	95 (90.5)	9 (8.6)
	Bone pain	40 (38.1)	64 (61.0)
	Thrombocytopenia	64 (61.0)	40 (38.1)
	Neurological disorder	65 (61.9)	39 (37.1)
	Respiratory difficulty	20 (19.0)	84 (80.0)
Fabry Disease	Neuropathic pain	31 (29.5)	73 (69.5)
	Renal problem	82 (78.1)	22 (21.0)
	Angiokeratoma	48 (45.7)	55 (52.4)
	Cardiac involvement	56 (53.3)	48 (45.7)
	Visual disturbance	45 (42.9)	59 (56.2)
Mucopolysaccharidoses	Coarse facial features	96 (91.4)	8 (7.6)
	Neurological findings	91 (86.7)	13 (12.4)
	Joint restriction	69 (65.7)	35 (33.3)
	Joint laxity	28 (26.7)	76 (72.4)
	Heart valve disease	43 (41.0)	61 (58.1)
	Autism features	12 (11.4)	91 (86.7)
	Organomegaly	37 (35.2)	66 (62.9)
	Hearing loss	46 (43.8)	58 (55.2)
	Corneal clouding	25 (23.8)	79 (75.2)
Pompe Disease	Muscle weakness	78 (74.3)	26 (24.8)
	Elevated CK	71 (67.6)	33 (31.4)
	Cardiomegaly	68 (64.8)	36 (34.3)
	Respiratory failure	40 (38.1)	64 (61.0)
Niemann-Pick A/B	Hepatosplenomegaly	69 (65.7)	34 (32.4)
	Neurological findings	83 (79.0)	21 (20.0)
	Cholestasis	39 (37.1)	65 (61.9)
	Thrombocytopenia	27 (25.7)	77 (73.3)
	Respiratory problems	28 (26.7)	76 (72.4)

81.9% felt inadequately informed about these conditions. Furthermore, the lack of a significant relationship between years of professional experience and awareness levels suggests that clinical experience alone is insufficient for acquiring adequate knowledge of these rare diseases, highlighting the need for targeted educational programs. Most inherited metabolic diseases are thought to present with clinical findings shortly after birth. Consequently, metabolic disorders that present

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with symptoms in late childhood or adulthood are often not initially suspected [4]. In our study, the low proportion of participants who selected the option indicating that LSD can present with symptoms at any age suggests that these conditions could be overlooked in adult patients. Furthermore, pediatric specialists were found to have a higher awareness of LSD than internal medicine specialists. These findings suggest that cases diagnosed in adulthood may be overlooked and emphasise the importance of raising awareness among adult physicians.

Participants most frequently reported a moderate level of knowledge about Gaucher disease, possibly because it is more commonly encountered than other LSDs. A survey study conducted in Japan found that hematologists had a greater awareness of Gaucher disease than gastroenterologists [5]. This difference was attributed to hematologists' tendency to work in larger hospitals, which provides them with greater opportunities for clinical experience and professional interaction related to the disease. By contrast, gastroenterologists, who often practise in smaller clinics, were reported to have more limited awareness of, and knowledge about, treatment. The lowest rate of "moderate level of knowledge" responses was observed for Niemann-Pick A/B disease, which presents with similar clinical features. This finding suggests that Gaucher disease is more frequently considered in the differential diagnosis of splenomegaly and thrombocytopenia, whereas Niemann-Pick A/B disease is often overlooked.

The higher correct response rates for common symptoms in MPS and Gaucher disease may reflect their more distinctive clinical features and more frequent clinical exposure. MPS cases often involve multiple systemic manifestations such as cardiac findings, corneal clouding, recurrent ear infections, hearing loss, hernias, and spinal deformities; therefore, patients are frequently referred to various medical specialties [6]. Therefore, it is crucial that not only pediatricians but also clinicians from other specialties recognize the characteristic manifestations of this disease. This highlights the need for broader studies assessing awareness across various medical disciplines. Coarse facial features are among the most frequent and diagnostic clinical signs of MPS. In our study, the majority of participants correctly identified this clinical feature associated with MPS. However, the literature indicates that diagnostic delays are common, particularly in patients with mild disease phenotypes. A survey by Bruni et al. emphasized that MPS type I is not widely recognised, and that training in differential diagnosis is needed to promote an earlier diagnosis [7]. Although a majority of participants correctly identified the most common clinical feature of MPS, 80% reported having 'no knowledge' or 'limited knowledge' of the disease. This suggests that physicians tend to focus primarily on the most obvious and typical symptoms of MPS, potentially overlooking milder or atypical symptoms.

It has been reported that there is usually a diagnostic delay of at least three years in Fabry disease, and the diagnosis is most often established after the age of 20 [8]. Bulbul et al. evaluated the awareness of physicians working in the province of Kırıkkale regarding Fabry disease and reported that only 22% of them considered Fabry disease as a differential diagnosis in patients presenting with compatible clinical findings [9]. In our study, 75.7% of the participants reported having no or limited knowledge about Fabry disease; this indicates insufficient awareness among physicians. It has been reported that peripheral neuropathic pain is the most common presenting complaint among patients with Fabry disease and is included as the first step in most diagnostic algorithms [10]. However, when Fabry-related symptoms were evaluated in our study, only 29.5% of participants reported peripheral neuropathic pain. This suggests that peripheral neuropathic pain is not widely recognized by physicians. Conversely, the results suggest that physicians are more familiar with the association between Fabry disease and renal complications in patients with diabetes. These findings suggest that awareness of the early clinical manifestations of Fabry disease is limited, with peripheral neuropathic pain , a characteristic symptom potentially, being overlooked.

The clinical presentation of Pompe disease is highly heterogeneous in terms of age at symptom onset, rate of disease progression, extent of organ involvement and overall clinical severity [11,12]. Clinical manifestations may range from the rapidly progressive classic infantile form, presenting with cardiomyopathy before the age of one, to the more slowly progressive late-onset forms. A registry study conducted by Kishnani et al. in 2013 demonstrated, as in previous reports, a significant diagnostic delay among patients with Pompe disease [13-15]. Although ERT has been available since 2006, and diagnostic methods such as enzyme activity measurement and genetic analysis are used, many patients still experience a prolonged interval between the onset of symptoms and diagnosis [12,16,17]. In our study, the overall level of knowledge about Pompe disease among participants was low. This suggests that awareness of Pompe disease is limited, which, consistent with the literature, may contribute to diagnostic delays.

A notable finding of our study is that despite the widespread use of enzyme and genetic analyses in current diagnostic processes, a significant proportion of physicians still believe that invasive methods (e.g., bone marrow aspiration and liver biopsy) play a role in diagnosis. This suggests that traditional approaches still influence the diagnosis of LSD and highlights the need to raise awareness of non-invasive, molecular-based methods. Regarding knowledge of treatment approaches, the majority of participants indicated that they believed treatment improves outcomes. While most physicians correctly identified ERT as a treatment option, fewer recognized substrate reduction therapies. This suggests that physicians are more familiar with ERT as the primary treatment for lysosomal storage diseases, while awareness of alternative approaches is relatively low. This finding is consistent with the results of A study was conducted among hematologists and

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gastroenterologists in Japan [5]. The aforementioned study found that a significant proportion of hematologists were familiar with ERT. However, awareness of SRT was lower. This suggests that knowledge regarding the diagnosis and treatment of rare metabolic diseases remains limited.

In our study, participants generally indicated that early diagnosis and treatment positively influence disease progression in lysosomal storage diseases. Furthermore, a clear emphasis was placed on the need for enhanced educational initiatives for physicians, which may facilitate earlier recognition in clinical practice.

Limitations

This study had several limitations. Firstly, the sample size was relatively small, comprising only pediatric and internal medicine specialists from a single centre, which limits the generalizability of the findings. The voluntary nature of participation may have resulted in a higher proportion of interested physicians enrolling, thereby introducing selection bias. Moreover, the survey was not pre-tested, which may have resulted in participants interpreting certain questions differently. Nevertheless, the findings highlight the need for educational initiatives to raise awareness of lysosomal storage diseases.

CONCLUSION

The data obtained in our study, therefore, highlight the importance of expanding educational programmes for physicians about LSD and of strengthening initiatives aimed at improving awareness of early diagnostic processes. Thus, early diagnosis may increase the treatability of these diseases and improve the patients' quality of life. Further support for these findings through larger, multicentre studies would help clarify existing uncertainties surrounding the early diagnosis and management of LSD.

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Ethics Committee Approval: This study was approved by the Adana City Training and Research Hospital Ethics Committee (Decision No: 478, Date: 10.04.2025).

Informed Consent: Written informed consent was obtained from all participants included in the study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that they have no conflict of interest.

Author Contributions: EB: Conception, Design, Supervision, Writing; MYÇ: Data Collection and/or Processing, Analysis and/or Interpretation; BK: Data Collection and/or Processing, Analysis and/or Interpretation. All the authors have read, edited, and approved the final version of the manuscript.

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Antibacterial activity of *Salvia officinalis* (sage) against *Streptococcus pyogenes* isolates from throat culture

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MAIN POINTS

- This study is the first to investigate the antibacterial effects of methanolic leaf and root extracts of *S. officinalis* against clinical *S. pyogenes* isolates.
- The MIC and MBC values were determined using the broth microdilution method, which indicated that leaf extracts had stronger antibacterial activity than root extracts.
- The results contribute to the standardization of plant-based antibacterial testing and support the potential use of *S. officinalis* as a complementary treatment against resistant bacterial strains.

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ABSTRACT

Aim: To investigate the antibacterial effects of methanolic extracts of *Salvia officinalis* (leaves and roots) against *Streptococcus pyogenes* isolates from throat cultures, based on the hypothesis that these extracts may exhibit inhibitory activity against clinical isolates, as suggested by previous reports of its antimicrobial potential.

Materials and Methods: After the plant was collected, it was separated into leaf and root parts, dried at 70°C for 24 hours, and then powdered. Dried plant samples were extracted in methanol for 24 hours. The broth microdilution method was used to determine antibacterial activity. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the extracts against the isolates were determined.

Results: MIC values of leaf and root extracts against isolates ranged from 7.8-125 µg/ml and 7.8-500 µg/ml, respectively; MBC values ranged from 7.8-125 µg/ml and 7.8-500 µg/ml, respectively. In general, lower MIC and MBC values were observed in leaf extracts than in root extracts.

Conclusion: Different methods are used in studies investigating the antibacterial effect of S. officinalis, and no standard method exists. If the safe use limits for sage are determined through cytotoxicity studies and a standard method is developed, *S. officinalis* can be used as a supplement for the treatment of infectious diseases caused by resistant strains.

Keywords: *Salvia officinalis*, Sage, *Streptococcus pyogenes*, Broth microdilution method, Antibacterial effect, Methanol extract

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INTRODUCTION

Streptococcus pyogenes is an important human pathogen that can cause both localized and life-threatening invasive infections. It usually colonizes the pharynx, anus, and genital mucosa. *S. pyogenes* is a Gram-positive bacterium that is catalase- and oxidase-negative. *S. pyogenes* grows well in 5%–10% carbon dioxide and on blood agar plates. According to the Lancefield classification, it is included in group A streptococci (GAS). The Type A antigen is a polysaccharide attached to a rhamnose polymer backbone and contains N-acetylglucosamine. The major surface protein in the cell wall of *S. pyogenes* is the M protein. Transmission routes include droplet transmission, contact with contaminated food, contaminated objects or surfaces, and skin contact with contaminated lesions [1].

S. pyogenes causes infections such as sepsis, septic arthritis, streptococcal toxic shock syndrome, scarlet fever, necrotizing fasciitis, pharyngotonsillitis, erysipelas, and impetigo. Late autoimmune reactions, such as acute rheumatic fever, rheumatic heart disease, and acute glomerulonephritis, are associated with GAS infections. According to current treatment guidelines, penicillin and β-lactams are recommended as the primary treatment options for uncomplicated GAS infections. Clindamycin is preferred in severe infections, in addition to β-lactam antibiotics. Macrolides and lincosamides are alternative options in patients with β-lactam allergy [2].

Studies report that *S. pyogenes*, which is sensitive to β-lactams (especially penicillin), is resistant to macrolides and lincosamides. Researchers attribute this situation to the increase in the number of prescribed antibiotics. [3,4]. Target

site modifications, overexpression of the efflux pump, drug inactivation, ribosomal protection are antibiotic resistance mechanisms [1].

Salvia officinalis (sage) is a perennial, shrubby plant in the family Lamiaceae. It has about 900 species and is the largest genus in the Lamiaceae family. Its prevalence extends to the Mediterranean and Middle East and occurs naturally in various countries worldwide. It is derived from the word "salvarem", which means to save or to treat. It is preferred as a spice in the food sector. In traditional medicine, its aboveground parts have been used for treatment for many years [5-7].

It has been reported in studies that secondary metabolites such as alkaloids, carbohydrates, flavonoids, phenolic acids, terpenes and terpenoids, especially in the leaf parts of sage, have anti-inflammatory, antioxidant, antimicrobial, anticancer, antimitogenic, hypoglycemic, hypolipidemic and antinociceptive effects [5,8,9]. It is predicted that sage essential oils damage the bacterial cell wall, the cytoplasmic membrane's double-layered phospholipid layer and membrane proteins, causing increased cytoplasmic membrane permeability and loss of cellular content [10]. Although many studies have evaluated the antibacterial potential of *S. officinalis* leaves or essential oils [11-13], comparative data on leaf and root extracts against clinical *S. pyogenes* isolates are scarce. This study uniquely compares these plant parts to highlight possible variations in antibacterial potency. In addition, the chemical composition and biological activity of medicinal plants such as *Salvia officinalis* may vary depending on geographical origin, climate, soil characteristics, and harvesting conditions. Therefore, region-specific studies are important to better understand the antibacterial potential of plant extracts obtained from locally grown specimens. This study aims to contribute data from Turkey by evaluating the antibacterial activity of methanolic extracts of *S. officinalis* leaves and roots against clinical *S. pyogenes* isolates, thereby providing regionally relevant evidence to the existing literature.

