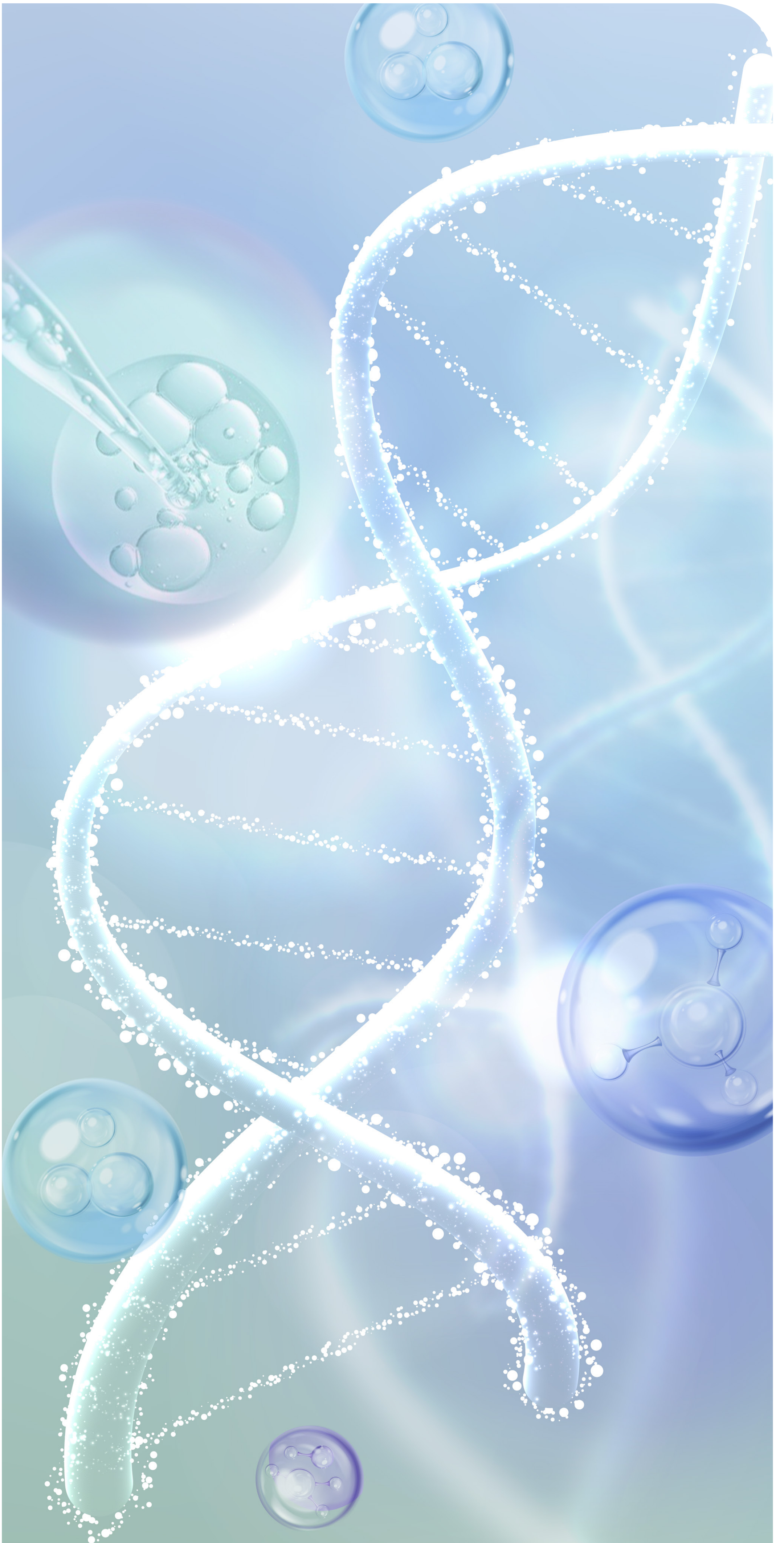


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Association Between Polypharmacy and Frailty in Elderly Patients Receiving Care at a Public Hospital in South India

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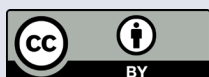
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ABSTRACT

OBJECTIVE This study analyses the impact of polypharmacy on frailty among the elderly patients visiting the public sector hospitals. The present study was done to assess the frailty status of the elderly patients and to see whether the individuals with polypharmacy exhibited more frailty when compared to those without polypharmacy.

METHODS This is a case-control study that included 488 participants age 60 years and above who visited the general medicine department of Public Hospital Ooty, India. Polypharmacy was defined as the concomitant use of 5 or more medications. Frailty status of the participants was assessed using the Tilburg Frailty Indicator.

RESULTS Polypharmacy was found to be present in 34.42% of cases. The prevalence of frailty was significantly higher in the polypharmacy group, affecting 41.07% of the study participants. Finally, three variables emerged as influential predictors of polypharmacy namely, 3 or more diseases (AOR 6.303, CI 4.009-9.911, $p < 0.0001$), unsatisfied sleep status (AOR 1.669, CI 1.068-2.606, $p = 0.01$), and people taking frailty causing drugs (AOR 4.328, CI 2.628-7.128, $p = 0.003$). Additionally, there was a moderate relationship between modified lifestyle (smoking, alcohol consumption) and polypharmacy.

CONCLUSIONS The results suggest that polypharmacy is linked with frailty among the elderly population, highlighting the importance of clinician awareness of these factors.

KEYWORDS elderly, frailty, polypharmacy, Tilburg Frailty Indicator, prevalence

INTRODUCTION

Across the world the elderly population is growing rapidly, both in absolute size and as a proportion of the total population. The term “geriatric” describes a population with a chronological age of at least 65 years in developed nations and at least 60 years in developing na-

tions (1). According to the population projection report of India, the population of people aged 60 and above is expected to expand from 101.5 million in 2011 to 227.4 million by 2036 (2).

Previous studies indicate that elderly individuals are at increased risk of taking multiple medications, primarily due to their higher prevalence

of chronic diseases. This is because in geriatrics the body physiology changes with time, further increasing the risk of both adverse drugs reactions and adverse outcomes (3). Polypharmacy—commonly defined as the use of five or more medications, including prescription, over-the-counter, and complementary products—is associated with adverse outcomes such as drug interactions, non-adherence to instructions for using the medications, reduced functional capacity, and frailty (4). A recent South Indian study reported the prevalence of hyper-polypharmacy (≥ 10 drugs), major polypharmacy (≥ 5 drugs), and minor polypharmacy (2–4 drugs) as 3.46%, 81.31%, and 15.22%, respectively, with higher rates among males (1).

Frailty, being a multifaceted disease, is highly correlated with aging and is marked by a depletion of reserves and an elevated risk of adverse consequences following the stressful events (5). A longitudinal cohort study in Italy found that the risk of frailty was 55.00% higher in participants taking 4–6 medications, and 147.00% higher in those taking ≥ 7 medications (6). Substantial evidence shows that frailty is strongly linked to negative health outcomes, including hospitalization, falls, disability, mortality, and higher health-care costs (4). Previous research suggests that frailty may arise from alterations across multiple interconnected physiological systems (7). Unsurprisingly, since the prevention of frailty among the elderly is a global priority, it is of the utmost importance to find the potential risk factor behind this. Several epidemiological studies have reported that frail or pre-frail elderly adults prescribed more than five medications are more likely to have emergency department visits (8–10). This study aims to evaluate the frailty status of elderly patients and to assess whether people with polypharmacy exhibit more frailty when compared to those without polypharmacy.

The link between polypharmacy and frailty is complex, and understanding it from both the clinical and the public health prospective has become the key challenge (11). Since polypharmacy is recognized as an important predictor of adverse outcomes in the elderly, our team decided to investigate this issue further. Moreover, there is very little research exploring the link between polypharmacy and frailty, particularly in India and within public health settings. Hence, it was

decided to determine whether polypharmacy is associated with frailty or not among the elderly patients visiting the public health sector.

METHODS

Study design

A prospective, case control study was conducted among an elderly population aged ≥ 60 years. This single-center study was performed in a public hospital in Ooty, India. Participants were recruited using consecutive sampling from the general medicine outpatient and inpatient departments.