MATERIALS AND METHODS

Ethical approval for this study was obtained from the Non-Interventional Clinical Research Ethics Committee of Kocaeli University Faculty of Medicine (Date: 27.03.2025, Approval number: KÜ GOKAEK-2025/07/13).

Collection of sage plant and obtaining extracts

Plant samples collected from the Nif Mountain region of Izmir Province in May 2023 were separated into leaves and roots, and dried at 70°C for 24 hours. Dried samples were powdered. Leaf powder (0.32 g) and root powder (0.40 g) were obtained. Plant powders dissolved in methanol were extracted in a soxhlet extraction device for 24 hours [14]. The obtained extracts were stored at -80°C.

Preparation of stock extract solutions

A 2 mg portion of each extract was weighed and dissolved in 1 ml of dimethyl sulfoxide (DMSO) for use in the broth microdilution test. The stock solution was obtained as 2 mg/ml [15]. All extract solutions were sterile-filtered through 0.22 µm membrane filters prior to antimicrobial testing.

Bacterial strains

A total of 18 *Streptococcus pyogenes* isolates from throat cultures processed at the Central Microbiology Laboratory of Kocaeli University Hospital between January 2023 and November 2024 were included in the study. Throat swab samples were plated on sheep blood agar (SBA). Pure colonies were identified as *S. pyogenes* using the Vitek MS system (BioMérieux, France). Bacteria were stored at −80°C until broth microdilution experiments were performed. *Escherichia coli* ATCC 25922 was used as the positive control in the broth microdilution test.

Broth microdilution test

Broth microdilution test used for determination of antibacterial activity of plant extracts was applied with modification of protocols suggested by Wijesundara et al. [13]. Sterile 96-well U-bottom microplates were used for the test. DMSO and *E. coli* ATCC 25922 were used as the negative and positive controls, respectively. 12 wells (one well for sterility control and one well for growth control) were examined for each bacterium. 100 µl of Mueller Hinton broth was added to all wells, and the extract was added on top of it. A serial dilution was performed, with an initial concentration of 1000 µg/ml and a final concentration of 1.95 µg/ml. Based on the two-fold serial dilution design, the final DMSO concentration decreased progressively across the wells and was maintained below 1% (v/v) under the assay conditions. 10 µl of the bacterial suspension, prepared in saline solution at 0.5 McFarland turbidity and diluted 1:10, was added to all wells except for the first well. Microplates were incubated at 37 °C for 18-24 hours in an atmosphere containing 5-10% CO_2. The lowest concentration with no visible growth was determined as the MIC [16]. To determine the minimum bactericidal concentration (MBC), 30 µL of the suspension containing medium, extract and bacteria in the wells where no growth was present was inoculated quantitatively to the blood agar plates [13]. The plates were incubated at 37 °C for 18-24 hours. The lowest concentration that reduced the number of bacteria in the plates after incubation by 99.9% (3 logarithms) was determined as the MBC value [17]. All experiments were performed in duplicate, and the results were consistent across replicates.

Statistical analysis

Statistical analyses were performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). Data distribution was assessed using the Shapiro–Wilk test. Since MIC

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Table 1. Physical properties and yield percentages of different parts of *S. officinalis*.

Solvent	Plant part	Physical properties	Biomass subjected to extraction (g)	Extraction yield (g)	Percentage yield (%)
Methanol	Leaf	Honey brown powder	8.00	0.32	4.00
	Root	Honey yellow powder	9.26	0.40	4.34

Table 2. MIC and MBC values of *S. officinalis* leaf and root extracts against *S. pyogenes* isolates.

	Leaf		Root	
Isolate Number	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
SP-1	62.5	62.5	62.5	500
SP-2	31.25	62.5	125	250
SP-3	31.25	62.5	125	500
SP-4	31.25	62.5	250	250
SP-5	125	125	500	500
SP-6	62.5	62.5	125	250
SP-7	125	125	125	250
SP-8	62.5	62.5	250	250
SP-9	31.25	62.5	125	250
SP-10	62.5	62.5	125	125
SP-11	31.25	31.25	62.5	125
SP-12	7.8	15.6	7.8	7.8
SP-13	31.25	31.25	7.8	62.5
SP-14	7.8	7.8	15.6	125
SP-15	7.8	7.8	15.6	125
SP-16	31.25	31.25	31.25	62.5
SP-17	15.6	15.6	31.25	62.5
SP-18	31.25	31.25	62.5	62.5

Note: SP; Streptococcus pyogenes.

and MBC values were not normally distributed, comparisons between leaf and root extracts were conducted using the Wilcoxon signed-rank test for paired samples. A p-value <0.05 was considered statistically significant. Additionally, a post-hoc power analysis was performed based on the observed effect size at a 95% confidence level.

RESULTS

The physical properties and percentage yields of different parts of the extracts

The highest extraction yield was obtained from the leaves of the plants. Table 1 shows the physical properties and percentage yields of different parts of the extracts.

Antibacterial activity of S. officinalis extracts

According to the broth microdilution test results, the MIC and MBC values of leaf and root extracts against *S. pyogenes* isolates were determined to be in the ranges of 7.8-125 µg/ml and 7.8-500 µg/ml, respectively. Overall, leaf extracts exhibited stronger antibacterial activity than root extracts. Leaf extracts exhibited lower MIC values in 13 of 18 isolates; MIC values were equal in 4 isolates; and root extracts exhibited lower MIC values in only 1 isolate. Similarly, MBC values of leaf extracts were lower than those of root extracts in 16 out of 18 isolates, with equal values observed in 1 isolate and root extracts showing lower MBC values in 1 isolate. These findings indicate that leaf extracts have higher inhibitory and bactericidal potential than root extracts. Detailed MIC and MBC values for each isolate are presented in Table 2.

Statistical comparison of MIC and MBC values

MIC values obtained from *S. officinalis* leaf extracts were significantly lower than those obtained from root extracts (Wilcoxon signed-rank test, p=0.002). Similarly, MBC values were significantly lower in leaf extracts than in root extracts (p<0.001). Post-hoc power analysis revealed that the achieved statistical power exceeded 0.80.

DISCUSSION

S. pyogenes isolates are susceptible to penicillin. However, resistance to other antibiotics preferred for treatment is developing. The increase in antibiotic resistance results in limited treatment options, increased morbidity and mortality, and a greater burden on health services. Therefore, it leads to the discovery and research into new antibacterial agents for treatment.

The World Health Organization (WHO) has declared that medicinal plants can be a main source for drug development. Accordingly, discovering new antimicrobial agents with diverse chemical structures and modes of action is critical to combating infectious diseases. Tannins, terpenoids, alkaloids, and flavonoids are abundant secondary metabolites in plants, and these compounds have antimicrobial effects. WHO has reported that plant extracts and their active ingredients are used in traditional medicine by 80% of the world's population [18,19].

S. officinalis is a perennial woody plant with a bitter taste and strong aromatic properties. Its leaf has antioxidant and antimicrobial properties. For this reason, its extracts and essential oils are widely used in the food, cosmetics and pharmaceutical industries [20].

With increasing interest in herbal medicine, sage has also attracted attention in traditional medicine because of its anti-inflammatory properties and diverse medicinal uses. The European Pharmacopoeia has recognized that leaf extracts of *S. officinalis* are a beneficial oral solution for reducing swelling and discomfort in inflamed gum tissues [21]. The antibacterial effects observed in this study may be attributed to phenolic and terpenoid compounds, such as thujone, rosmarinic acid, and camphor, which are commonly found in

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S. officinalis. These bioactive molecules can disrupt bacterial cell membranes, interfere with energy metabolism, and inhibit protein synthesis. The higher activity of leaf extracts may result from higher concentrations of essential oils and flavonoids in leaf extracts than those in root parts, as reported in phytochemical analyses of sage species.

Antibacterial and antibiofilm effects of *S. officinalis* extracts and essential oils against clinical isolates such as *E. coli*, *Pseudomonas aeruginos*a, *Klebsiella pneumonia*e, *K. oxytoca*, *Staphylococcus aureus*, *Salmonella enteritidis*, *Bacillus cereus*, *Mycoplasma gallisepticum* and *Pasteurella multocida* were investigated [22-25].

In dentistry, studies have investigated the antibacterial effects of *S. officinalis* extracts and essential oils against bacterial species that cause dental infections. A gargle obtained from *S. officinalis* leaves has been shown to reduce counts of *Streptococcus mutans* isolated from dental caries. [26]. In a study using the broth microdilution method, *S. officinalis* essential oil was reported to exhibit antibacterial activity against *S. mutans* and *Porphyromonas gingivalis*. Researchers emphasized that sage essential oil is a promising source of antimicrobial agents that can prevent dental caries and other oral infectious diseases [27]. In addition, antibacterial activity of sage essential oil has been reported against *S. salivarius*, *S. mitis*, and *S. sanguinis* isolated from individuals with oral disease [28,29].

Few studies have specifically investigated the antibacterial activity of *S. officinalis* against *S. pyogenes*. In a study conducted in Canada, ethanolic leaf extracts of *S. officinalis* were tested against two standard S. pyogenes strains (ATCC 19615 and ATCC 49399) and one clinical isolate using the broth microdilution method. The MIC and MBC values were reported as 62.5 µg/ml and 125 µg/ml, respectively [13]. In a related study by the same authors, sage essential oil exhibited MIC values of 250–500 µg/ml and MBC values of 500 µg/ml against standard and clinical *S. pyogenes* strains [12]. In another study, the antibacterial activity of essential oils, obtained from the aboveground parts of *S. officinalis* by hydrodistillation, was evaluated using the disk diffusion method against standard strains, including *S. pyogenes* ATCC 19615, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853. The researchers reported moderate antibacterial activity of sage essential oil against *S. pyogenes* and other Gram-positive strains, while lower activity was observed against *P. aeruginosa* [11].

In this study, the antibacterial effects of methanolic extracts of *S. officinalis* leaves and roots against clinical *S. pyogenes* isolates were evaluated using the broth microdilution method, and MIC and MBC values were determined. The antibacterial activity observed is in line with previous reports demonstrating the efficacy of *S. officinalis* extracts and essential oils against a broad spectrum of bacterial species [22–29]. In several studies, Gram-positive bacteria have been reported to exhibit lower MIC and MBC values than Gram-negative bacteria, which may be attributable to differences in cell wall structure and membrane permeability. The background literature provides a contextual framework for interpreting the activity observed against *S. pyogenes* isolates in the present study.

The variation in MIC and MBC values observed among *S. pyogenes* isolates may reflect strain-specific biological differences and inherent variability in susceptibility. Such inter-isolate variability has been reported in previous studies evaluating plant-derived extracts against clinical bacterial isolates. The reliability of the results is supported by duplicate testing and standardized broth microdilution principles, based on CLSI methodology and EUCAST definitions.

In this study, the MIC values for leaf extracts ranged from 7.8 µg/ml to 125 µg/ml, and for root extracts from 7.8 µg/ml to 500 µg/ml. The MBC values in leaf extracts were determined to range from 7.8 µg/ml to 125 µg/ml, and in root extracts from 7.8 µg/ml to 500 µg/ml. While the MIC values determined in our study are similar to the study conducted by Wijesundara et al., the MBC values differ. In our study, MIC values for the isolates were lower than those reported in many other studies. These differences may be attributable to factors such as the geographical areas where the plants were collected, extraction methods, and solvent purity. Unlike most previous studies that primarily focused on leaf extracts, the inclusion of root extracts constitutes a notable strength of this study. We believe that, in our study, determining the MIC values of root extracts and observing their antibacterial activity will provide preliminary information and inform future studies. In addition, the relatively large number of clinical *S. pyogenes* isolates analyzed provides preliminary data that may contribute to future standardization of plant-based antibacterial testing.

Limitations

Limitations of this study include the lack of phytochemical profiling and cytotoxicity testing. Nevertheless, the findings provide preliminary evidence that *S. officinalis* may serve as a natural adjuvant or as a complementary therapeutic agent for mild throat infections, pending further in vivo validation. Routine antimicrobial susceptibility testing is not recommended for *S. pyogenes* due to its predictable susceptibility to penicillin, as stated in CLSI guidelines [30]. Therefore, the present study focused on evaluating the antibacterial activity of *S. officinalis* extracts rather than correlating extract activity with antibiotic resistance phenotypes.

CONCLUSION

In this study, it was observed that *S. officinalis* extracts, especially leaf extracts, could be effective against *S. pyogenes*. The pain-relieving effect of sage, which is frequently used by the public to treat throat infections, may be due to its antibacterial and anti-inflammatory properties. In addition, methods that determine the antibacterial activities of plant extracts need to be standardized, and herbal treatments cannot be used instead of medical treatment. As with other herbal products,

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uncontrolled use of *S. officinalis* should be avoided. The antibacterial effects, mechanisms of action, and cytotoxic effects of *S. officinalis* should be investigated more extensively.

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Informed Consent: Written informed consent was obtained from all individual participants included in the study.

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Prognostic significance of PIK3CA immunoexpression and mutation status in triple-negative breast cancer

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MAIN POINTS

- PIK3CA immunoexpression was predominantly low in TNBC, with only 20% of cases showing moderate–high expression, indicating that overexpression is limited to a distinct biological subset.
- Moderate–high PIK3CA expression was significantly associated with improved overall survival, while all deaths occurred in the negative-expression group (p=0.043).
- No meaningful correlation was found between PIK3CA expression and conventional clinicopathological parameters.
- PIK3CA mutations were detected in 33.3% of high-expression cases, indicating only a partial alignment between protein expression and mutation status.
- The observed association between PIK3CA expression and overall survival should be interpreted cautiously and requires validation in larger, adequately powered cohorts.

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ABSTRACT

Aim: Triple-negative breast cancer (TNBC) is an aggressive subtype with limited targeted therapies. This study investigated PIK3CA protein expression in TNBC and, exploratorily, assessed PIK3CA mutations in a subset of tumors with high expression.