Study population

Sample size was calculated using the EPITOOLS software. Based on a previous meta-analysis of Indian studies (2002–2020), the expected prevalence of polypharmacy among controls was 49.00% (12). The sample size for each group was determined to be 133, and the overall sample size for a case control study was found to be 266 at a power of 0.8 with the confidence interval of 95% (95%CI).

Elderly patients of either sex, aged ≥ 60 years and with polypharmacy were considered as case groups and patients of either sex, aged ≥ 60 years without polypharmacy were considered as the control group. Elderly patients with physical disability who had been treated at wards other than general medicine were excluded from the study. Prior to the data collection, written informed consent was obtained from all eligible participants. This study was conducted over a period of 6 months, from December 2023 to May 2024, in South India. During the course of study, data was collected from 488 patients, exceeding the initially determined sample size of 266.

Ethical consideration

The protocol approval for this study was issued by the Institutional Ethics Committee of Government Medical College and Hospital, The Nilgiris (Ref no IRBGMCO033). Confidentiality of the participant data was strictly maintained.

Study measures

The Tilburg frailty indicator questionnaire

The Tilburg Frailty Indicator (TFI) is a self-reporting questionnaire consists of 15 set of

questions covering physical, psychological and social components which was used for assessing the multidimensional frailty among the study participants. A TFI score of ≥ 5 is considered to be Frail as per previous research studies (13).

Modified Kuppuswamy Socioeconomic Scale 2023

Scoring of the 2023 edition of the Modified Kuppuswamy socioeconomic scale is primarily based on education, occupation and monthly income. The Modified Kuppuswamy Scale categorizes socioeconomic status into five distinct groups, ranging from lower to upper class, based on a scoring system of between 3 and 29. The score is then calculated and analysed to determine the socioeconomic class of each subject (14).

Data collection

The research work was explained and the consent form was given to the elderly patients who met the study criteria. Once voluntary consent was obtained, participants received a brief explanation about the items to be filled in the data collection form. The patient's sociodemographic, socioeconomic status, medical history, medication history, including a detailed list of their current drugs including over-the-counter medications, prescription drugs, and dietary supplements, lifestyle factors and comorbidities were collected from the medical records and from interviews with the participants. Data was analysed using appropriate statistical tests. The elderly participants were then subjected to a 'frailty assessment' with the help of the TFI. All test observations were documented in the data collection form. The information related to frailty was suitably noted in the data collection form for further segregating of the elderly into frail and non-frail categories.

Statistical analysis

The statistical analysis was performed using SPSS Version 16.0. Polypharmacy was considered the dependent variable, and demographics and socioeconomic status were considered as the independent variables. For the comparison of variables, Mann-Whitney U test and Pearson χ^2 test were used for continuous and categorical variables, respectively. Characteristics of the polypharmacy group, being a continuous variable, are

presented as mean $\pm$ standard deviation. The impact of demographic variables on polypharmacy was evaluated using bivariate analysis, and adjusted associations were subsequently identified through regression analysis, with polypharmacy as the dependent variable. Additionally, Pearson's correlation was used to examine the relationship between the number of medications and TFI scores. Statistical significance was defined as a "p" value of less than 0.05.

RESULTS

Table 1 shows the demographic and clinical characteristics of the 488 participants. Out of those participants, 51.43% were male and 48.56% were female. Among the study participants who had polypharmacy, 41.07% were frail and 58.92% were non-frail.

Overall, the prevalence of polypharmacy was higher among males ($p = 0.06$), former alcoholics ($p = 0.02$), participants aged ≥ 75 years ($p = 0.08$), past smokers ($p = 0.004$), those with poor sleep satisfaction ($p = 0.01$), individuals with ≥ 4 comorbidities ($p = 0.0001$), and those with body mass index (BMI) < 18.5 ($p = 0.09$) (Table 2).

When comparing frailty scores (TFI), participants with polypharmacy had a higher mean score (5.64 ± 3.45) than those without polypharmacy (4.48 ± 2.51), a statistically significant difference. This indicates that participants with polypharmacy were more likely to be frail than those without.

DISCUSSION

Geriatric syndrome is a multifactorial health condition resulting from the accumulation of impairments that affect multiple body systems and their functions. Frailty is defined as a geriatric syndrome that leads to a decrease in physical reserve and increased vulnerability to stressors. Aging is significantly associated with comorbid chronic conditions, which place the older population at increased risk of polypharmacy (3). Frailty affects social well-being and leads to increased utilisation of health care and greater health care costs (15).

Polypharmacy is generally defined as the concurrent use of five or more medications, whether clinically necessary or not (16). Studies worldwide have shown that elderly individuals typically use an average of 2 to 9 medications per day (17). In

Table 1. Demographic distribution (N = 488)

Characteristics	n (%)
Age	
60-64	201 (41.14)
65-69	114 (23.36)
70-74	90 (18.44)
≥ 75	83 (17.00)
Body mass index	
< 18.5	33 (6.76)
18.5-24.9	219 (44.87)
25-29.9	175 (35.86)
≥ 30	61 (12.50)
Living condition	
Own home	479 (98.15)
Nursing home	8 (1.63)
Senior care home	1 (0.20)
Marriage status	
Widow/divorced	203 (41.59)
Married	280 (57.37)
Unmarried	5 (1.02)
Physical activity	
Yes	427 (87.50)
No	61 (12.50)
Alcoholic	
Yes	36 (7.37)
No	356 (72.95)
Former alcoholic	96 (19.67)
Smoking	
Yes	42 (8.60)
No	353 (72.33)
Former smoker	93 (19.05)
Sleep (satisfaction)	
Yes	197 (40.36)
No	291 (59.63)
SES	
Lower (< 5)	214 (43.85)
Upper lower (5-10)	240 (49.18)
Lower middle (11-15)	23 (4.71)
Upper middle (16-25)	9 (1.84)
Upper (26-29)	2 (0.40)
Disease	
1	149 (30.53)
2	196 (40.16)
3	101 (20.69)
≥ 4	42 (8.60)
Gender	
Male	251 (51.43)
Female	237 (48.56)

SES, Socioeconomic status (Modified Kuppaswamy Scale 2023)

this population, polypharmacy is influenced by factors such as age, gender, education level, and the type and number of comorbidities. Several earlier studies have reported that the prevalence

of polypharmacy among older adults ranged from 23.10% to 59.10% (18-20). Polypharmacy can lead to negative health outcomes such as increased morbidity, higher economic burden, more hospitalizations, decreased quality of life, and higher rates of readmission. As the number of prescribed medications increases, the potential for drug-drug interactions also rises, further exacerbating adverse outcomes. Age-related changes in renal clearance and metabolism contribute to this problem by increasing drug exposure and susceptibility to drug side effects (21). For that reason, polypharmacy is recognized as a significant and growing concern in clinical practice.

The study team chose to focus on the impact of polypharmacy on frailty because of the growing elderly population, the high prevalence of polypharmacy in this group, and its complex relationship with frailty. In our study, frailty was assessed using the self-reported TFI questionnaire.

We chose to use the TFI scale over other assessment instruments because it conceptualizes frailty as a multidimensional construct, encompassing physical, psychological, and social domains of functioning in the elderly. The TFI scale is widely regarded as a tool with strong validity, reliability, and internal consistency. Individuals with a score of ≥ 5 were considered frail in our study, according to standard scoring criteria. There are only a few studies from India reporting the use of the TFI.

A long-term cohort study in Italy examining the association between polypharmacy and frailty showed that, at the 11-year follow-up, the probability of frailty increased with each additional medication reported at baseline (6). Similarly, a study conducted in Australia found that each additional medication was associated with a 13.00% higher risk of frailty, an 8.00% higher risk of disability, and a 9.00% higher risk of mortality (22).

Most of the studies pertaining to polypharmacy have been reported from private hospitals and very few from public hospitals. Ours is one such study reporting this important aspect from a public hospital.

Several classes of drugs have been associated with worsening health outcomes, such as falls and frailty. We conducted a PubMed search of previously published studies reporting drugs linked to frailty and falls (23-25). Medications found to exacerbate or contribute to frailty included

Table 2. Bivariate analysis of risk factors associated with polypharmacy

Subject characteristic	OR	(95%CI)	p-value
Sex			
Male	1.41	(0.97-2.06)	0.060
Female	1	(Reference)	
Age			
60-64	1	(Reference)	0.405
65-69	0.81	(0.49-1.32)	
70-74	0.91	(0.54-1.54)	
> 75	0.66	(0.41-1.05)	
Alcohol intake			
Former alcoholic	1.60	(1.06-2.42)	0.020
Not Alcoholic	1	(Reference)	
Smoking habit			
Smoker	1.81	(1.206-2.72)	0.004
Non-smoker	1	(Reference)	
Sleep (satisfaction)			
Satisfied	1	(Reference)	0.010
Not Satisfied	1.63	(1.10-2.42)	
Frailty			
Frail	1.7012	(1.15-2.51)	0.008
Non-Frail	1	(Reference)	
NO. of diseases			
1-2	1	(Reference)	< 0.0001
≥ 3	5.81	(3.80-8.87)	
Drugs and frailty			
Drug induced frailty	4.61	(1.68-12.6)	0.003
Non-drug induced frailty	1	(Reference)	
Body mass index			
< 18.5	1.89	(0.89-3.97)	0.090
18.50-24.99	1	(Reference)	
25.00-29.99	1.21	(0.79-1.85)	
> 30.00	1.57	(0.87-2.82)	

benzodiazepines, antipsychotics, anticholinergics, antiepileptic drugs, NSAIDs, and antihistamines. We further verified whether patients in both groups had been prescribed these drugs. In our study, patients in the frail group were more commonly prescribed with drugs such as ibuprofen, fluoxetine, diclofenac, chlorpheniramine maleate, and diphenhydramine, which have been linked to frailty. Benzodiazepines, such as diazepam and clonazepam, commonly used as sedatives, can lead to falls and contribute to the development or worsening of frailty in older adults. The study results demonstrated that patients in the polypharmacy group were more likely to be prescribed these drugs compared to those without polypharmacy. This relationship was also found to be statistically significant.

According to several studies, polypharmacy is a significant risk among the elderly and is commonly associated with factors such as age over

65 years, long-term illnesses, chronic diseases, frequent healthcare visits, and hospitalizations. In our study, we observed several factors to be associated with polypharmacy, including alcohol use, smoking, poor sleep satisfaction, having more than two diseases, and frailty.

With respect to alcoholism, alcohol can damage the liver, impairing its ability to metabolize drugs and lead to increased drug concentrations which may cause liver toxicity and other adverse effects. Additionally, alcohol can exacerbate the side effects of some medications, often necessitating the prescription of additional drugs, thus further compromising overall health in the elderly (26).

This case-control study demonstrated a higher prevalence of polypharmacy among current and former smokers, with an odds ratio of 1.81 (95%CI: 1.206-2.72). Similar results were reported in a 4.7-year follow-up study conducted in Australia, which also showed similar statistically

significant findings (27). Smoking induces elevation of certain liver enzymes, thereby metabolizing drugs more rapidly than in non-smokers. This reduction in drug concentration and efficacy can lead to subtherapeutic dosing, in which case higher doses of medication may be required to achieve the desired therapeutic effects while potentially increasing the risk of adverse effects or toxicity (28).

Sleep disruption becomes more common with age. Older adults often experience altered sleep architecture, an increased risk of sleep disorders, shifted circadian rhythms, and various mental and physical comorbidities related to sleep quality. Quality of life is significantly affected by age-related sleep problems, particularly among those exposed to polypharmacy (29). In comparing individuals with and without polypharmacy, there was a significant difference in the proportion of participants with polypharmacy reporting poor sleep satisfaction. Overall, poor sleep satisfaction was more frequent among participants with polypharmacy.

Our study reported that the prevalence of polypharmacy was higher among participants with ≥ 2 diseases compared to those with fewer than 2 diseases. Managing multiple chronic conditions often necessitates the use of multiple medications to control symptoms, slow disease progression, and reduce complications. Additionally, the use of medications from different therapeutic classes increases the risk of drug-drug interactions, potentially further worsening the well-being of the elderly (30, 31). The longitudinal Midlife in the United States (MIDUS) study found comparable statistically significant associations between polypharmacy and the prevalence of chronic diseases (16).

The polypharmacy group in this study had a considerably higher prevalence of frailty (41.07%) compared with the non-polypharmacy group (29.06%). Polypharmacy may contribute to the onset of frailty, regardless of the presence of multiple chronic conditions, likely due to the adverse effects of the medications. It is postulated that age-related changes in pharmacodynamics and pharmacokinetics increase the likelihood and severity of adverse drug reactions (ADRs) in older individuals, resulting in more frequent drug-drug and drug-disease interactions (27).

When examining the relationship between frailty and the number of prescribed drugs using Pearson's correlation, a weak positive correlation was observed ($r = 0.233$), which suggests that the risk of frailty increases with the number of drugs prescribed.

Five variables (alcohol intake, smoking status, sleep satisfaction, having three or more diseases, and the use of frailty-associated drugs) were included in the binary logistic regression model after being identified as significant in the bivariate analysis. Finally, three variables emerged as significant predictors of polypharmacy: having three or more diseases (absolute odds ratio (AOR) 6.303, CI 4.009–9.911, $p < 0.0001$), poor sleep satisfaction (AOR 1.669, CI 1.068–2.606, $p = 0.01$), and use of frailty-associated drugs (AOR 4.328, CI 2.628–7.128, $p = 0.003$).

However, such a study has not been conducted or reported in India. Our study is among the limited number of studies that examine the link between polypharmacy and frailty. Its generalizability is limited, as it was conducted in a single hospital in a specific region of South India.

The association between polypharmacy and frailty could be bidirectional, as demonstrated in previous systematic reviews. These studies indicate that frail individuals often develop multiple chronic conditions requiring complex treatment regimens, with frailty being linked to higher medication use as a result of functional decline, multimorbidity, and increased healthcare interactions (11). This highlights the importance of longitudinal studies to determine causality and support interventions such as regular medication reviews in frail populations.

CONCLUSIONS

The overall prevalence of polypharmacy in our study was lower than that reported in other studies. Several predictors were identified, consistent with findings from international research. A weak positive correlation ($r = 0.233$) between the polypharmacy and frailty scores suggests that frailty risk increases proportionally with medication burden. These results highlight the critical need for regular medication reviews, cautious prescribing practices that consider both the number and type of medications, and the implementation of targeted interventions for elderly patients. In

conclusion, our findings demonstrate that polypharmacy is associated with increased frailty in older adults. Future multicenter studies across India are needed to validate these results and inform standardized guidelines for medication management in elderly populations.

By observing the results of the present study, we are in the opinion that, in a large country like India with limited electronic health records (EHR) integration within the public health sector, hospitals have a challenges of improved coordination and sharing of medical records between institutions which could help minimize the occurrence and adverse consequences of poly pharmacy.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

S.T.: methodology, validation, investigation, formal analysis, writing-original draft, data curation; M.V.V.S.: methodology, validation, investigation, formal analysis, writing-original draft, data curation; G.A.: methodology, validation, investigation, formal analysis, writing-original draft, data curation; N.N.: methodology, validation, investigation, formal analysis, writing-original draft, data curation; S.B.: resources, supervision, project administration, visualization, writing-review & editing; S.A.: supervision, project administration, visualization, writing-review & editing; S.P.: supervision, project administration, writing-review & editing, visualization; H.N.V.: conceptu-

alization, methodology, data curation, software, project administration, writing-review & editing.

All authors have read and agreed to the published version of the manuscript

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The protocol approval for this study was issued was responsible Institutional Ethics Committee of Government Medical College and Hospital, The Nilgiris (Ref no IRBGMCO033).

INFORMED CONSENT STATEMENT

Written informed consent was obtained from all participants. Confidentiality of the participant data was strictly maintained.

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Efficacy of Lidocaine/Prilocaine Cream During Dry Needling of Upper Trapezius Myofascial Trigger Points: A Double-blinded Randomized Controlled Trial

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ABSTRACT

OBJECTIVE This study aimed to evaluate the efficacy of a eutectic mixture of local anesthetics (EMLA) cream in reducing pain during dry needling (DN) of active myofascial trigger points (MTrP) in the upper trapezius muscle.

METHODS This prospective, randomized, double-blind, placebo-controlled trial was conducted with participants aged 20 years and older who had active MTrP in the upper trapezius muscle. Recruitment occurred from 11 July 2023 to 4 March 2024. Participants were randomly assigned to receive either EMLA cream (n = 32) or a placebo cream (n = 32) which was applied over the active MTrP 60 minutes prior to the DN procedure. After the DN procedure, patients rated their pain using a 100-mm visual analog scale during DN (VAS_{TrP}) as the primary outcome and during the needle's skin penetration (VAS_{skin}) as the secondary outcome.