Materials and Methods: Sixty-five TNBC cases were retrospectively analyzed. PIK3CA expression was assessed by H-score and grouped for survival analysis as negative versus moderate–high. Six high-expression cases underwent mutation analysis. Associations with clinicopathological parameters, overall and disease-free survival were evaluated using Kaplan–Meier and log-rank tests.

Results: PIK3CA expression was negative in 80.0% (52/65) of cases, while moderate–high expression was observed in 20.0% (13/65). No significant associations were observed between PIK3CA immunohistochemical expression and clinicopathological features (p>0.05). PCR-based mutation analysis was performed on the moderate–high expression subgroup. 33.3% (2/6) of the tested high-expression cases harbored PIK3CA mutations. All deaths occurred in the negative-expression group; overall survival was significantly better in the moderate–high expression group (p = 0.043). However, no death events were observed in the moderate–high subgroup (n = 13), and this finding should be interpreted cautiously because of the low number of events. No significant difference in disease-free survival was found between groups (p = 0.588).

Conclusion: PIK3CA protein expression was not associated with conventional clinicopathological prognostic parameters in TNBC. Nevertheless, moderate–high PIK3CA expression was associated with better overall survival, indicating a hypothesis-generating rather than an independent prognostic effect. Moreover, despite the absence of significant differences at the immunohistochemical level, PIK3CA mutations were detected by PCR in a limited subset of high-expression cases, highlighting a potential discordance between protein expression and molecular alterations. These findings warrant validation in larger, multicenter studies.

Keywords: TNBC, PIK3CA, Immunohistochemistry, Prognosis, Survival

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INTRODUCTION

Breast cancer is one of the most common malignancies among women worldwide. According to the 2022 GLOBOCAN data, it accounts for 23.8% of all cancer types in women, with an annual incidence of 46.8 per 100,000 women and a mortality rate of 12.7 per 100,000 women. Breast cancer remains a major public health concern globally, affecting women in both developed and developing countries [1].

Triple-negative breast cancer (TNBC), an aggressive subtype of breast carcinoma, accounts for approximately 15–25% of all breast cancer cases [2]. Compared with other molecular subtypes, TNBC is characterized by a more aggressive clinical course, with a higher risk of distant metastasis and poorer prognosis [2]. Under current treatment approaches, the me-

dian overall survival is approximately 10.2 months; the five-year survival rate is reported as 65% for patients with regional disease, dropping to 11% in cases with distant metastatic spread [3,4].

Despite advances in diagnostic techniques and the development of targeted therapeutic options, the biologically heterogeneous nature of the disease continues to necessitate a personalized treatment approach. In this context, the identification of novel molecular biomarkers that may enhance our understanding of tumor biology is of great importance for improving clinical management.

Activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a pivotal role in regulating key cellular processes, including cell growth, proliferation, survival, and metabolism. The PIK3CA gene encodes the p110α catalytic subunit of class IA PI3Ks and frequently harbors oncogenic mutations. PIK3CA mutations are commonly detected in breast cancer, with higher prevalence in estrogen receptor-positive (ER+) tumors, HER2-negative tumors, and tumors with low proliferation. However, the relationship between PIK3CA gene mutations and protein-level expression remains insufficiently clarified, and current evidence in the literature shows inconsistent findings [5–7]. Importantly, mutation status does not necessarily translate into protein abundance detectable by immunohistochemistry, as PIK3CA expression may be influenced by transcriptional and post-transcriptional regulation, protein stability, and intratumoral heterogeneity. This discordance may be particularly relevant in TNBC, where available data are more limited and heterogeneous. Therefore, assessing PIK3CA at the protein level by immunohistochemistry may provide complementary information beyond mutation status, including the extent and spatial distribution of expression within the tumor.

Evidence in the literature suggests that PIK3CA mutations may be associated with a more favorable prognosis in certain breast cancer subgroups; however, this effect appears to vary depending on tumor subtype, treatment modality, and additional molecular parameters [8]. Moreover, a substantial proportion of studies reporting favorable prognostic associations are derived from hormone receptor–positive cohorts, which may limit the direct applicability of these findings to TNBC. In this context, evaluating PIK3CA not only at the genetic level but also in terms of its immunohistochemical expression profile may provide valuable prognostic and predictive insights.

The present study aimed to evaluate PIK3CA protein expression in TNBC using immunohistochemistry and to examine its associations with clinicopathological characteristics and survival outcomes, including overall and disease-free survival. PIK3CA mutation analysis was also performed in a subset of tumors exhibiting high protein expression to preliminarily assess the relationship between protein expression and mutation status. These findings may generate hypotheses regarding the prognostic significance of PIK3CA in TNBC.

MATERIALS AND METHODS

Patient selection

This study included 65 patients who were histopathologically diagnosed with triple-negative breast cancer (TNBC) (ER−, PR−, HER2−) at our center between 2015 and 2024 and who had available formalin-fixed, paraffin-embedded (FFPE) tissue samples suitable for immunohistochemical evaluation. Eligible cases were required to have sufficient clinicopathological data and follow-up information in the hospital records and to have undergone surgical and/or systemic treatment at our institution.

Patients diagnosed with breast cancer of molecular subtypes other than TNBC (i.e., Luminal A, Luminal B, or HER2-positive) [9], those lacking adequate tissue for immunohistochemical or molecular analysis, cases with incomplete clinical data or follow-up information, patients diagnosed solely with in situ carcinoma, and those presenting with metastatic disease at initial admission were excluded from the study. Patients with a history of any other malignancy prior to diagnosis—except for non-melanoma skin cancer—were not included in the study.

Ethical approval for this study was obtained from the Pamukkale University Non-Interventional Clinical Research Ethics Committee (Date: 09.05.2024; Approval No: E-60116787-020-523657).

Immunohistochemical and molecular analysis

PIK3CA expression was evaluated immunohistochemically and scored using the H-score method, with staining intensity graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The H-score is obtained by multiplying the percentage of tumor cells showing each staining intensity by the corresponding intensity value. The total score ranges from 0 to 300. For categorical interpretation, scores were classified as follows: negative (0–30), moderate (31–100), and high (101–300) according to previously published cut-off values [10] to ensure methodological consistency and comparability with the existing literature. To increase statistical power, scores were additionally regrouped into two categories for analysis: negative (0–30) and moderate–high (31–300) by combining the moderate and high groups to avoid sparse subgroup sizes and improve the robustness of statistical analyses [10].

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissues using the cobas® DNA Sample Preparation Kit in accordance with the manufacturer's instructions. PCR-based mutation analysis was performed to detect hotspot mutations in the PIK3CA gene. Specifically, mutations in exon 9 (E542K, E545K) and exon 20 (H1047R) were assessed.

The PCR reaction was prepared in a total volume of 25 µL and consisted of DNA template, primers, dNTP mix, $MgCl_2$, Tris-HCl buffer, and Taq DNA polymerase. Thermal cycling conditions included an initial denaturation step, followed by

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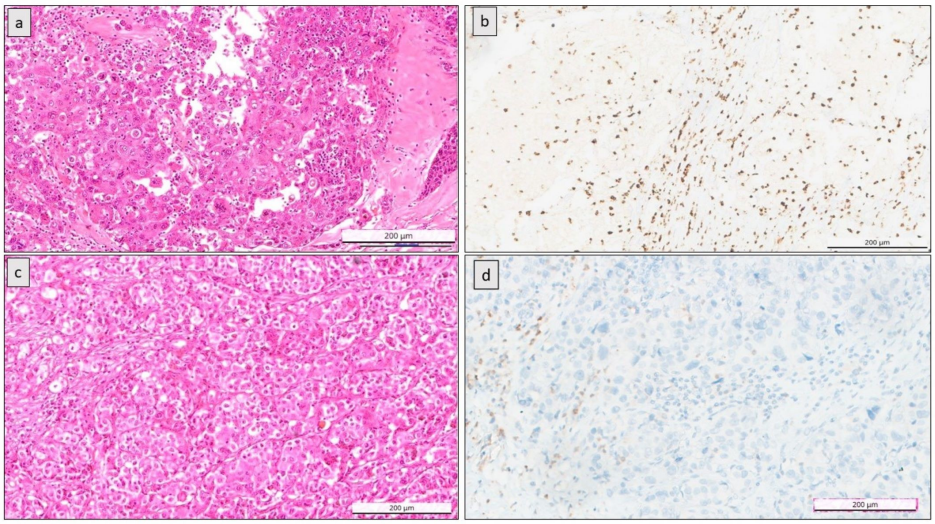

Figure 1. Representative histopathological and immunohistochemical features of triple-negative breast carcinoma. (a) High-grade invasive carcinoma (H&E, ×100). (b) PIK3CA-positive nuclear immunohistochemical staining (×100). (c) High-grade invasive carcinoma (H&E, ×100). (d) PIK3CA-negative immunohistochemical staining (×100).

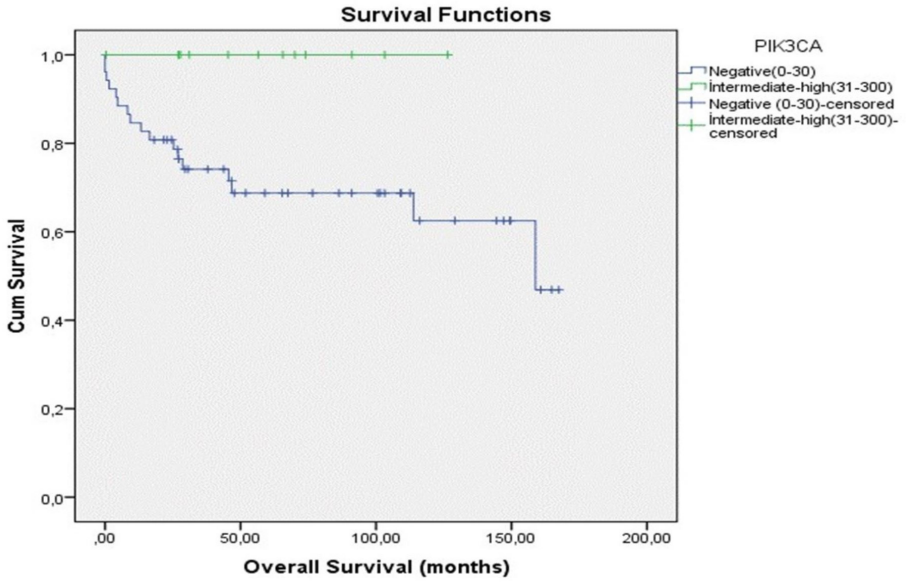

Figure 2. Overall survival curves according to PIK3CA expression levels. Patients with moderate–high expression (31–300) showed better survival than those with negative/low expression (0–30) (Log-rank, χ^2=4.106; df=1; p=0.043).

35 cycles of denaturation, annealing, and extension, and a final extension step optimized for target amplification.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA). The Chi-square test or Fisher's exact test was used to compare categorical variables, as appropriate. Survival analyses were conducted using Kaplan–Meier survival curves, and differences between curves were assessed using the log-rank test. A p-value of <0.05 was considered statistically significant. Multivariable Cox regression analysis was considered but not performed due to the limited number of events and complete separation.

RESULTS

Clinicopathological characteristics

The mean age was 52.4 ± 13.3 years (range: 28–87 years), and 67.7% were postmenopausal.

Based on age distribution, 60% of the cases were aged ≥55 years and 40% were <55 years. The mean overall survival time was 63.3 ± 49.8 months, and the median overall survival was 46.8 months.

Histopathologically, 90.8% (n = 59) of cases were diagnosed as invasive carcinoma of no special type (NST), 3.0% (n=2) as invasive lobular carcinoma, and 6.2% (n=4) as metaplastic carcinoma.

Tumors were located in the right breast in 46.9% of cases and

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Table 1. Clinicopathological characteristics of the study cohort.

Parameter		n (%)
Age groups	<55 years	26 (40.0)
	≥55 years	39 (60.0)
Histologic type	Invasive carcinoma of no special type (NST)	59 (90.8)
	Invasive lobular carcinoma	2 (3.1)
	Metaplastic carcinoma	4 (6.2)
Type of surgery	Mastectomy	31 (47.7)
	Excision	31 (47.7)
	Tru-cut biopsy only	3 (4.6)
Tumor size	1--20 mm	19 (29.2)
	21--50 mm	37 (56.9)
	≥50 mm	9 (13.8)
Histologic grade	Grade 2	23 (36.5)
	Grade 3	40 (63.5)
Tumor localization	Right breast	30 (46.9)
	Left breast	34 (53.1)
Lymphovascular invasion	Absent	31 (49.2)
	Present	14 (22.2)
	Unknown	18 (28.6)
Extranodal extension	Absent	48 (73.8)
	Present	7 (10.8)
	Unknown	10 (15.4)
Perineural invasion	Absent	53 (81.5)
	Present	3 (4.6)
	Unknown	9 (13.8)
Ductal carcinoma in situ (DCIS)	Absent	34 (52.3)
	Present	23 (35.4)
	Unknown	8 (12.3)
Neoadjuvant therapy	No	28 (43.1)
	Yes	14 (21.5)
	Unknown	23 (35.4)
Multicentricity	Absent	34 (52.3)
	Present	10 (15.4)
	Unknown	21 (32.3)
Tumor focus	Unifocal	43 (66.2)
	Bifocal	15 (23.1)
	Multifocal	7 (10.7)
Sentinel lymph node metastasis	Absent	31 (49.2)
	Present	14 (22.2)
	Unknown	18 (28.6)
Survival status	Alive	48 (73.8)
	Deceased	17 (26.2)

in the left breast in 53.1% of cases. The mean tumor size was 1.78 ± 0.88 cm. 66.2% (n=43) of patients had unifocal tumors, 23.1% (n=15) had bifocal tumors, and 10.7% (n=7) had multifocal tumors.