RESULTS Patients in the EMLA group reported significantly lower scores for VAS_{TrP} and VAS_{skin} than those in the placebo group ($p < 0.05$). Specifically, 53.1% of patients in the EMLA group reported 'moderate to severe pain' according to VAS_{TrP}, significantly lower than the 78.10% in the placebo group ($p = 0.035$). Moreover, 65.63% of patients in the EMLA group reported 'no pain' on the VAS_{skin} scale, compared to just 15.63% in the placebo group ($p < 0.001$). Importantly, no significant adverse events associated with EMLA were observed.

CONCLUSION EMLA cream is effective in providing analgesia during DN of an active MTrP in the upper trapezius muscle.

KEYWORDS EMLA, local anesthetic, upper trapezius, dry needling, myofascial trigger point

INTRODUCTION

Myofascial pain syndrome (MPS) is a disorder characterized by pain stemming from myofascial trigger points (MTrP). These trigger points are hyperirritable nodules found within taut muscle bands. When these points are palpated, they can cause a local twitch response (LTR), a quick con-

traction of muscle fibers accompanied by pain radiating from the area (1). MTrP can lead to patterns of pain that are referred to other areas, as well as autonomic symptoms, motor dysfunction, and a reduced range of motion. MTrPs are categorized into two types: active MTrPs, which cause spontaneous pain complaints, and latent MTrPs,

which are usually inactive but can become painful when stimulated (2). Previous studies indicate that between 30.00% and 85.00% of patients with musculoskeletal pain are affected by MPS (3). According to epidemiological studies conducted in Thailand, MPS was diagnosed in 36.00% of patients with musculoskeletal pain (4), and the trapezius is the most commonly found MTrP (5).

One of the well-known and effective treatments for MTrP is dry needling (DN), which consists of inserting a thin, solid, dry needle into the muscle where the MTrPs are located and moving it in and out using a circular fan-like and fast-in and fast-out technique (2, 6-8). Common adverse effects of DN include bruising, bleeding, pain during treatment, and post-needling soreness (PNS). During the intervention, most patients do not feel the insertion of the needle but sometimes have a painful sensation from the LTR that lasts less than a second. Some patients describe this as a little electric shock, while others consider it a cramping sensation. However, LTRs generated by DN have been linked to pain relief, enhanced range of motion, and decreased levels of nociceptive and inflammatory mediators at MTrP (9). Although the pain during DN is mild, it has been linked to patient dissatisfaction, decreased adherence to treatment, and an increase in PNS (9-11). While various strategies and therapies have been studied to reduce PNS, to the best of our knowledge, no research has focused on methods to alleviate pain during DN (10, 11).

A eutectic mixture of local anesthetics (EMLA) cream, which combines 2.50% lidocaine and 2.50% prilocaine, provides safe and effective local analgesia for several invasive procedures, including venipuncture, vaccination, totally implantable venous access devices (TIVADs), fine-needle aspiration biopsy, and spinal needle insertion (12-17). Despite the limited penetration depth, many studies have found that it effectively reduces the pain of both subcutaneous and intramuscular injections (14, 18-21).

Because of uncertainty about whether EMLA can effectively relieve pain associated with the DN procedure, this study aimed to evaluate the effectiveness of EMLA cream compared to a placebo in alleviating pain during DN of an active MTrP in the upper trapezius muscle. This study hypothesized that EMLA cream is effective in decreasing pain during the DN procedure.

METHOD

Study design and participants

This study was a prospective, randomized, double-blind, placebo-controlled analysis conducted at the Department of Rehabilitation Medicine at Phramongkutklao Hospital in Bangkok, Thailand. Patients with MPS affecting the upper trapezius muscle were recruited between 11 July 2023 and 4 March 2024.

The inclusion criteria for the study were as follows: patients must be 20 years or older, consent to participate, and present with at least one active MTrP in the upper trapezius muscle. The diagnosis of an active MTrP was based on the following criteria: 1) the presence of a palpable taut band in the muscle; 2) the existence of a hyper-sensitive tender spot within this taut band; 3) the presence of spontaneous pain; and 4) increased pain in response to intense digital pressure which mimicked the patient's original pain experience (2, 22, 23).

Exclusion criteria were any of the following: 1) presence of a contraindication for DN, such as needle phobia, coagulopathy, thrombocytopenia, or psychiatric disorder; 2) previous use of analgesic or non-steroidal anti-inflammatory drugs (NSAIDs) within 24 hours; 3) inability to comprehend questions; 4) presence of a contraindication for EMLA cream such as hypersensitivity to local anesthetics of the amide type or any ingredients in EMLA cream, or congenital or idiopathic methemoglobinemia (24); 5) pregnant or breast-feeding women; 6) the presence of denuded or broken skin over the MTrPs site.

Randomization

Patients were randomly assigned to one of two groups: the EMLA group or the placebo group. A block of four randomization processes ensured that both groups had a balanced number of participants. The randomization sequence was created by an independent physiatrist, and allocation concealment was achieved using opaque, sealed envelopes.

Ethical considerations

The study protocol received approval from the Institutional Review Board, Royal Thai Army Medical Department (R062h/66), and was registered with the Thai Clinical Trials Registry

(TCTR20240323002). All subjects provided written informed consent for their participation in the study.

Sample size calculation

Based on data from a previous study on the effectiveness of EMLA cream in reducing pain associated with vaccination (14), the Visual Analog Scale (VAS) was chosen as the primary outcome measure. Considering a power of 0.8 and an alpha level of 0.05, the target population per group was calculated to be 32.

Study procedures

The placebo cream in the study was Siriraj Daily Cream, a white lotion similar in appearance and consistency to EMLA cream. A total of 1 mL of either EMLA cream or placebo cream was measured using a 1 mL disposable syringe. This amount was then uniformly applied in a thick layer over the active MTrP of each subject 60 minutes prior to the DN procedure.

To begin, the first researcher marked the DN site over the most painful active MTrP in the upper trapezius muscle using a circular template 3.5 cm in diameter. The cream was applied within this marked circle and secured with a Tegaderm patch. Importantly, the physiatrist performing the DN was not involved in applying the creams, ensuring that neither the patient nor the physiatrist knew which cream had been used.

The Tegaderm patch was removed immediately before the needle insertion, and the first researcher wiped off the remaining cream. The physiatrist was then called on to perform the DN. Before the needle insertion, the patient was positioned face down and informed about the possibility of sharp pain and muscle twitching.

After disinfecting the area, the DN procedure was performed using a single 0.25 × 40 mm solid filament needle (EACU™, Maanshan Bond Medical Instruments Co., Ltd., Maanshan City, China) using a circular fan-like and fast-in-and-fast-out technique (2, 8) for 10 repetitions. When the needle was withdrawn, a cotton ball was pressed on the treatment area for approximately 90 seconds. The number of LTRs and side effects from the cream or DN were assessed and recorded. In this study, seven physiatrists with at least five years' experience in DN who were staff members in the

Department of Rehabilitation Medicine at Phramongkutklao Hospital, performed the procedures.

Outcome measures

The primary outcome was pain intensity during DN. The most painful intensity during DN occurred during needling manipulation using a fanning technique. The secondary outcome was pain intensity while the needle penetrated the skin. Because of its high reliability for assessing acute pain intensity (25), both outcomes were assessed using a 100 mm vis VAS with a range of 0 mm (no pain) to 100 mm (worst imaginable pain). Before performing the DN procedure, the patient was instructed to complete the pain survey form, in which the patient was asked to rate his or her pain intensity during DN (VAS_{Trp}) and while the needle penetrated the skin (VAS_{skin}) using a VAS. Immediately after completing the DN procedures, patients were asked to mark the horizontal 100 mm VAS line representing their pain intensity.

Baseline patient characteristics were collected, including age, sex, MPS pain intensity in the upper trapezius muscle using a 100 mm VAS, and history of DN, in addition to the number of needle insertions, number of LTRs, and any adverse events from the DN or the creams.