Histological grade was assessable in 63 cases: 63.5% were grade 3 and 36.5% were grade 2; no grade 1 tumors were identified. A ductal carcinoma in situ (DCIS) component was present in 35.4% of cases. Lymphovascular invasion (LVI) and perineural invasion (PNI) were observed in 26.2% and 4.6% of cases, respectively.

Axillary lymph node status was available for 45 patients. Of these, 68.9% (n=31) had no lymph node metastasis, and 31.1% (n=14) had lymph node metastasis. Extranodal extension was detected in 10.8% of cases.

Based on immunohistochemical evaluation, PIK3CA expression was negative (0–30) in 80% of cases, moderate (31–100) in 6.2% of cases, and high (101–300) in 13.8% of cases (Figure 1).

Regarding surgical characteristics, mastectomy was performed in 47.7% of patients, excisional biopsy in 47.7%, and core needle biopsy alone in 4.6%. Both core needle and surgical excision specimens were available for evaluation in 63.1% of patients. Sentinel lymph node biopsy was performed in 30.8% of cases, with an average of two nodes removed. Neoad-

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Table 2. Comparison of clinicopathological parameters according to PIK3CA expression level in breast carcinoma.

Parameter	PIK3CA Negative (0--30) n (%)	PIK3CA Moderate--high (31--300) n (%)	p
Histologic Type†			
Invasive carcinoma of no special type (NST)	48 (73.8)	11 (16.9)	
Invasive lobular carcinoma	2 (3.1)	0 (0)	
Metaplastic carcinoma	2 (3.1)	2 (3.1)	
Age (years)			0.167
≥55	29 (44.6)	10 (15.4)	
<55	23 (35.4)	3 (4.6)	
Tumor Size (mm)			0.385
1--20	13 (20.0)	6 (9.2)	
21--49	32 (49.2)	5 (7.7)	
≥50	7 (10.8)	2 (3.1)	
Lymph Node Metastasis			0.846
Absent	25 (38.5)	6 (9.2)	
Present	11 (16.9)	3 (4.6)	
Unknown	15 (23.1)	3 (4.6)	
Stage			0.190
Early stage	29 (44.6)	9 (13.8)	
Advanced stage	6 (9.2)	0 (0)	
Menopausal Status			0.412
Premenopausal	16 (24.6)	5 (7.7)	
Postmenopausal	36 (55.4)	8 (12.3)	
Extranodal Extension			0.868
Absent	38 (58.5)	10 (15.4)	
Present	6 (9.2)	1 (1.5)	
Unknown	8 (12.3)	2 (3.1)	
DCIS Component			0.427
Absent	25 (38.5)	9 (13.8)	
Present	21 (32.3)	2 (3.1)	
Unknown	6 (9.2)	2 (3.1)	
Local Recurrence			0.632
Absent	47 (72.3)	11 (16.9)	
Present	2 (3.1)	1 (1.5)	
Unknown	3 (4.6)	1 (1.5)	
PNI			0.727
Absent	43 (66.2)	10 (15.4)	
Present	2 (3.1)	1 (1.5)	
Unknown	7 (10.8)	2 (3.1)	
Multicentricity			0.837
Absent	27 (41.5)	7 (10.8)	
Present	9 (13.8)	1 (1.5)	
Unknown	16 (24.6)	5 (7.7)	
Neoadjuvant Therapy			0.296
No	24 (36.9)	4 (6.2)	
Yes	11 (16.9)	3 (4.6)	
Unknown	17 (26.2)	6 (9.2)	

† p value not calculated due to uneven distribution of histologic subtypes.

juvant therapy was administered to 21.5% of patients (Table 1).

Among the 44 patients with available staging data, 86.4% had early-stage disease (Stage I–II) and 13.6% had advanced-stage disease (Stage III–IV). Local recurrence was observed in 4.6% (n=3) of patients. Among the 48 patients with available postoperative metastasis data, 25% developed distant metastasis. Metastatic sites were the liver (72.7%; n=8), the brain (9.1; n=1), the colon (9.1; n=1), and the vertebrae (9.1; n=1).

Association between PIK3CA expression and clinicopathological variables

No statistically significant association was found between PIK3CA immunostain expression levels and various clinicopathological parameters, including age group, tumor laterality, histological grade, lymph node metastasis, tumor size, menopausal status, type of surgery, sentinel lymph node

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Table 3. Basic clinicopathological features and PIK3CA mutation status of the breast carcinoma cases.

Case	Age (years)	Tumor size (cm)	Sentinel LN metastasis	Local recurrence	PIK3CA mutation status
1	53	5.0	No	No	Positive (H1047R)
2	50	1.6	No	No	Negative
3	38	5.7	No	No	Positive (E545K)
4	40	2.5	Yes	No	Negative
5	50	1.2	No	No	Negative
6	57	1.5	No	Unknown	Negative

Notes: - Sentinel LN: Sentinel lymph node; - "Unknown" indicates unavailable data.

biopsy status, neoadjuvant therapy, multicentricity, number of tumor foci, tumor lymphovascular invasion (LVI), perineural invasion (PNI), extranodal extension, presence of ductal carcinoma in situ (DCIS), and local recurrence (all $p>0.05$) (Table 2).

Molecular findings

PIK3CA mutation analysis was performed by PCR on six cases exhibiting high PIK3CA expression levels (101–300). Mutations were detected in 33.3% (n=2) of these cases, and 66.7% (n=4) were mutation-negative. Statistical comparison across expression categories could not be performed because mutation testing was limited to the high-expression subgroup. Clinicopathological features of these cases are summarized in Table 3.

Survival analysis

The impact of PIK3CA expression levels on survival outcomes was assessed using the Kaplan–Meier method, and differences between groups were analyzed using the log-rank test. For analytical robustness, PIK3CA expression scores were dichotomized into two groups: negative (0–30) and moderate–high (31–300). In the negative-expression group, 32.7% of patients were deceased, whereas 0% (n = 0/13) in the moderate–high-expression group were deceased. Analysis of overall survival stratified by PIK3CA expression level (0–30 vs. 31–300) revealed a statistically significant difference between the groups (Log-Rank test: $\chi^2 = 4.106$, df = 1, $p = 0.043$). Patients with moderate–high PIK3CA expression demonstrated better overall survival compared with those with negative expression (Figure 2). However, this finding should be interpreted with caution, given the small sample size of the moderate–high expression subgroup and the absence of death events in this group, which may limit the robustness of the survival comparison and increase the likelihood of an unstable estimate. Accordingly, multivariable Cox proportional hazards regression, adjusting for established prognostic factors, was considered but could not be performed reliably because of the limited number of events and complete separation (i.e., zero death events in the moderate–high expression group). Therefore, these results should be regarded as hypothesis-generating and warrant validation in larger, adequately powered cohorts.

With respect to disease-free survival (DFS), three recurrences were recorded in total: two occurred in the negative-expression group and one in the moderate-expression group. No recurrences were observed among patients with high expression levels. The DFS analysis based on PIK3CA expression categories (0–30 vs. 31–300) did not show a statistically significant difference between the groups (Log-Rank [Mantel–Cox] test: $\chi^2 = 0.293$, df = 1, $p = 0.588$). The mean DFS time was 159.8 ± 5.3 months (CI: 149.4–170.3) for the PIK3CA-negative group (0–30), and 116.8 ± 9.3 months (CI: 98.5–135.1) for the moderate–high expression group (31–300). Median DFS could not be estimated due to censoring of long-term survivors. No statistically significant association was observed between PIK3CA expression level and disease-free survival.

DISCUSSION

Chemotherapeutic agents that induce DNA damage, which are frequently used in the treatment of TNBC, have been shown to stimulate AKT phosphorylation (Protein Kinase B, PKB) through DNA damage–responsive protein kinases. In this context, several studies have demonstrated that the pro-apoptotic effects of DNA-damaging drugs are synergistically enhanced when combined with PI3K inhibitors [11–13]. These findings suggest that mutations within the PI3K pathway may shed light on the molecular basis of chemoresistance in TNBC, particularly with respect to resistance mechanisms that evolve in response to conventional chemotherapy.

Our cohort had a predominantly postmenopausal, older-age profile. While prior studies (mostly not specific to TNBC) have reported higher PIK3CA mutation rates in older and postmenopausal women, the extent to which this observation applies to TNBC remains less well established [14,15].

In the present study, the mean age of the 65 patients included was 52.4 ± 13.3 years, with approximately two-thirds (67.7%) being postmenopausal. Moreover, 60% of the patients were aged 55 years or older. This demographic profile aligns with previous reports indicating that PIK3CA mutations occur more frequently in older and postmenopausal women. Previous studies in breast cancer (not specifically TNBC) have reported higher PIK3CA mutation rates in older and postmenopausal women [14,15].

In TNBC, the reported frequency of PIK3CA mutations

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generally ranges between 5% and 16% [5], although rates exceeding 20% have been described in enriched, single-center cohorts—such as the 23.7% mutation rate reported in a PLOS ONE cohort [13]. For broader, non-selected TNBC populations, the most reliable clinically accepted prevalence remains within the 8–16% range [5].

Literature indicates that activating mutations in the PIK3CA gene are observed in approximately 18–40% of breast tumors, predominantly clustered within exon 9 and exon 20 hotspot regions [14,16]. Although these prevalence estimates are largely derived from mixed breast cancer cohorts rather than TNBC-specific series, they provide a general framework for the distribution of PIK3CA hotspot mutations. In the present study, PIK3CA mutations in exon 9 were detected in two mutation-positive cases. These alterations are known to activate the PI3K/AKT signaling pathway, thereby promoting tumor development [16,17]. Findings related to endocrine resistance (e.g., associations with anti-estrogen therapy resistance in Luminal B disease) are not directly applicable to TNBC, given the lack of hormone receptor expression; therefore, these reports are mentioned here primarily to highlight subtype-specific clinical consequences of PI3K pathway activation [15]. Similarly, evidence from HER2-positive breast cancer regarding improved efficacy with combined PI3K inhibition and HER2-targeted therapy reflects a non-TNBC treatment context [18]. In TNBC, where standard management relies heavily on chemotherapy (and, in selected settings, immunotherapy), PI3K pathway alterations may instead be most relevant to chemotherapy response and resistance mechanisms, warranting TNBC-focused validation.

In TNBC, emerging evidence suggests that chemoresistance may be linked to PI3K/AKT pathway activation, and synergistic effects have been reported when PI3K inhibitors are combined with chemotherapy [19,20]. Nevertheless, the precise prognostic significance of PIK3CA mutations and the PI3K/AKT pathway across different molecular subtypes remains unclear [21]. Overall, the PI3K/AKT pathway is recognized as both a prognostic and predictive biomarker and is increasingly gaining prominence in the landscape of targeted therapeutic strategies [22].

In our study, the mean overall survival was 63.3 months and the median survival was 46.8 months, broadly consistent with previously published estimates. For contextual reference, the SOLAR-1 trial reported overall survival of 53–64 months in patients with PIK3CA-mutant, hormone receptor–positive/HER2-negative metastatic breast cancer [23]. Given the HR-positive population and endocrine-therapy backbone of SOLAR-1, these outcomes should be interpreted as background rather than a direct comparator to a TNBC cohort. Histologically, most cases (90.8%) were invasive ductal carcinoma (IDC), consistent with the distribution reported in the literature [24]. The relatively low frequency of lobular carcinoma (3%) and the higher proportion of metaplastic carcinoma (6.2%) may be attributable to the TNBC-focused cohort in this study. The notably elevated rate of metaplastic carcinoma likely reflects its well-established association with triple-negative phenotype and inherently aggressive biological behavior [2].

When tumor characteristics were examined, the rates of multifocality and bilaterality were 13.8% and 15.4%, respectively. In the literature, multifocality has been reported in the range of 10–15%, while bilaterality has been reported between 2–11% [22]. The high proportion of grade 3 tumors (63.5%) in our cohort further supports the inherently aggressive biology of TNBC. The lymph node metastasis rate of 22.2% was at the lower end of the range reported in previous studies [25], whereas the extranodal extension rate of 10.8% was clinically remarkable. Notably, 86.4% of patients were diagnosed at an early stage (Stage I–II), which is higher than the 70–80% reported in the literature [3], and may reflect improved access to healthcare services and increased awareness. The local recurrence rate was 4.6%, which was lower than previously reported rates, whereas distant metastasis developed in 25% of patients. This finding underscores the high metastatic potential of TNBC, even in cases diagnosed at an early stage, likely due to the biologically aggressive nature of certain subtypes [26].

Based on immunohistochemical evaluation, PIK3CA expression was negative (H-score 0–30), moderate (H-score 31–100), and high (H-score 101–300) in 80%, 6.2%, and 13.8% of TNBC cases, respectively. This distribution indicates that PIK3CA overexpression is restricted to a relatively small subset of TNBC rather than representing a universal molecular feature of this subtype. Previous studies have predominantly linked PIK3CA overexpression to biologically aggressive breast cancer phenotypes, particularly basal-like, HER2-positive, and triple-negative non-basal tumors [10]. PIK3CA protein expression does not necessarily correlate with PIK3CA gene mutation status, which may be explained by post-transcriptional and post-translational regulatory mechanisms influencing protein expression and stability [13]. Interestingly, our findings demonstrated a more favorable overall survival among patients with moderate-to-high PIK3CA expression, contrasting with studies reporting associations with adverse outcomes [24]. This suggests that PIK3CA expression may identify a biologically distinct, potentially more indolent TNBC subset with prognostic relevance, in line with recent evidence demonstrating survival heterogeneity across TNBC subgroups [19]. Collectively, these data highlight that the prognostic significance of PIK3CA may vary across breast cancer subtypes and underscore its potential utility as a stratification biomarker in TNBC. Validation in larger, multi-center cohorts is warranted.

This study investigated the impact of PIK3CA protein expression levels on tumor biology and the disease course of breast cancer. Our findings demonstrated that PIK3CA expression was low in most cases, with only 13 of 65 patients (20%) show-

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ing moderate or high expression. Notably, no mortality or recurrence was observed among patients with high PIK3CA expression; however, given the small subgroup size and the low number of events, this observation should be interpreted cautiously and considered hypothesis-generating rather than evidence of a definitive favorable prognostic effect. The statistically significant difference in mortality rates between the negative and moderate–high expression groups further reinforces this observation.

In our study, PIK3CA expression did not show a significant association with conventional clinicopathological variables, including age, tumor size, stage, histological grade, menopausal status, lymph node involvement, surgical approach, or recurrence. This finding suggests that PIK3CA expression may provide additional prognostic information beyond traditional clinicopathological parameters and could reflect aspects of the tumor's molecular behavior; however, its independent prognostic value could not be established in the absence of multivariable analyses.

Although the detection of PIK3CA mutations in a subset of TNBC cases with high immunohistochemical expression supports a potential molecular association, the limited sample size precludes definitive conclusions; therefore, these should be interpreted as hypothesis-generating. Previous studies have demonstrated that PIK3CA protein expression does not consistently correlate with underlying gene mutations and may arise through mutation-independent mechanisms [10,19,20]. PCR-based mutation analysis was performed on six cases exhibiting high PIK3CA expression to preliminarily investigate the association between protein expression and mutation status. Due to technical constraints at the time of analysis, PIK3CA mutation testing could only be performed in these six cases. This was not a pre-specified selection strategy. Therefore, this mutation analysis should be considered exploratory and interpreted cautiously given the limited sample size and restricted generalizability.

In the present study, PIK3CA mutations were identified in only two cases exhibiting high protein expression, suggesting that elevated PIK3CA expression in TNBC is not uniformly driven by genetic alterations. Alternative regulatory mechanisms, including post-translational modifications, altered protein stability, and activation of the PI3K/AKT pathway independent of direct genetic mutations, have been reported in breast cancer [11,21]. Therefore, the clinical relevance of PIK3CA in TNBC should be evaluated through an integrated assessment of protein expression and molecular alterations rather than mutational status alone [19,20].

Limitations

This study has several limitations. First, the relatively small sample size and uneven distribution of cases across PIK3CA expression subgroups reduced statistical power. In particular, the moderate–high expression subgroup was small, and the absence of deaths in this group may have led to an unstable survival estimate; therefore, the observed overall survival difference should be interpreted with caution. Survival analyses were limited to univariate Kaplan–Meier estimates, as multivariable Cox proportional hazards regression adjusting for established prognostic factors could not be reliably performed due to the limited number of events and complete separation (i.e., zero events in one group). Second, its single-center, retrospective design limits the generalizability of the findings and may introduce selection bias.

Due to the retrospective nature of the study, the possibility of missing clinical or pathological data cannot be excluded. Although PIK3CA mutation analysis was performed, it was limited to cases with high expression levels. Importantly, mutation testing could be performed in only six patients due to limited technical resources at the time of analysis. Mutation testing was restricted to a small subset of high-expression cases as an exploratory, feasibility-driven assessment. Therefore, the lack of mutation testing in low-expression cases limits comparisons across expression subgroups and may introduce selection bias in mutation–expression analyses. Furthermore, the potential discordance between PIK3CA protein expression and gene mutation status adds complexity to the interpretation. Finally, some comparisons in the Discussion necessarily drew on evidence from non-TNBC breast cancer cohorts, which may limit the direct applicability of those contextual comparisons to TNBC. The inclusion of less common histological TNBC subtypes, such as lobular and metaplastic carcinomas, may have introduced biological heterogeneity. Although their inclusion reflects real-world clinical practice and does not materially alter the main findings, future studies focusing exclusively on invasive carcinoma of no special type (NST) that include larger, histologically homogeneous cohorts are warranted to validate and refine these observations. Therefore, the findings should be considered preliminary and hypothesis-generating, requiring validation in larger, multicenter prospective studies with comprehensive molecular profiling and adequately powered multivariable survival modeling [10,20].

Taken together, our findings underscore the potential utility of PIK3CA expression as a biomarker for more refined risk stratification in TNBC and further support the incorporation of molecular markers into routine pathological evaluation to facilitate more precise and personalized therapeutic decision-making. However, its independent prognostic value and clinical utility require confirmation in larger, well-characterized cohorts.

CONCLUSION

In conclusion, alterations in PIK3CA and the PI3K/AKT pathway play a key role in breast cancer. In this study, PIK3CA expression was predominantly low in TNBC cases, with high expression observed only in a small subset of patients. Notably, no mortality was detected among patients with moderate-to-high expression, suggesting a potential as-

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sociation with improved survival.

The absence of correlation between PIK3CA expression and conventional clinicopathological parameters indicates that this biomarker may be linked to more complex molecular mechanisms. Furthermore, the mismatch between protein expression and mutation status highlights that gene analysis alone may not be sufficient.

These findings support the potential prognostic value of PIK3CA expression in TNBC; however, larger multicenter studies are needed to validate these results.

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Informed Consent: Informed consent was waived due to the retrospective nature of the study.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Experiences of orthopaedic and traumatology surgeons with fasciotomy in the 2023 Kahramanmaras earthquake in Türkiye

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MAIN POINTS

- Surgical Burden in Disaster Settings: During the Kahramanmaraş earthquakes, fasciotomies were frequently performed outside the operating room under limited sterile conditions.
- Lack of Standardization: The high rate of inadequate decompression (86%) and variability in incision techniques indicate the need for standardized fasciotomy protocols.
- Impact of Surgical Environment: Fasciotomies performed in operating rooms had significantly lower complication rates, highlighting the importance of appropriate surgical infrastructure.
- Need for Preparedness: Regular training programs and the development of national and international disaster surgery guidelines are essential to improve preparedness and reduce complications.

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ABSTRACT

Aim: February 6, 2023 Kahramanmaras earthquakes were among the most devastating disasters in Türkiye, causing numerous musculoskeletal injuries. This study evaluated orthopedic surgeons' experiences with fasciotomy in disaster zones, focusing on surgical practices, complication rates, challenges, and the adequacy of training.

Materials and Methods: Between March–April 2023, an 18-item online survey was conducted among orthopedic surgeons serving in the earthquake-affected region. A total of 114 surgeons participated. The questionnaire covered demographics, surgical experience, fasciotomy techniques, complications, and training perceptions. Subgroup analyses were performed by experience level, surgical setting, incision preference, and prior fasciotomy exposure.

Results: Fasciotomies were mainly performed in the operating room (74%; n=85), followed by the emergency department (12%; n=14), field hospitals (7%; n=8), intensive care units (4%; n=5), and temporary areas (3%; n=3). For leg fasciotomies, double incisions were preferred in 81% (n=92) and single incisions in 16% (n=18). For forearm fasciotomies, volar incisions were used in 74% (n=84), and combined volar+dorsal incisions were used in 26% (n=30). The overall complication rate was 43% (n=49); the most common complications were bleeding (16%; n=18) and wound problems (12%; n=14). Inadequately decompressed compartments were reported in 86% of cases (n=98). Complications were more frequent among surgeons with 1–5 years' experience (58%; n=18) compared with those with >10 years' experience (35%; n=17) (p=0.03), and were more frequent when procedures performed outside the operating room (55%; n=16) than within it (29%; n=25) (p=0.01). Surgeons with prior fasciotomy experience reported higher confidence (76%; n=73) than those without (41%; n=7) (p<0.01).

Conclusion: The fasciotomy experiences of orthopedic surgeons during the Kahramanmaraş earthquake revealed deficiencies in standardization and competency in disaster surgery. Outcomes depended on the surgeon's experience and on the surgical conditions. Strengthening disaster preparedness requires structured training, simulation-based exercises, national and international guidelines.

Keywords: Earthquake, Fasciotomy, Orthopedic surgeon, Compartment syndrome, Disaster surgery

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INTRODUCTION

The earthquakes centered in Kahramanmaraş on February 6, 2023, were among the most devastating disasters in Türkiye's recent history, resulting in thousands of deaths and tens of thousands of injuries [1]. Earthquakes differ from other natural disasters as they cause a large number of traumatic injuries within a short period and can paralyze healthcare systems [2,3].

Earthquake-related injuries predominantly involve the musculoskeletal system. Previous studies have shown that a significant proportion of these injuries consist of soft tissue damage, long bone fractures, and crush injuries [2-4]. The management of crush injuries requires a multidisciplinary approach, as they may lead to acute compartment syndrome (ACS). If not properly managed, ACS can result in limb loss or mortality [2,4,5]. The most effective method to relieve increased intracompartmental pressure in ACS is urgent fas-

ciotomy [2,6,7]. However, in disaster conditions, controversies exist regarding the timing, incision preference, and sterilization standards of fasciotomy, though the first 24 hours represent a critical time window [5-8].

Earthquakes in Türkiye and other parts of the world can generate a patient burden that challenges or even exceeds healthcare system capacity [9,10]. In such situations, the preparedness level of the health system directly influences survival and rehabilitation outcomes [11,12]. Globally, earthquake-related deaths have averaged around 27,000 annually since the 1990s, with higher mortality and morbidity reported in low-income countries [3].

This study aimed to evaluate the experiences of orthopedic and traumatology specialists who were actively working in disaster zones during the Kahramanmaraş earthquake, focusing on fasciotomy practices, surgical technique preferences, complication rates, and perceptions of training adequacy. Our study aims to provide novel contributions that may support practical approaches and guide educational planning in disaster surgery.

MATERIALS AND METHODS

This study was a cross-sectional, descriptive survey. The study population consisted of orthopedic and traumatology specialists who actively served in disaster zones during the earthquakes that were centered in Kahramanmaraş on February 6, 2023. Inclusion criteria were: working in regional healthcare facilities or field hospitals during the earthquake, and completing the questionnaire in full. Physicians who partially completed the survey or belonged to non-orthopedic specialties were excluded. This cross-sectional survey study was reported in accordance with the strengthening the reporting of observational studies in epidemiology (STROBE) statement [13]. No randomization or allocation was performed because this was a cross-sectional, descriptive survey; groups were defined based on participants' characteristics for comparative analyses.

Primary outcome measure of this study was the self-reported occurrence of fasciotomy-related complications (yes/no) and the distribution of complication types. Secondary outcomes included the frequency of inadequately decompressed compartments and surgeons' self-perceived competence and training adequacy regarding fasciotomy in disaster settings.

Data were collected using an 18-item online questionnaire developed by the researchers based on a literature review of disaster surgery and expert opinion. The survey included questions on demographics, surgical experience, practices, complications, and perceptions of training adequacy. The demographic section assessed age, gender, workplace, years of specialty practice, and type of institution. The surgical practice section included experience with fasciotomy before and after the earthquake, the number of fasciotomies performed, surgical settings (operating room, emergency department, field hospital), and types of incisions. The final section addressed the frequency and types of complications, the adequacy of sterilization conditions, and the self-assessment regarding disaster surgery training.

The questionnaire was prepared using Google Forms and distributed to orthopedic surgeons via email and professional communication groups. It remained open for four weeks, from March to April 2023, and was completed by 114 orthopedic surgeons. Participants were recruited by non-probability convenience sampling via professional communication groups and e-mail lists; participation was voluntary. Data confidentiality was ensured: no personal identifiers were recorded, and results were reported anonymously. No formal a priori sample size calculation was performed due to the descriptive survey design and the disaster context. The questionnaire was distributed to all eligible orthopaedic surgeons serving in the earthquake zone and remained open for four weeks. The final sample size was determined by the number of complete responses received during this period (n=114).

Subgroup analyses were performed based on predefined variables: incision type during leg fasciotomy (double vs. single), sterile conditions (sterile vs. non-sterile), years of surgical experience (1–5 years, 6–10 years, >10 years), prior fasciotomy experience (yes vs. no), and work environment (state or university hospital vs. field hospital). These groups were compared in terms of complication rates, perceptions of training adequacy, incision preferences, and fasciotomy case volume.

Ethics approval for the study was obtained from the Clinical Research Ethics Committee of Mersin University on 17/09/2025 (decision no: 16/1003); informed consent was obtained online from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA; licensed to our institution). Descriptive statistics for continuous variables were presented as mean ± standard deviation (SD) for normally distributed data and as median (interquartile range, IQR) for non-normally distributed data. Categorical variables were expressed as number (n) and percentage (%). Normality was assessed using visual methods (histograms) and the Shapiro–Wilk test. For group comparisons, Student's t-test was used for normally distributed continuous variables, and the Mann–Whitney U test was used for non-normally distributed variables. Categorical variables were compared using the chi-square test or Fisher's exact test, depending on expected cell counts. A two-sided p-value < 0.05 was considered statistically significant.

RESULTS

Total of 114 orthopedic surgeons participated in the study. Of these, 27% (n=31) had 1–5 years of experience, 14% (n=16) had 6–10 years, 44% (n=50) had more than 10 years, and 15%

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Table 1. Subgroup analyses of complications and self-perceived competence.