Statistical analysis

Analysis was performed on an intention-to-treat basis. Descriptive statistics were used to analyze demographic and clinical characteristics. Continuous data are presented as mean and standard deviation or median and interquartile range (IQR), and categorical data are presented as numbers and percentages. Differences in age, pain intensity of MPS, number of LTRs, VAS_{Trp}, and VAS_{skin} were compared between groups using the Mann-Whitney U test. The chi-square test or Fisher's exact test was used to compare categorical data, including gender and history of DN. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using STATA version 14.

RESULTS

Eighty-one patients were evaluated for eligibility. Of these, 17 were excluded because they met at least one of the exclusion criteria: fear of needles ($n = 4$), previous use of NSAIDs within 24 hours ($n = 3$), and denial of participation due

to the time-consuming process ($n = 10$). A total of 64 patients were randomized into either the EMLA group ($n = 32$) or the placebo group ($n = 32$) (Figure 1). As shown in Table 1, there were no statistically significant differences in the patient's sex, age, history of DN, or pain intensity of MPS (VAS_{MPS}) between the two groups. However, the number of LTRs was significantly lower in the placebo group ($p = 0.021$).

Both pain intensity during DN (VAS_{TrP}) and while the needle penetrated the skin (VAS_{skin}) were significantly lower in the EMLA group than

in the placebo group ($p < 0.05$) (Figure 2). Category scales for pain intensity are presented in Table 2. There was a statistically significant difference in VAS_{TrP} categorized by severity between groups ($p = 0.016$), with almost 80% of patients in the placebo group reporting 'moderate to severe pain'. In contrast, only half of the EMLA group rated this pain as moderate to severe. Correspondingly, for VAS_{skin} , the percentage of patients reporting 'no pain' in the EMLA group (65.63%) was significantly higher than that in the placebo group (15.63%) ($p < 0.001$). Additionally, none of the participants

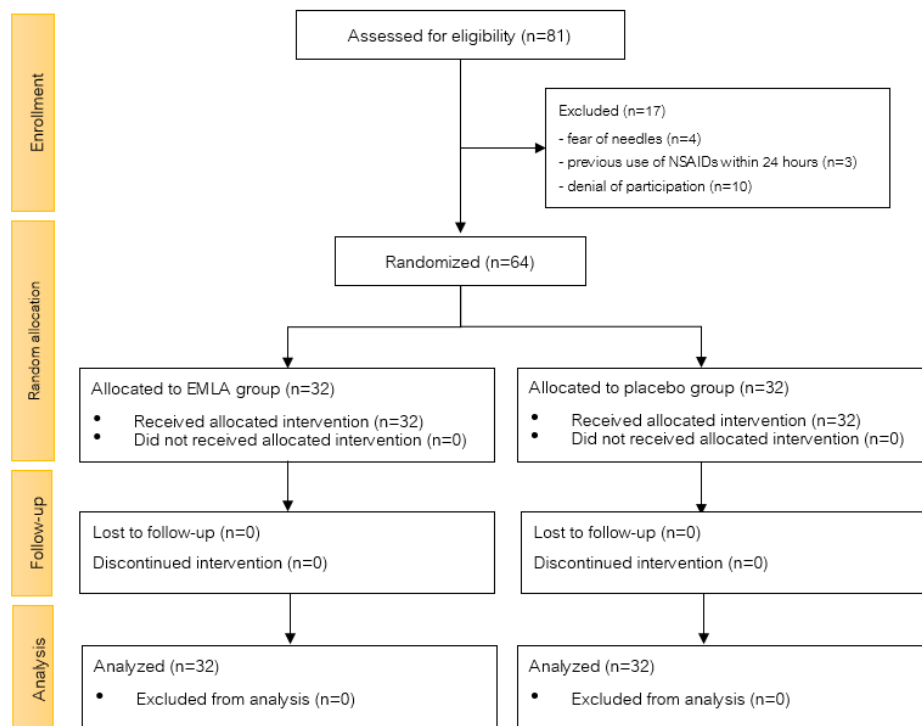


Figure 1. CONSORT flow diagram of the present study

Table 1. Demographic characteristics of participants

	EMLA group (n = 32) n (%) Median (IQR)	Placebo group (n = 32) n (%) Median (IQR)	p-value
Gender			
Female	21 (65.63)	19 (59.38)	0.606
Age (years)	29 (23.0–40.0)	28 (22.0–50.5)	0.598
History of DN			
(Yes)	18 (56.25)	24 (75.00)	0.114
VAS_{MPS} (mm)	50 (40–60)	55 (40–65)	0.353
Number of LTRs	5 (4–8.5)	4 (3–6)	0.021*

EMLA, eutectic mixture of local anesthetics; DN, dry needling; VAS_{MPS} , Visual Analog Scale for myofascial pain syndrome; LTRs, local twitch responses; n, number of participants; IQR, inter-quartile range

Mann-Whitney U test for continuous outcomes and Chi-square test or Fisher's exact test for categorical data were performed.

*Statistically significant ($p < 0.05$)

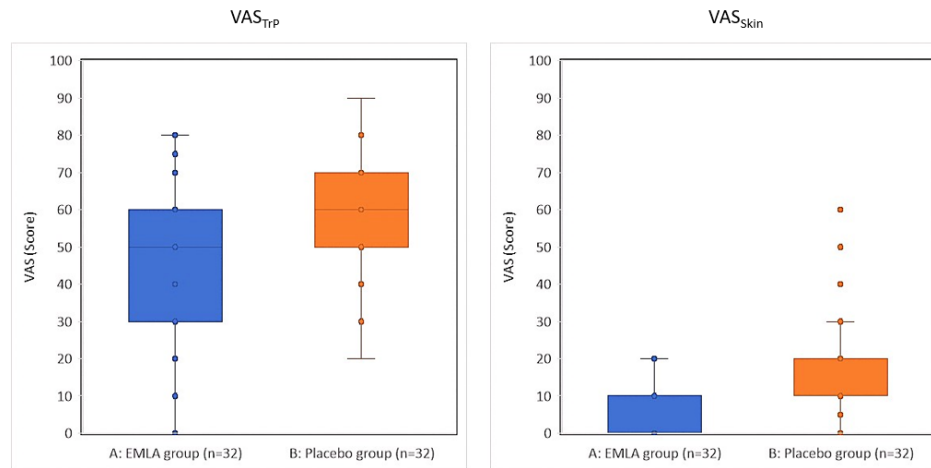


Figure 2. Box plots comparing pain intensity during DN (VAS_{TrP}) and pain while the needle penetrated the skin (VAS_{skin}) between the EMLA and placebo groups

Table 2. Pain intensity during DN (VAS_{TrP}) and pain while the needle penetrated the skin (VAS_{skin}) between the EMLA and placebo groups

	EMLA group (n=32)	Placebo group (n=32)	p-value
VAS_{TrP}			
Moderate to severe pain (45–100 mm), n (%)	17 (53.1)	25 (78.1)	0.035*
Median (IQR)	50 (30–60)	60 (50–70)	0.016*
VAS_{skin}			
No pain (0–4 mm), n (%)	21 (65.63)	5 (15.63)	< 0.001*
Median (IQR)	0 (0–10)	10 (10–20)	< 0.001*

EMLA, eutectic mixture of local anesthetics; VAS_{TrP} , Visual Analog Scale for pain during dry needling; VAS_{skin} , Visual Analog Scale for pain while the needle penetrated the skin; n, number of participants; IQR: inter-quartile range; Mann-Whitney U test for continuous outcomes and Chi-square test or Fisher's exact test for categorical data were performed; *Statistically significance ($p < 0.05$)

in the EMLA group reported 'moderate to severe pain' while the needle penetrated the skin.

Observed adverse effects from EMLA cream included pallor ($n = 3$), a tingling sensation ($n = 2$), and erythema ($n = 2$) at the site of cream application, all of which resolved spontaneously within 5 minutes. No adverse events from DN were observed.

DISCUSSION

The present study demonstrates that using EMLA cream effectively reduces pain during DN and while the needle has penetrated the skin. The current study observed a significant reduction in VAS_{TrP} and VAS_{skin} in the EMLA group compared with the placebo group. The efficacy of EMLA cream was shown by the statistically significant difference in frequency distribution in pain severity between groups. For VAS_{TrP} , the percentage of patients with 'moderate to severe pain' in the EMLA

group was markedly significantly lower than that in the placebo group. Similarly, the majority of patients in the EMLA group reported 'no pain' and none reported 'moderate-to-severe pain' for VAS_{skin} .