Variable	Group	Complications (n, %)	p value	Self-perceived competence (n, %)	p value
Experience	1–5 years (n=31) >10 years (n=50)	18 (58%) 17 (35%)	0.030*	19 (61%) 39 (79%)	0.080
Sterile conditions	Sterile (n=73) Non-sterile/partially sterile (n=41)	23 (32%) 25 (61%)	0.004*	--	--
Working environment	Operating room (n=85) Emergency department/field hospital/intensive care unit (n=29)	25 (29%) 16 (55%)	0.010*	65 (77%) 19 (66%)	0.090
Previous fasciotomy experience	Yes (n=96) No (n=18)	39 (41%) 9 (50%)	0.210	73 (76%) 7 (41%)	<0.001*
Incision preference leg	Double incision (n=92) Single incision (n=10)	More skin problems More bleeding	0.110	-- --	-- --
Incision preference forearm	Volar only (n=84) Volar + dorsal (n=30)	Lower complications Higher bleeding/skin issues	0.090	-- --	-- --
Adequacy of prior training/experience	Adequate/partly adequate (n=85) Inadequate (n=29)	26 (31%) 16 (54%)	0.040*	69 (81%) 11 (39%)	<0.001*

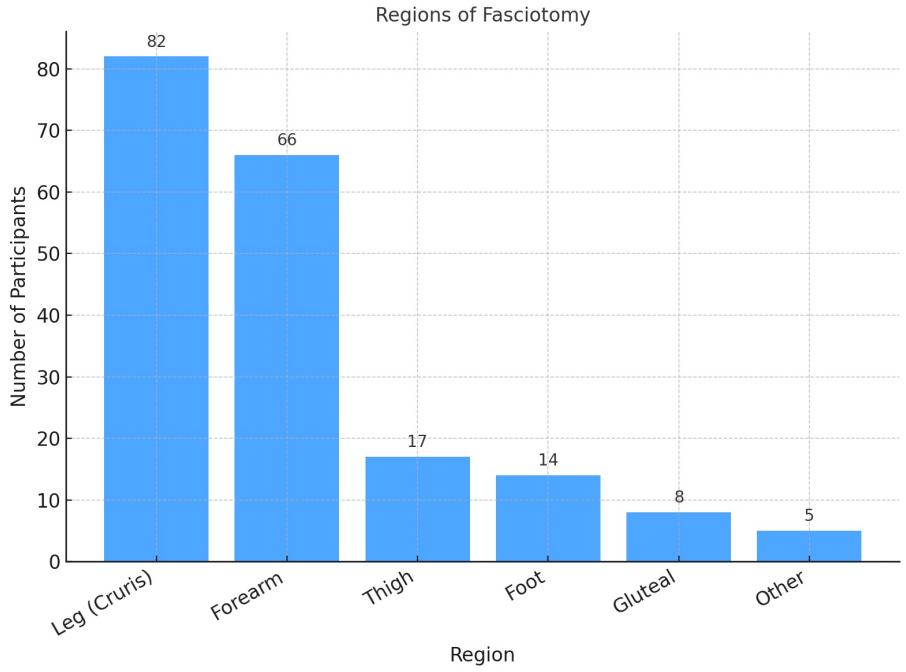

Figure 1. Regions of fasciotomy.

(n=17) were residents. Prior to the earthquake, 84% (n = 96) of participants reported a prior fasciotomy. Fasciotomies were most frequently performed in operating rooms (74%; n=85), followed by emergency departments, field hospitals (7%; n=8), intensive care units (4%; n=5), and temporary surgical areas (3%; n=3). For leg fasciotomies, 81% (n=92) preferred double incisions, 16% (n=18) preferred single incisions, while 2% (n=2) reported using both methods, and 2% (n=2) had not performed a fasciotomy in this region. For forearm fasciotomies, 74% (n=84) used a single volar incision and 26% (n=30) used combined volar and dorsal incisions.

The most common fasciotomy site was the leg (72%; n=82), followed by the forearm (58%; n=66), the thigh (15%; n=17), the foot (12%; n=14), and the gluteal region (7%; n=8). A smaller proportion (4%; n=5) reported fasciotomies in other areas, such as the hand or upper arm (Figure 1). In terms of patient volume, 55% (n=63) operated on 1–5 patients, 28% (n=32) operated on 6–10, 12% (n=14) operated on 11–20, and 5% (n=5) operated on more than 20 patients.

Regarding complications, 43% (n=49) reported none. The most frequent complications were bleeding (16%; n=18) and wound problems (12%; n=14). Combined bleeding and wound complications occurred in 14% (n=16), while nerve or vascular injuries or multiple complications occurred in 10% (n=11) (Figure 2). Regarding self-assessment, 31% (n=35) reported feeling fully competent, 52% (n=59) partially compe-

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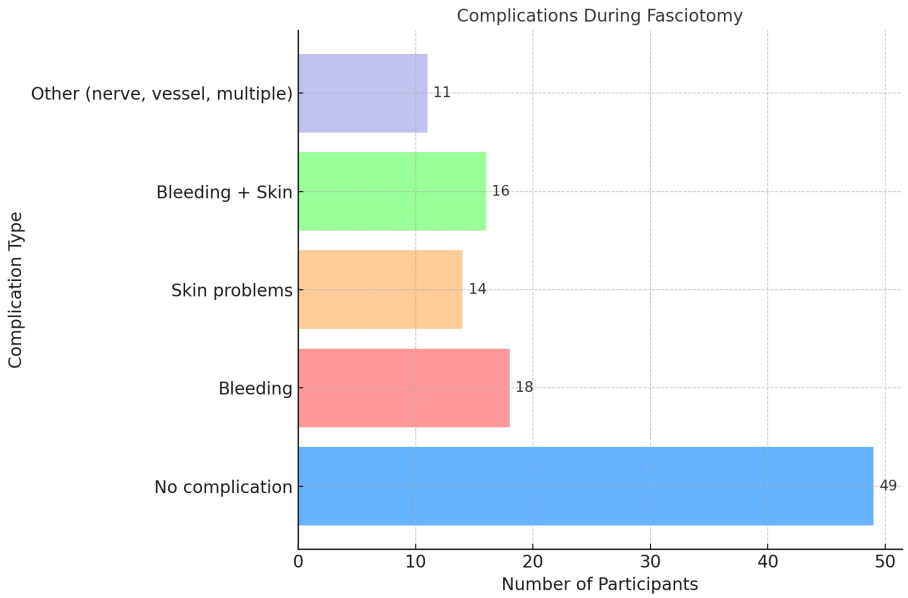

Figure 2. Complications during surgery.

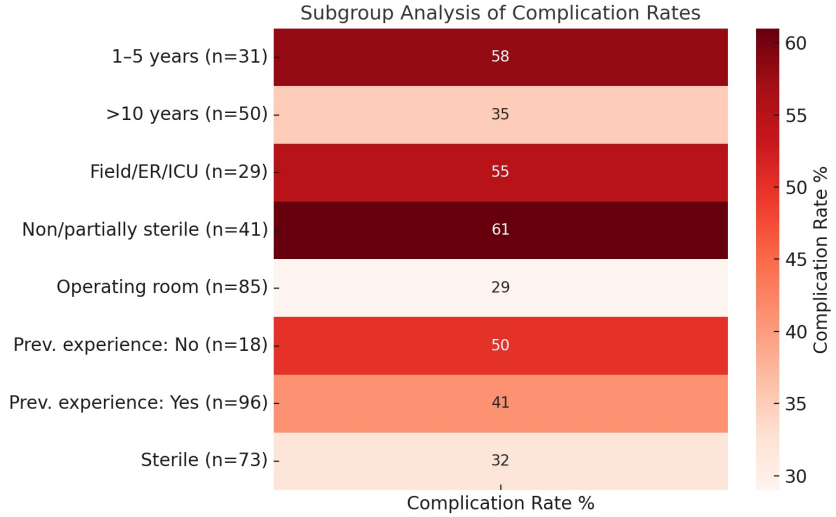

Figure 3. Subgroup analysis complication rates.

tent, and 17% (n=20) inadequate.

Subgroup analyses revealed a significant relationship between years of experience and complication rates. Among surgeons with >10 years of experience, 17 of 50 (35%) reported complications, compared with 18 of 31 (58%) among surgeons with 1–5 years of experience (p=0.03). Complication rates also differed by surgical setting: 25 of 85 fasciotomies (29%) performed in operating rooms had complications, compared with 16 of 29 (55%) performed in emergency departments, field hospitals, or ICUs (p=0.01) (Figure 3). Surgeons with prior fasciotomy experience reported greater confidence (76%; n=73) than those without such experience (41%; n=7) (p<0.01). However, complication rates did not differ significantly between the two groups (p=0.21). Incision preference was not associated with statistically significant differences in complication rates (p>0.05). These comparisons are presented in Table 1.

A majority of participants (86%; n=98) reported encountering at least one inadequately decompressed compartment during the earthquake. The most common reasons were short skin incisions, failure to open certain compartments, and insufficient fascial release. These three factors occurred together in 38% of cases (n=43). Only 14%(n=16)reported no such issues. Regarding skin closure, 57% (n=65) stated they used sutures when necessary, 29% (n=33) never used them, 11% (n=12) always applied sutures, and 2% (n=2) reported initially suturing but later continuing with dressings.

Suggested improvements included increasing training and course offerings (61%; n=70) and developing disaster surgery protocols (52%; n=59). Other recommendations included preparation of surgical equipment (47%; n=54), interdepartmental coordination (36%; n=41), strengthening communi-

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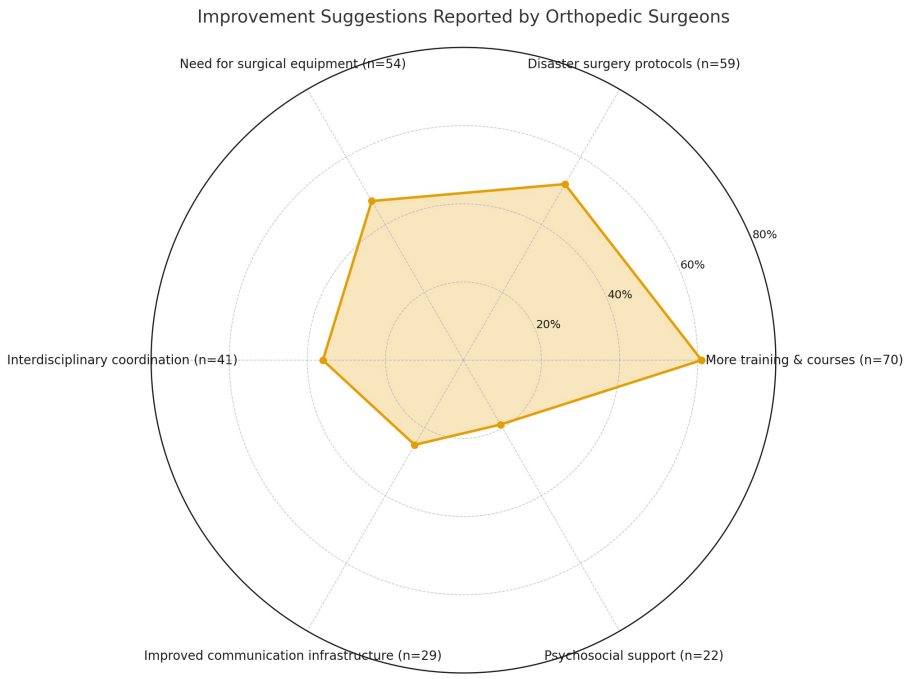

Figure 4. Improvement suggestions reported by orthopedic surgeon.

cation infrastructure (25%; n=29), and psychosocial support (19%; n=22) (Figure 4).

DISCUSSION

This study is among the first to systematically evaluate orthopaedic surgeons' experiences performing fasciotomy while working under earthquake conditions. Although previous studies have addressed the epidemiology and surgical management of earthquake-related orthopaedic injuries [13–15], surgeons' preferences, technical approaches, complications, challenges, and training needs related to fasciotomy in disaster settings have not been sufficiently explored. Therefore, our findings provide important insights into surgical decision-making and practice variability under extraordinary circumstances.

A key finding of this study is the lack of standardization in fasciotomy practice during disaster response. Surgeon experience significantly influenced outcomes; higher complication rates among participants with 1–5 years of experience suggest that technical proficiency and clinical judgement are critical determinants of success. This aligns with Lu-Ping et al. [16], who highlighted better outcomes in disaster injury management by experienced teams.

The surgical environment was another major determinant. Fasciotomies performed outside the operating room were associated with higher complication rates, consistent with prior reports indicating increased infection risk in non-standard settings, including field hospitals [17]. Similarly, organized triage systems and access to appropriate operating room infrastructure have been shown to reduce adverse outcomes in earthquakes [18]. Collectively, these findings emphasize the need for logistical preparedness and structured surgical systems in disaster medicine. These findings are consistent with earthquake-related orthopaedic reports from Türkiye, which emphasize the overwhelming patient load, disruption of hospital logistics, and the critical importance of organized triage and prioritization of urgent procedures (e.g., fasciotomy) during the early disaster response period [19,20].

With regard to technique, double-incision leg fasciotomy and volar forearm fasciotomy were the most commonly used approaches. Although incision type was not significantly associated with complications, heterogeneity in surgical practice was evident. As noted in the literature, multiple fasciotomy techniques may yield favourable results when correctly performed; therefore, the focus should remain on adequate decompression and adherence to trauma principles, including damage control surgery [21–24].

Notably, most participants reported encountering inadequately decompressed compartments, primarily due to short incisions, unopened compartments, or insufficient fascial release. This finding is clinically relevant, as incomplete decompression can increase morbidity and negatively affect long-term functional outcomes. In disaster settings where systematic monitoring and serial reassessment are limited, the risk of technical inadequacy may be higher.

Decision-making for fasciotomy remains challenging in earthquake zones. In the absence of compartment pressure monitoring, clinical examination becomes the main diagnostic tool; when serial assessments are not feasible, early fasciotomy may be considered despite an increased risk of infection [25]. In our cohort, the high frequency of fasciotomy, the complication burden, the frequent reports of inadequate decompression reflect the complexities and constraints of disaster surgery.

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Finally, surgeons with prior fasciotomy experience reported greater confidence in both decision-making and technical execution, supporting the importance of structured training. Standardized disaster surgery courses, simulation-based training, and field exercises may improve preparedness and reduce complication rates in future disasters [14,17,23,26].

This study has limitations. Its survey-based design may introduce recall and self-reporting biases, and complications may not be objectively verifiable. Nevertheless, the strength of this study lies in capturing the experiences of a large cohort of orthopaedic surgeons working under unprecedented earthquake conditions.

In conclusion, our findings reveal substantial variability in fasciotomy practice during the Kahramanmaraş earthquake and highlight the influence of surgeon experience, surgical environment, and training adequacy on outcomes. These results may inform the development of disaster-oriented training programs and contribute to establishing standardized protocols for fasciotomy in mass casualty settings.

CONCLUSION

This study highlighted the experiences of orthopedic surgeons in performing fasciotomy during the Kahramanmaraş earthquakes. The findings revealed that many surgeons were compelled to perform fasciotomies outside the operating room, frequently encountered inadequately decompressed compartments, and experienced complications at a considerable rate. The lack of standardization in disaster surgery influenced surgeons' approaches to patient management and led to variations in treatment protocols.

Our results indicate that patient management and surgical success in disaster settings depend not only on individual experience but also on appropriate surgical conditions, logistical support, and pre-established standardized protocols. Therefore, regular training programs, field exercises, and the development of standardized national and international disaster surgery guidelines are critically important for future disaster preparedness.

Ethics Committee Approval: Ethical approval for the study was obtained from the Mersin University Ethics Committee (Date: 17.09.2025, Approval number: 16/1003).

Informed Consent: Informed consent was obtained online from all participants.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The authors declare no conflicts of interest.

Author Contributions: Conceptualization: ÖK, FG, İD, BS; Methodology: ÖK, FG; Formal analysis and investigation: ÖK, FG, İD, BS; Writing - original draft preparation: ÖK; Writing - review and editing: ÖK, FG, İD, BS; Funding acquisition: ÖK, İD; Resources: ÖK, BS; Supervision: ÖK, FG, İD, BS.

Financial Disclosure: None.

Artificial Intelligence Disclosure: In this study, an artificial intelligence–based tool (ChatGPT, OpenAI, GPT-5) was used. This tool was used solely for language editing, translation, and improving the clarity and readability of the text. It was not used in the stages of study design, data evaluation, analysis of results, or generation of scientific interpretations. All parts of the manuscript, including data collection, statistical analysis, and interpretation of findings, were carried out entirely by the authors. All text revised with the assistance of artificial intelligence was carefully reviewed, revised, and validated by the authors. We confirm that the authors take full responsibility for the content of the manuscript and that the use of artificial intelligence complies with the ethical guidelines of the journal.

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Clinical course and reaction profile of patients undergoing venom immunotherapy

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MAIN POINTS

- Systemic reactions occurred in 22.6% of patients undergoing venom immunotherapy (VIT), with a higher frequency in the honeybee group, consistent with prior data.
- Most systemic reactions occurred during the first 1–6 weeks and were successfully managed with dose reduction, dose splitting, or omalizumab support when necessary.
- Severe (Grade 3) index reactions were common, while adrenaline use during initial sting reactions was notably insufficient.
- Omalizumab facilitated safe continuation of VIT in selected patients who developed early high-grade systemic reactions.
- Real-world data highlight the importance of close monitoring and individualized management strategies during VIT.

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ABSTRACT

Aim: Bee venom allergy is a major cause of anaphylaxis in adults. Venom Immunotherapy (VIT) is the only proven curative treatment, but the frequency and management of local and systemic reactions during therapy are essential considerations for safety. This study aimed to evaluate the demographic and clinical characteristics of patients undergoing VIT and to analyze reaction rates and management strategies.

Materials and Methods: This retrospective single-center study included 31 patients (18 honeybee, 13 vespid)who received VIT at the Ankara Bilkent City Hospital Allergy and Clinical Immunology Clinic between October 2024 and September 2025. All patients received a standard premedication protocol. Demographic, clinical, and laboratory data were obtained from medical records. Systemic reactions were classified according to the Ring–Messmer grading system, and management strategies for VIT-related reactions were analyzed.

Results: The mean age of the patients was 41.5 ± 15.3 years, and 61.3% were male. A history of beekeeping was significantly more common among honeybee-allergic patients (p = 0.009). Index sting reactions were often; Grade 3 reactions were observed in 58.1% of cases. Respiratory (77.4%) and cardiovascular (64.5%) involvement were the most common clinical manifestations; adrenaline was administered in 54.8% of index reactions. During VIT, 12 patients (38.7%) developed reactions, of which 7 (22.6%) were systemic. Systemic reactions occurred more frequently in the honeybee VIT group than in the vespid group, although this difference was not statistically significant (p = 0.101). Most systemic reactions were mild to moderate and occurred early during treatment. In selected cases, VIT was successfully continued with dose adjustment and adjunctive omalizumab therapy.

Conclusion: Systemic reactions during VIT were more frequent among honeybee venom–allergic patients but were generally mild to moderate and manageable. With close monitoring, appropriate dose modifications, and biologic support when indicated, VIT can be safely continued in most patients.

Keywords: Venom immunotherapy, Bee venom allergy, Honeybee allergy, Vespid allergy, Anaphylaxis, Omalizumab

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INTRODUCTION

Hymenoptera stings are very common, and data show that 56.6-94.5% of the general population will be stung at least once in their lifetime [1]. Approximately 3% of the general population will develop a systemic reaction following a Hymenoptera sting during their lifetime [2,3].

The only known effective treatment for preventing life-threatening systemic allergic reactions in patients with Hymenoptera venom allergy is venom immunotherapy (VIT). VIT is highly effective in preventing systemic sting reactions in both children and adults and significantly improves quality of life [1,4].

However, as with all immunotherapies, VIT carries certain risks of side effects and adverse events. Although these re-

actions that may occur during treatment are generally mild, transient local symptoms, serious systemic reactions may rarely occur. Systemic adverse events have been reported to be more frequent with immunotherapies targeting bee venom. The patient's age, concomitant cardiovascular diseases or mastocytosis, use of ACE inhibitors and/or beta-blockers, and the application protocol (e.g., rush or ultra-rush) may be considered decisive factors in these side effects [1,2]. A recent multicentre study reported that ACEI and beta-blocker use did not increase the risk of systemic reactions during vitiligo treatment [5].

Therefore, studies on the safety of bee VIT, the frequency of side effects, the associated risk factors, and their management are critical for the clinical application of the treatment.

Although the efficacy of VIT is well known, real-world data on the frequency, severity, and management of adverse reactions occurring during VIT in routine clinical practice are limited. Furthermore, evidence regarding patient-related risk factors and management strategies for systemic reactions occurring during VIT is still developing, highlighting the need for additional observational studies.

The primary aim of our study is to evaluate, using real-world data, the clinical course and reaction profiles of patients treated with VIT. By examining the side-effect profile of venom immunotherapy vaccines, which are newly introduced and have no alternatives in our country, we aim to provide guidance to those who will use these vaccines. Secondary objectives include comparing the characteristics and frequencies of reactions associated with honeybee and wasp VIT, evaluating strategies for managing VIT-related reactions, and examining the use of omalizumab in patients who develop systemic reactions during immunotherapy.

MATERIALS AND METHODS

Study design

Our study is a retrospective observational study of patients who started bee VIT at the Immunology and Allergy Clinic of Ankara Bilkent City Hospital between October 2024 and September 2025. A total of 31 patients who started treatment with VIT were included in the study. Patients were identified from electronic medical records. All consecutive patients who received VIT at our center during the study period and who met the inclusion criteria were included in the analysis. The Inclusion criteria included patients with a documented history of systemic reactions to Hymenoptera stings who received VIT. No eligible patients were excluded. Written informed consent was obtained from all patients by the treating physicians at the time of initiation of VIT as part of routine clinical practice. Due to the retrospective design, the study was conducted using existing clinical data in accordance with ethical principles.

The primary outcome measure was the occurrence of systemic reactions during venom VIT. Secondary outcome measures included the frequency and types of VIT-related reactions (local, large local, and systemic); the severity of systemic reactions according to the Ring–Messmer classification; organ involvement; the need for adrenaline administration; the timing of reactions; and management strategies, including dose adjustment and use of omalizumab.

Data from patients who developed a systemic reaction following a honeybee or vespid sting and who were indicated for VIT according to the European Academy of Allergy and Clinical Immunology (EAACI) guidelines were analyzed. The study was initiated following approval by the local ethics committee of Ankara Bilkent City Hospital (Date: 15.10.2025; Approval no: TABED 2-25-1572) in accordance with the tenets of the Declaration of Helsinki.

Data collection

Patients' demographic, clinical, and laboratory data were obtained from the hospital electronic record system and patient files. These data included age, gender, occupation, place of residence, comorbidities, use of beta-blockers or ACE inhibitors, the type of stinging insect, and characteristics of the index reaction. Organ involvement (respiratory, gastrointestinal, cardiovascular, cutaneous, and neurological) during reactions was also recorded. Among patients who developed a reaction during VIT administration, the reaction type (local, extensive local, systemic), severity, involved organs, and use of adrenaline were recorded. In cases where a systemic reaction was detected, the dose at which the reaction developed, the week of occurrence, tryptase levels, response to subsequent doses, and continuation of treatment were examined. The severity of index reactions and those experienced during VIT was graded according to the Ring–Messmer systemic reaction classification. The Ring–Messmer systemic allergic reaction classification defines the clinical severity of anaphylactic reactions in four stages. Grade I is characterized by skin involvement, such as urticaria and itching. Grade II refers to the condition in which, in addition to skin manifestations, mild to moderate cardiovascular and respiratory symptoms are present. Grade III encompasses serious, life-threatening reactions and is characterized by marked hypotension, bronchospasm, and loss of consciousness. Grade IV represents the most severe condition, defined by cardiac and/or respiratory arrest, and requires emergency intervention [6].

Diagnostic process

Sensitivity in adult patients was evaluated using in vitro tests measuring honeybee- and vespid-specific IgE levels. Due to the unavailability of the prick test solution for bee venom in our clinic, the evaluation of bee allergy in the patient was performed using in vitro diagnostic methods. IgE levels for honeybee and vespid sp. were analyzed using the Immulite 2000 system (Siemens Healthcare Diagnostics, Tarrytown, NY). A sp IgE level $\geq$0.35 kU/L was considered positive for venom

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sensitivity. Component-specific testing was performed in patients with dual positivity. Component-specific testing allows evaluation of IgE antibodies against individual allergen components (e.g., Api m 1 and Api m 2 for honeybee; Ves v 1 and Ves v 5 for vespid) rather than at the extract level. This method helps distinguish cross-sensitivity from true dual sensitivity [7].

VIT application process

All patients received the standard premedication protocol as outlined in the EAACI guidelines: on the day of the procedure, an additional dose of cetirizine (10 mg) was administered 1 hour prior to the procedure [1].

VIT was administered to all patients using the same allergen extract (Roxall®, Roxall Medizin GmbH, Germany) in the conventional format. Venom extracts from the honey bee (Apis mellifera) and vespid wasps (Vespula) extracts were used in the treatment. The conventional Roxall protocol is based on the principle of increasing doses. The applications were performed subcutaneously, with the dose increased weekly. Starting with low doses, patients were gradually titrated to the target maintenance dose of 100 μg. After the initial phase was completed within 8 weeks, the maintenance phase was initiated. During the maintenance phase, patients received injections of the relevant venom (honeybee or vespid) at a dose of 100 μg every 4 weeks. All injections were performed in clinical settings with access to emergency intervention for potential local or systemic reactions. Patients were observed for at least 2 hours after each administration. Any local or systemic reactions that occurred were recorded in the patient's file.

In cases in which extensive local reactions developed during treatment, cold application was performed in accordance with the EAACI recommendations. In patients who developed a systemic reaction (SR), adrenaline, antihistamine (Avil), systemic corticosteroid (Prednol), and intravenous fluid therapy were administered according to the severity of the reaction; patients were kept under observation. In subsequent applications, a step reduction was performed in accordance with the EAACI guidelines. If the systemic reaction recurred despite the dose reduction, or if a systemic reaction developed at the first dose and was assessed as high-grade, premedication with omalizumab was administered. Dose adjustments during VIT were implemented for patients who experienced systemic reactions or clinically significant large local reactions, based on clinical judgment and current guideline-based practice. In cases of recurrent systemic reactions despite dose reduction, adjunctive treatment with omalizumab was initiated to improve tolerability and enable continuation of VIT [1].

Systemic reactions were defined and graded according to the Ring and Messmer classification. Local reactions were defined as erythema and swelling limited to the injection site, whereas large local reactions were defined as swelling exceeding 10 cm in diameter and persisting for more than 24 hours. All VIT-related reactions were prospectively recorded during treatment visits and classified by clinical presentation and organ involvement.

Statistical analysis

Due to the retrospective nature of the study, no a priori sample size calculation was performed before data collection, and all eligible patients who met the inclusion criteria were included in the final analysis. All data were analyzed using SPSS Statistics version 25.0 (Armonk, NY: IBM Corp.). The normality of continuous variables was assessed using the Kolmogorov–Smirnov test and visual inspection of histograms and Q–Q plots. Continuous variables are expressed as mean ± standard deviation, while categorical variables are expressed as counts and percentages. For comparisons between groups, the Chi-square test or Fisher's exact test were used for categorical variables, and the Student's t-test or Mann–Whitney U test were used for continuous variables. A significance level of $p<0.05$ was accepted for all statistical analyses.

RESULTS

General characteristics of the study population

A total of 31 patients who started VIT were included in this study. Bee immunotherapy was initiated in 18 (58%) patients, and vespid immunotherapy was initiated in 13 (42%) patients. Nineteen participants (61.3%) were male, and the mean age of all patients was 44.6 ± 13.18 years. Twenty-nine patients (93.5%) lived in urban centers, and 2 (6.5%) lived in rural areas. Individuals involved in beekeeping or living in near beekeeping activities were significantly more common in the group receiving honeybee VIT (p=0.009). No significant difference was observed between the two groups in terms of the presence of comorbidities (p=0.463). Sixteen patients (51.6%) had no chronic diseases. Chronic spontaneous urticaria was present in 4 patients (12.4%), and allergic rhinitis/asthma in 2 patients (6.5%) (Table 1).

Evaluation of index reaction severity and features

Grade-3 reactions (n:18, 61.5%) were the most frequently observed with respect to index reaction severity (p=0.567). There was no significant difference in the severity of index reactions between vespid and honeybee stings (p=0.567). The systems most frequently affected during index reactions were the respiratory system (77.4%), the cardiovascular system (64.5%), and the skin (64.5%). The hematology service performed a bone marrow biopsy to investigate mast cell disorders in a patient with a basal tryptase value of 13. Hematology did not consider mastocytosis in the patient because the bone marrow biopsy result was normal (Table 2).