The pain-relieving effect of EMLA was more pronounced when the needle penetrated the skin than when it penetrated the MTrP, possibly due to the limited depth of penetration of EMLA cream (26). Despite its limited penetration depth, some studies have suggested EMLA effectively reduces intramuscular injections' underlying muscle spasms and pain (14, 18–20). Possible mechanisms of reduction of pain associated with intramuscular insertion include: 1) EMLA may reach deeper tissues than previously reported and may block nociceptors for muscle stretch; 2) blockage of cutaneous nociceptors may in some way modify afferent signals from the stretching of underlying muscle; 3) because it was difficult to distinguish

pain associated with a skin prick from that associated with muscle insertion, subjects reported a reduction in overall pain perception as their score for muscle insertion (19, 20).

Bijur PE et al. found that a change of 10 mm or more in the VAS indicates an actual change in most patients' acute pain experience (25). In the present study, the results showed both statistically significant and clinically significant differences regarding the median difference of VAS_{TrP} and VAS_{skin} between the EMLA and placebo groups.

According to Martín-Sacristán, L. et al., there is a statistically significant association between the number of LTRs and the symptoms suffered by DN (27). In the current study, although the number of LTRs was significantly lower in the placebo group, pain during DN and while the needle penetrated the skin was higher in this group than in the EMLA group. These findings support the efficacy of EMLA cream in reducing pain from the DN procedure.

The primary barrier to the routine use of EMLA cream for reducing pain in invasive procedures is its delayed onset of action of approximately 60 minutes (12, 14-16, 18, 28). This disadvantage was also observed in our study, in which approximately 60.00% of the excluded subjects declined to participate due to the time-consuming process. To reduce this obstacle, the researcher suggests two less time-consuming alternatives: first, the development of a protocol for cream application upon arrival at OPD by nurses for patients with chronic MPS who have an appointment for DN, and second, providing patients with validated instructions on how to apply the cream prior to visiting the OPD.

In the present study, only mild adverse effects from EMLA cream were recorded in 22% of subjects, including pallor, tingling sensation, and erythema, at the site of cream application which resolved spontaneously within 5 minutes.

Several limitations of our study should be mentioned. First, pain is a subjective phenomenon highly dependent on the individual's perception of its severity. Although VAS was a valid outcome assessment tool with high intra- and inter-rater reliability to assess pain severity in a clinical population (7, 25, 29), other objective outcomes should be assessed as well, e.g., blood pressure and heart rate rising after DN. Second, DN

was applied to only one MTrP of the selected upper trapezius muscle; therefore, the results from this study cannot be applied to DN used on several MTrPs and/or different muscles. Third, DN techniques were applied with specific characteristics regarding the number of needle insertions and needle diameter, so the application form limited the generalizability of the results. Fourth, pain during DN was significantly correlated with PNS, which is also an important adverse effect of DN, causing limitation of activities of daily living and reducing therapeutic adherence (11). Further studies should investigate the efficacy of EMLA cream in reducing PNS. Lastly, DN is operator-dependent which may influence pain perception during the procedure. However, all seven physiatrists performing the DN were trained at the same institution. Additionally, the upper trapezius is a relatively accessible muscle for DN, and the number of needle manipulations was standardized.

CONCLUSIONS

EMLA cream effectively reduces pain during DN and while the needle is penetrating the skin. In addition, no significant side effects were observed except for local reactions, which resolved spontaneously within a few minutes. EMLA cream is a painless, effective, and noninvasive option for reducing pain from DN procedures.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

P.F.: contributed to concept and design the study, acquisition and analysis of the data, draft the manuscript; T.P.: contributed to concept and design the study, analysis and interpretation of the data and critically revise the manuscript; C.P.: contributed to concept and design the study, analysis and interpretation of the data and critically revise the manuscript.

All authors approved the final manuscript as submitted.

DATA AVAILABILITY STATEMENT

The data supporting this study's findings are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board, Royal Thai Army Medical Department (Research code: R062h/66, approved on 7 June 2023).

INFORMED CONSENT STATEMENT

Written informed consent was obtained from all participants involved in the study.

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Biochemical and Genetic Determinants of Early Cardiovascular and Hepatic Dysfunction: A Focus on *ADIPOQ* Expression

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ABSTRACT

OBJECTIVE Non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) frequently coexist due to common metabolic dysfunctions such as insulin resistance, chronic inflammation, and dyslipidemia. Adiponectin, encoded by the *ADIPOQ* gene, is an anti-inflammatory adipokine vital for glucose and lipid regulation. Reduced adiponectin levels and certain *ADIPOQ* gene variants (e.g., rs266729, rs2241766) have been linked to both NAFLD and CVD progression. This study aims to evaluate the association between *ADIPOQ* gene expression and early biochemical markers of hepatic and cardiovascular dysfunction.

METHODS A case-control study (n = 300) was conducted in Thiruvananthapuram, Kerala, India (2022-2024), analyzing blood samples for fasting blood sugar (FBS), lipid profile, malondialdehyde (MDA), interleukin-6 (IL-6), and *ADIPOQ* expression (RT-PCR). Statistical analyses included receiver operating characteristic (ROC) curves and quantile regression using Stata 17.0.

RESULTS Cases exhibited significantly elevated FBS, triglycerides (TG), low-density lipoprotein (LDL), MDA, and IL-6, along with reduced high-density lipoprotein (HDL) and adiponectin levels ($p < 0.05$). *ADIPOQ* gene expression was markedly downregulated, particularly in individuals with both NAFLD and CVD. A positive correlation was observed between *ADIPOQ* expression and HDL, and a negative correlation with IL-6. Reduced *ADIPOQ* expression showed an association with risk in unadjusted analysis, but this association was not statistically significant after adjustment (OR = 0.0220). Nevertheless, ROC analysis indicated potential diagnostic utility.

CONCLUSIONS Reduced *ADIPOQ* expression is significantly associated with early metabolic and inflammatory markers in NAFLD and CVD. These findings highlight the potential utility of *ADIPOQ* as a biomarker for early detection and targeted prevention strategies in at-risk populations.

KEYWORDS *ADIPOQ* gene expression, adiponectin, non-alcoholic fatty liver disease, cardiovascular disease, inflammation

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) are among the leading chronic health burdens globally; both are closely linked to impaired metabolic balance. They frequently coexist due to shared pathophysiological drivers such as insulin resistance, low-grade chronic inflammation, obesity, and dyslipidemia (1). Early detection of molecular disruptions underlying both hepatic and cardiovascular pathology is critical to preventing progression to irreversible stages, including myocardial infarction or cirrhosis.

Adiponectin is a key anti-inflammatory and insulin-sensitizing adipokine encoded by the *ADIPOQ* gene, playing a central role in linking NAFLD and CVD. It regulates crucial metabolic processes such as glucose and lipid metabolism and supports vascular endothelial function. Notably, low circulating adiponectin levels are frequently observed in individuals with NAFLD and CVD, sometimes even before overt symptoms appear, suggesting a role in the early onset of these diseases (2). The *ADIPOQ* gene, located on chromosome 3q27, is tightly regulated at both transcriptional and post-transcriptional levels. Genetic variants, particularly single nucleotide polymorphisms (SNPs) such as rs266729 and rs2241766, have been associated with altered adiponectin levels and an increased risk of metabolic dysfunction. These SNPs may influence gene expression or protein activity, thereby diminishing adiponectin's protective effects and facilitating the development of NAFLD and CVD (3).

Biochemically, reduced adiponectin levels are closely linked to a pro-atherogenic lipid profile, heightened systemic inflammation, and oxidative stress, all of which synergistically contribute to endothelial dysfunction, hepatic fat accumulation, and fibrosis. One of the key indicators of metabolic disruption, FBS provides a direct measure of glucose regulation. Elevated FBS often reflects insulin resistance, a common pathophysiological driver of both NAFLD and CVD. Persistent hyperglycemia promotes endothelial damage and hepatic steatosis by enhancing *de novo* lipogenesis and impairing lipid oxidation (4). Dyslipidemia, manifested as elevated triglycerides (TG), total cholesterol (TC), and low-density lipoprotein (LDL) levels, coupled with decreased high-density li-

poprotein (HDL), is a hallmark of both CVD and NAFLD. Notably, oxidized LDL (ox-LDL) serves as a potent inflammatory stimulus, contributing to atherogenesis and vascular dysfunction (5). Malondialdehyde (MDA), a well-established biomarker of lipid peroxidation and oxidative stress, is significantly elevated in patients with NAFLD and CVD. High MDA levels reflect an increased oxidative burden and indicate ongoing endothelial injury and hepatic dysfunction (6). IL-6, a key pro-inflammatory cytokine, acts at the intersection of immune dysregulation and metabolic stress. Elevated IL-6 levels are associated with exacerbated insulin resistance, hepatic inflammation, and adverse cardiovascular outcomes, underscoring its central role in the pathogenesis of both NAFLD and CVD (7).