Management of reactions occurring during VIT

A total of 12 patients (38.7%) developed reactions during VIT. Of these reactions, 22.6% were systemic, and 16.1% were local or extensive. Systemic reactions were more common in

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Table 1. Patients' demographic features.

Feature	Vespid VIT (n=13)	Honeybee VIT (n=18)	Total (n=31)	p value
Gender (male), n (%)	7 (53.8)	12 (66.7)	19 (61.3)	0.710
Comorbidities, n (%)				
-None	5 (38.5)	11 (61.1)	16 (51.6)	
-Chronic spontaneous urticaria	1 (7.7)	3 (16.7)	4 (12.4)	0.463
-Asthma and / or allergic rhinitis	1 (7.7)	1 (5.6)	2 (6.5)	
-Chronic illness existence	7 (53.8)	3 (16.7)	10 (32.5)	
Age (mean± SD)	46.84 ±15.79	43.11 ± 11.16	44.6 ± 13.18	0.182
Place of residence, n (%)				
-City	13 (100)	16 (88.9)	29 (93.5)	0.497
-Rural	0	2 (11.1)	2 (6.5)	
Profession, n (%)				
-Beekeeping (self or relatives)	0 (0)	6 (33.3)	6 (19.4)	**0.009**
-Field work with exposure risk	1 (7.7)	5 (27.8)	6 (19.4)	
-Other	2 (92.3)	7 (38.9)	19 (61.3)	
Beta blocker therapy, n (%)	2 (15.4)	1 (5.6)	3 (9.7)	0.364
ACE inhibitor therapy, n (%)	1 (7.7)	1 (5.6)	2 (6.5)	0.671

Abbreviations: VIT: venom immunotherapy; ACE: angiotensin converter enzyme; SD: standard deviation.

Table 2. Index reaction and its properties.

Variables	Vespid	Honeybee	Total	P
Number of bee stings, n (%)				
-1 time	8 (61.6)	9 (50)	17 (54.8)	0.638
-2 time	3 (23.1)	2 (11.1)	5 (16.1)	
-3 or more	2 (15.4)	7 (38.9)	9 (29.1)	
Number of previous reactions, n (%)				
-1 time	9 (69.2)	15 (83.3)	24 (77.4)	0.635
-2 time	3 (23.1)	2 (11.1)	5 (16.1)	
-3 or more	1 (7.7)	1 (5.6)	2 (6.5)	
Severity of index reaction, n (%)				
-Grade-1	0	1 (5.6)	1 (3.2)	
-Grade-2	5 (38.5)	7 (38.9)	12 (38.7)	0.567
-Grade-3	8 (61.5)	10 (55.6)	18 (58.1)	
-Grade-4	0	0	0	
Index reaction organ involvement, n (%)				
-Respiratory System	10 (76.9)	14 (77.8)	24 (77.4)	
-Gastrointestinal system	2 (15.4)	4 (22.2)	6 (19.4)	0.811
-Skin (Urticaria,angioedema)	8 (61.5)	12 (66.7)	20 (64.5)	
-Cardiovascular	9 (69.2)	11(61.1)	20 (64.5)	
-Neurological system	1 (7.7)	1 (5.6)	2 (6.5)	
Adrenaline use in index reaction, n (%)				
-Yes	6 (46.2)	11(61.1)	17 (54.8)	0.393
-No	2 (15.4)	4 (22.2)	6 (19.4)	
-Unknown	5 (38.5)	3 (16.7)	8 (25.8)	
Laboratory Parameters, median(min-max)				
-Specific IgE Honeybee	0.1 (0.1-0.35)	21.9 (0.35-100)	1.05 (0.1-100)	**0.001**
-Specific IgE Vespid	3.1 (0.7-83.6)	0.11 (0.1-52.2)	1.01 (0.1-83.6)	**0.001**
-Total IgE (IU/ mL)	36.5 (2-219)	58.5 (1-780)	48 (2-780)	0.187
-Tryptase (ng / mL)	5.48 (2.5-20)	5.42 (3.3-15.7)	5.48 (2.5-20)	0.433

*Ring -Messmer Systemic Reaction Rating.

the group receiving bee VIT. Table 3 shows, for patients who developed reactions during VIT application, the type of reaction (local, extensive local, systemic), severity, affected organs, and use of adrenaline.

Systemic reactions developed in 7 (22.6%) patients. Upon examination of the clinical course of these patients, the reaction was observed to develop after the first dose in 3 (9.7%) patients. In two of these cases, VIT treatment was continued after omalizumab premedication, while one patient discontinued treatment at their own request after anaphylaxis devel-

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Table 3. Venom Reactions in patients receiving immunotherapy.

Variables	Vespid	Honeybee	Total	P
Premedication before VIT, n (%)	13 (100)	18 (100)	31	1
History of reaction during VIT,n (%)	4 (30.8)	8 (44.4)	12 (38.7)	0.484
Type of reaction, n (%)				
-Local	3 (23.1)	0	3 (9.7)	0.101
-Large local	0	2 (11.1)	2 (6.5)	
-Systemic	1 (7.7)	6 (33.3)	7 (22.6)	
Adrenaline use in VIT reactions*, n (%)	1 (7.7)	5 (27.8)	6 (19.4)	0.359
Severity of systemic reactions during VIT, n (%)				
-Grade-1	0	3 (16.7)	3 (9.7)	0.860
-Grade-2	1 (7.7)	1 (5.6)	2 (6.5)	
-Grade-3	0	2 (11.1)	2 (6.5)	
-Grade-4	0	0	0	
Organ involvement, n (%)				
-Respiratory System	0	1 (5.6)	1 (3.2)	NA
-Gastrointestinal system	0	3 (16.7)	3 (9.7)	
-Skin (Urticaria,angioedema)	1 (7.7)	5 (27.8)	6 (19.4)	
-Cardiovascular	1 (7.7)	2 (11.1)	3 (9.7)	
-Neurological system	0	0	0	

Abbreviations: VIT: Venom immunotherapy. *Ring -Messmer Systemic Reaction Rating.

Table 4. Clinical course of patients with systemic reactions to VIT.

Pt	Gender	Age	Received VIT	Systemic Reaction Grade*	Systemic Reaction Adrenaline	Reaction dose	Timing of reaction	How to Proceed After the Reaction	Did the reaction repeat?
1	Woman	52	honeybee	3	Applied twice	1st dose	Week 1	The patient discontinued treatment	-
2	Woman	53	honeybee	1	Applied once	4th dose	Week 4	The dose was continued by decreasing to a lower dose level.	Subsequent doses were well tolerated; maintenance therapy was initiated
3	Woman	41	vespid	2	Applied once	1st dose	1st dose	Omalizumab was started because he had a reaction to his first dose	Subsequent doses were well tolerated; maintenance therapy was initiated
4	Male	36	honeybee	3	Applied once	1st dose	1st dose	Omalizumab was started because he had a reaction to his first dose	VIT initial treatment is administered under omalizumab
5	Male	36	honeybee	1	Applied once	6th dose	Week 6	The dose was continued by decreasing to a lower dose level	Subsequent doses were well tolerated; maintenance therapy was initiated
6	Male	47	honeybee	2	Applied twice	5th dose	Week 5	The dose was continued by decreasing to a lower dose level	Subsequent doses were well tolerated; maintenance therapy was initiated
7	Male	54	honeybee	1	Not used	2nd dose	Week 2	The dose was continued by decreasing to a lower dose level	Initial treatment continues

Abbreviations: VIT: Venom immunotherapy, Pt: Patient. *Ring -Messmer Systemic Reaction Rating.

oped during the first dose. Omalizumab treatment was initiated in 2 patients who experienced a systemic reaction during the first dose of VIT. After obtaining off-label approval for omalizumab treatment, the patient received 300 mg of omalizumab monthly for 3 months, and the first VIT dose was repeated 1 week after the third omalizumab dose. Omalizumab treatment was continued until maintenance therapy was initiated. Most reactions were observed between weeks 1 and 6.

In most cases in which reactions developed, treatment was continued by reducing the dose and administering it in di-

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vided doses. No recurrence of systemic reactions was observed in these patients after subsequent doses. The characteristics of patients who experienced systemic reactions associated with VIT are shown in Table 4.

DISCUSSION

In our study, we evaluated the general characteristics of patients receiving bee VIT, the characteristics of the index reaction, the frequency and types of reactions that developed during treatment, and the management strategies. Roxall®, a product of Roxall Medizin GmbH used for VIT in our country, includes 1-year follow-up results for conventional VIT. Given that Roxall, recently introduced in our country, is the only vaccine currently used in bee venom immunotherapy and the only vaccine evaluated for its reaction profile, this study aims to create a guideline for clinical practice by identifying the intervention approaches applied to patients who develop a reaction during bee venom immunotherapy and the situations in which anti-IgE therapy is initiated as prophylaxis.

A post hoc power analysis was performed based on the observed rate of systemic reactions during venom immunotherapy. Systemic reactions occurred in 7 of 31 patients (22.6%). Using a reference incidence of 7.5% reported in previous studies and a two-sided α level of 0.05, the statistical power obtained was approximately 57% at a 95% confidence level. Although the achieved statistical power was moderate because of the limited sample size, the substantially higher observed reaction rate supports the clinical relevance of the findings.

Among patients indicated for VIT, demographic characteristics such as bee species distribution, occupational exposure, comorbidities, and cardiovascular medication use were generally similar between patients receiving honeybee VIT and patients receiving vespid VIT; however, honeybee sensitivity was significantly higher among patients engaged in beekeeping (p=0.009). This finding is consistent with the literature, which indicates a higher risk of re-stinging and a higher incidence of systemic reactions, particularly among beekeepers [2,8,9].

In our study, the rates of β-blocker and ACE inhibitor use were low, and no differences were observed between groups. In a multicenter, prospective study involving 1342 patients, these drugs did not increase the frequency of systemic adverse events during VIT or worsen the severity of shock-related reactions. This finding supports our assessment that cardiovascular treatments alone are not contraindicated for VIT in our clinical practice [5].

In our study, systemic reactions associated with both honeybee and vespid stings were predominantly grade 2–3, consistent with the VIT indication, and involved the respiratory, skin, and cardiovascular systems; this distribution is consistent with studies in the literature [1,2].

The rate of adrenaline administration during the index event was 54.8%. The literature emphasizes that adrenaline should be administered intramuscularly as a first-line treatment and as early as possible; however, real-world data show that adrenaline use is insufficient. This finding has also been highlighted in numerous studies demonstrating the underuse of adrenaline [10-12].

In many multicenter studies, the incidence of systemic reactions during VIT ranges from 8% to 20%. The primary risk factor is treatment involving honeybee venom. The risk of systemic adverse reactions associated with bee venom therapy has been reported to be 3.1 to 6 times higher [1]. In our study, the systemic reaction rate was determined to be 22.6%, which is similar to the rates reported in previous similar studies [13-16]. Findings in the literature indicating a higher frequency of systemic reactions, particularly with immunotherapies targeting bee venom, are also consistent with our results [1,2,16].

In most of our patients, basal tryptase levels are within the normal range despite the development of SR; this is consistent with guidelines and review data showing that elevated basal serum tryptase and mast cell disease are strong predictors of SR risk, but normal tryptase does not rule out SR [1,2]. Additionally, the literature emphasizes that systematic screening for clonal mast cell diseases, such as mast cell activation syndrome, may be necessary even if the baseline tryptase level is normal, particularly in patients with severe anaphylaxis. Routine screening is recommended for patients with Hymenoptera venom allergy [17,18].

In patients experiencing systemic reactions, the strategy of continuing treatment by stepping down to a lower dose after the reaction is a commonly used practice that enhances safety [1,2,19].

In two patients, omalizumab was added to VIT after an SR developed following the first dose. Omalizumab neutralizes free IgE and reduces FcεRI expression, thereby decreasing mast cell and basophil activity and reducing mast cell degranulation upon exposure to venom allergens. There is a large body of literature indicating that VIT can be safely continued with short-term omalizumab in selected cases; however, this use is off-label, and there is no standard dose-timing regimen [20-23].

Limitations and strengths

Our study has several limitations. First, its retrospective, single-center design and relatively small sample size may limit both the statistical power of the analyses and the generalizability of the findings. Second, because only the conventional Roxall protocol was evaluated, comparisons with alternative VIT protocols were not possible. Because the study focused on short-term outcomes, long-term maintenance responses and field testing outcomes could not be assessed. Consequently, the applicability of the results to long-term clinical practice may be limited. Finally, venom sensitization was assessed using in vitro diagnostic methods, including measurement of venom-specific IgE levels. Skin testing could not be

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performed during the study period due to the unavailability of standardized skin test solutions; therefore, diagnostic evaluation relied solely on in vitro testing and clinical history.

One strength of this study is its reliance on real-world data from the Allergy-Immunology Clinic of a large urban hospital in Turkey. It features a clinical report evaluating the management of systemic reactions developing during VIT administration. Furthermore, adding omalizumab to treatment when systemic reactions develop has shown that safely continuing treatment is a practical and feasible strategy in daily clinical practice.

CONCLUSION

In conclusion, our study shows that the frequency of systemic reactions (SR) is consistent with the literature and that honeybee VIT is associated with a higher risk of SR. Inadequacies in the use of epinephrine for the management of index reactions have been demonstrated. Systemic reactions during VIT can be managed by adding omalizumab or by stepping down the dose, depending on the severity of the reaction and the dose at which it developed. Dissimilarities between our results and those reported in other studies may be related to the study's retrospective, real-world design, the relatively small sample size, differences in patient characteristics, and variations in venom type distribution and immunotherapy protocols.

Ethics Committee Approval: The study was initiated after approval from the local ethics committee of Ankara Bilkent City Hospital (Date: 15.10.2025; Approval no: TABED 2-25-1572).

Informed Consent: Due to the retrospective design, informed consent was not obtained from patients.

Peer-review: Externally peer-reviewed.

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