The complex interplay between biochemical markers and genetic determinants is pivotal in the early development of cardiovascular and hepatic dysfunction. Gaining insights into these relationships is essential for improving risk assessment and advancing personalized treatment strategies. This study investigates the association between *ADIPOQ* gene expression and early biochemical markers of hepatic and cardiovascular dysfunction. By unravelling these molecular and metabolic connections, the study seeks to contribute to the development of targeted, precision-based interventions for the prevention and effective management of these closely linked metabolic disorders.

METHODS

Study design and ethical considerations

This case-control study was conducted over a period of two years, from December 2022 to December 2024, adhering strictly to the ethical principles outlined in the Declaration of Helsinki (1964) and its subsequent revisions. Ethical approval was obtained from the Institutional Ethics Committee of Genetika, Centre for Advanced Genetic Studies (Reference No: 09/2022/IECG). Written informed consent was obtained from all participants before recruitment, ensuring full voluntary participation and confidentiality.

Study setting and participant recruitment

Participants were recruited from multiple sources, including the Hridayalaya Heart and the Robotic Research Centre, Lords Hospital, World

Heart Day Medical Camp, and several affiliated blood collection centers across Thiruvananthapuram, Kerala, India. All laboratory investigations, including biochemical and molecular analyses, were conducted at the Genetika, Centre for Advanced Genetic Studies, ensuring methodological consistency.

Participant characteristics

A total of 300 individuals (age 18–45 years) were enrolled: 150 individuals with NAFLD and/or cardiovascular risk factors (cases), and 150 healthy controls, age- and sex-matched. Recruitment from the same geographic region minimized selection bias (Table 1).

Data collection and sample processing

Detailed demographic, lifestyle, and clinical data were collected using pre-validated structured questionnaires. Approximately 6–8 mL of fasting venous blood was drawn from each participant under aseptic conditions into both plain and EDTA-coated tubes for downstream biochemical and molecular assessments.

Biochemical analyses

Fasting blood glucose levels were estimated using the glucose oxidase-peroxidase (GOD-POD) enzymatic method. The serum lipid profile, including TG, TC, HDL, and LDL, was analyzed using standard enzymatic colorimetric assays with commercially available diagnostic kits. Lipid peroxidation was quantified by measuring serum MDA levels using a competitive enzyme-linked

immunosorbent assay (ELISA). Additionally, systemic inflammation was assessed by measuring serum IL-6 concentrations using a high-sensitivity ELISA kit according to the manufacturer's protocol.

Gene expression analysis

Total RNA was extracted from whole blood using a commercially available RNA extraction kit, and its quantity and purity were assessed using bio spectrophotometry. Complementary DNA (cDNA) was synthesized from 50 ng of RNA using reverse transcriptase. Real-time quantitative PCR (qPCR) was conducted using the CFX Opus 96 System (Bio-Rad, Hercules, CA, USA) and SYBR Green Master Mix (Lot No: 103024 RMM). The expression of the *ADIPOQ* gene was evaluated using gene-specific primers:

Forward: 5'-CAGGCCGTGATGGCAGAGATG-3'

Reverse: 5'-GGTTTCACCGATGTCTCCCTTAG-3'

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed as the internal house-keeping gene. The primer sequences were as follows:

Forward: 5'-CCATGGAGAAGGCTGGGG-3'

Reverse: 5'-CAAAGTTGTCATGGATGACC-3'

Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Sample size determination

The minimum sample size was estimated using the formula for comparing two independent means:

$$N = \frac{(Z\alpha/2 + Z\beta)^2 \times (S_1^2 + S_2^2)}{(\mu d)^2}$$

Table 1. Participant characteristics and eligibility criteria

Item	Case group (NAFLD and/or CV risk, n = 150)	Control group (healthy, n = 150)
Age range	18–45 years	18–45 years
Sex matching	Matched with controls	Matched with cases
Clinical condition	Diagnosed NAFLD and/or established CV risk factors	No known chronic/systemic illnesses
Inclusion criteria	Confirmed NAFLD Presence of CV risk conditions	Healthy individuals without chronic/systemic disease
Exclusion criteria	- Age < 18 or > 45 - Chronic systemic illnesses - Malignancies - Long-term pharmacological treatment - Prior exposure to ionizing radiation, chemotherapy, or mutagenic agents	Same as case group

NAFLD, non-alcoholic fatty liver disease; CV risk, cardiovascular risk factors

Where:

- S1 = Standard deviation in group 1 (NAFLD)
- S2 = Standard deviation in group 2 (controls)
- μd = Expected mean difference between the

two groups

- α = significance level (type I error)
- β = type II error (1 - Power)

Based on available prevalence estimates and to ensure adequate statistical power, the final sample size was fixed at 150 cases and 150 controls.

Statistical analysis

Descriptive statistics were used to summarize demographic, clinical, and laboratory data. Independent t-tests were applied to compare mean values between case and control groups for variables with normal distribution. Receiver operating characteristic (ROC) curve analysis was employed to assess diagnostic performance metrics, including the area under the curve (AUC), sensitivity, and specificity. Quantile regression was used to evaluate the associations between *ADIPOQ* gene expression ($2^{-\Delta\Delta CT}$) and clinical parameters, while accounting for potential heteroscedasticity in the data. For variables violating the assumption of normality, bootstrap methods were applied to derive robust *p*-values. All statistical analyses were performed using Stata version 17.0 (StataCorp LLC, College Station, TX, USA).

RESULTS

Participant characteristics

The study enrolled 300 young adults aged 18-45, including 150 individuals with clinically diagnosed CVD and/or NAFLD and 150 age- and sex-matched healthy controls. Comprehensive

demographic, anthropometric, physiological, clinical, and lifestyle data were collected, as well as biochemical and genetic analyses.

Biochemical profiles

Table 2 summarizes the descriptive statistics of biochemical parameters. FBS levels were significantly higher in cases (mean: 129.1 mg/dL) compared to controls (90.3 mg/dL), with a broader range suggesting higher prevalence of hyperglycemia. Serum TG were also elevated in cases (147.1 mg/dL vs. 114.1 mg/dL in controls), with greater variability indicating frequent dyslipidemia. TC levels were markedly higher in cases (232.8 mg/dL) than in controls (182.2 mg/dL), while HDL cholesterol levels were substantially lower in cases (38.2 mg/dL) compared to controls (54.3 mg/dL). LDL cholesterol followed a similar pattern, with elevated mean levels in cases (165.1 mg/dL) versus controls (105.1 mg/dL).

Inflammatory and oxidative stress markers

Cases exhibited significantly higher MDA levels (4.3 mg/L vs. 1.7 mg/L in controls), indicating increased oxidative stress. IL-6 levels were also elevated in cases (7.9 pg/mL) compared to controls (3.8 pg/mL), highlighting systemic inflammation. Conversely, adiponectin gene (*ADIPOQ*) expression was markedly reduced in cases (0.59 μ g/mL) relative to controls (1.01 μ g/mL), suggesting impaired protective metabolic regulation.

Comparative statistical analysis

Table 3 presents the results of bootstrap t-tests with Levene's test for variance. Significant differences were observed for fasting blood

Table 2. Descriptive statistics of the biochemical parameters for cases and controls

Variable name	Cases		Controls	
	Min - Max	Mean $\pm$ SD	Min - Max	Mean $\pm$ SD
Fasting blood sugar (mg/dL)	75.0-261.0	129.1 $\pm$ 30.5	70.7-134.0	90.3 $\pm$ 13.2
Serum triglycerides (mg/dL)	90.6-220.0	147.1 $\pm$ 30.5	80.6-156.1	114.1 $\pm$ 21.1
Serum total cholesterol (mg/dL)	148.0-358.0	232.8 $\pm$ 35.1	147.1-245.0	182.2 $\pm$ 20.5
Serum HDL cholesterol (mg/dL)	24.0-68.0	38.2 $\pm$ 7.1	30.2-79.0	54.3 $\pm$ 8.3
Serum LDL cholesterol (mg/dL)	57.6-286.4	165.1 $\pm$ 33.2	61.6-179.8	105.1 $\pm$ 24.1
Malondialdehyde (nmol/mL)	1.24-6.2	4.3 $\pm$ 1.2	0.5-4.0	1.7 $\pm$ 0.6
Interleukin-6 (pg/mL)	0.126-15.2	7.9 $\pm$ 2.8	0.126-12.1	3.8 $\pm$ 2.6
Adiponectin encoding gene (<i>ADIPOQ</i>) ($2^{-\Delta\Delta CT}$)	0.066-1.60	0.59 $\pm$ 0.31	0.350-1.66	1.01 $\pm$ 0.2

All values are presented as mean $\pm$ standard deviation (SD). Min-max represents the range of values.

Statistical comparison between cases and controls was performed using Student's t-test for normally distributed variables or Mann-Whitney U test for non-normally distributed variables.

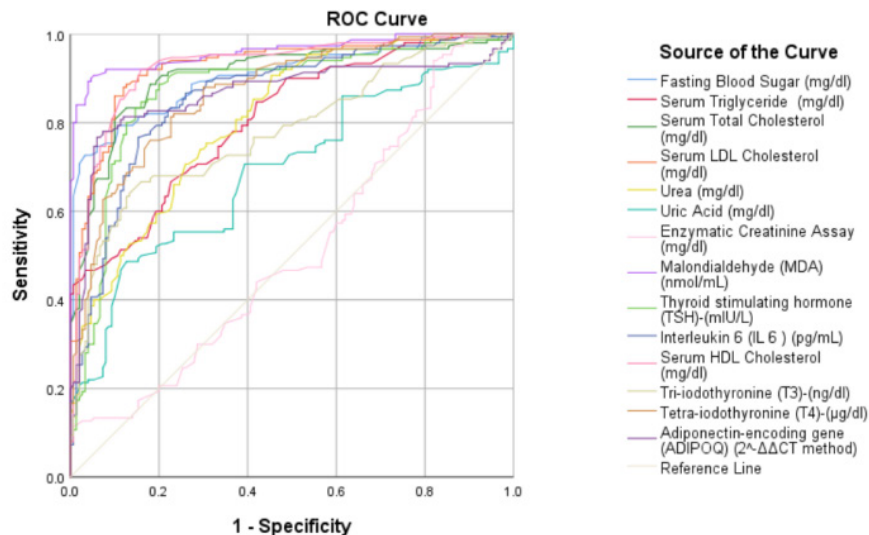
HDL, high-density lipoprotein; LDL, low-density lipoprotein

Table 3. Comparative analysis of clinical investigation parameters between cases and control (Levene's test with Bootstrap t-test)

Variable name	Levene's Test p-value	Bootstrap t-test p-value
Fasting blood sugar (mg/dL)	$p < 0.01$	$p < 0.01$
Serum triglyceride (mg/dL)	$p < 0.01$	$p < 0.01$
Serum total cholesterol (mg/dL)	$p < 0.01$	$p < 0.01$
Serum HDL cholesterol (mg/dL)	0.155	$p < 0.01$
Serum LDL cholesterol (mg/dL)	0.002	$p < 0.01$
Malondialdehyde (MDA) (nmol/mL)	$p < 0.01$	$p < 0.01$
Interleukin 6 (IL 6) (pg/mL)	0.802	$p < 0.01$
Adiponectin-encoding gene (ADIPOQ) ($2^{-\Delta\Delta CT}$)	$p < 0.01$	$p < 0.01$

Data presentation: All biochemical values in the study are presented as mean $\pm$ standard deviation (SD). Levene's Test was used to assess the equality of variances between cases and controls. Bootstrap p -values were calculated to assess robust significance, accounting for non-normality where applicable. A p -value < 0.05 was considered statistically significant

HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde

**Figure 1.** Receiver operating characteristic (ROC) curve for various clinical parameters

sugar, TG, TC, HDL cholesterol, LDL cholesterol, MDA, IL-6, and ADIPOQ expression (all $p < 0.01$). HDL cholesterol remained consistently lower in cases despite equal variance, while LDL cholesterol showed significance with unequal variance. These findings reinforce the roles of hyperglycemia, dyslipidemia, oxidative stress, and inflammation in disease status.

Diagnostic utility of biomarkers

Figure 1 illustrates ROC curves for the biochemical and genetic markers. MDA demonstrated the highest discriminative power (AUC = 0.9605), followed by LDL cholesterol (AUC = 0.9262), HDL cholesterol (AUC = 0.9259), TC (AUC = 0.9063), and FBS (AUC = 0.9046). IL-6 (AUC =

0.8589) and ADIPOQ expression (AUC = 0.8654) showed strong diagnostic performance, while TG (AUC = 0.8087) demonstrated moderate predictive capacity.

Correlation analyses

Scatter plots (Figures 2-8) illustrate associations between ADIPOQ expression and biochemical markers. Negative correlations were observed with fasting blood sugar, TG, TC, LDL cholesterol, MDA, and IL-6, indicating reduced ADIPOQ expression alongside metabolic and inflammatory disturbances. In contrast, a positive correlation was observed with HDL cholesterol, suggesting its protective role in maintaining adiponectin activity.

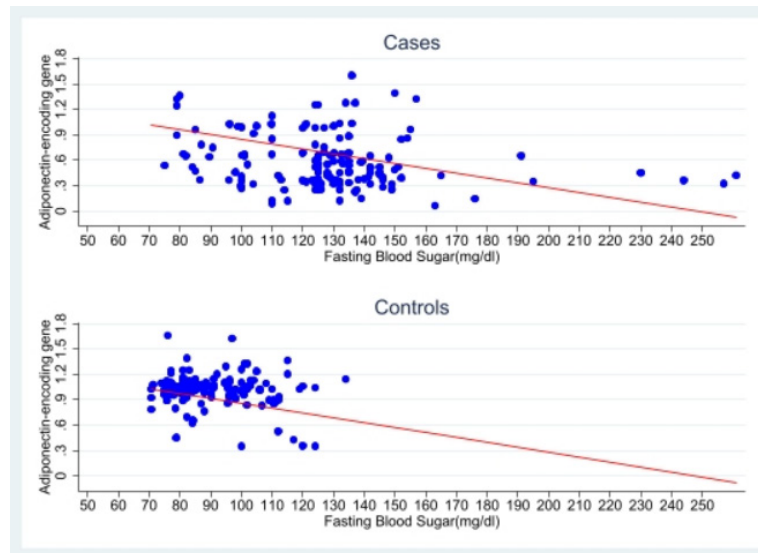


Figure 2. Scatterplot of adiponectin-encoding gene expression and fasting blood sugar in cases and controls

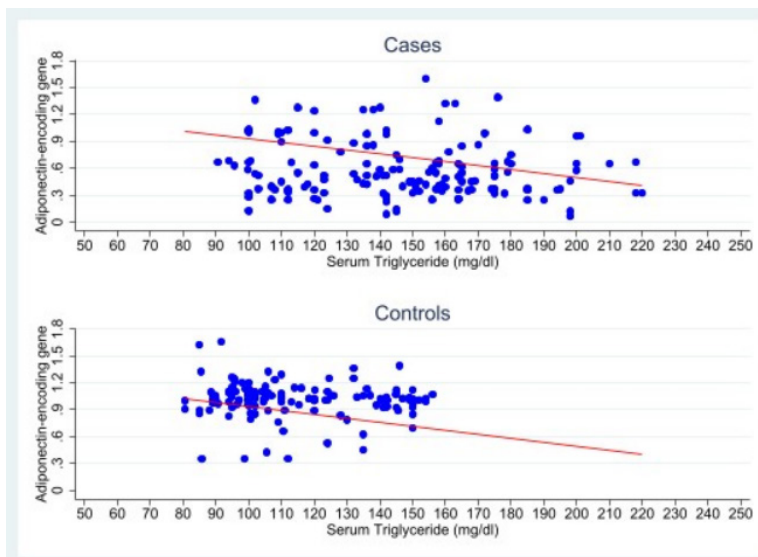


Figure 3. Scatterplot of adiponectin-encoding gene expression and serum triglyceride in cases and controls

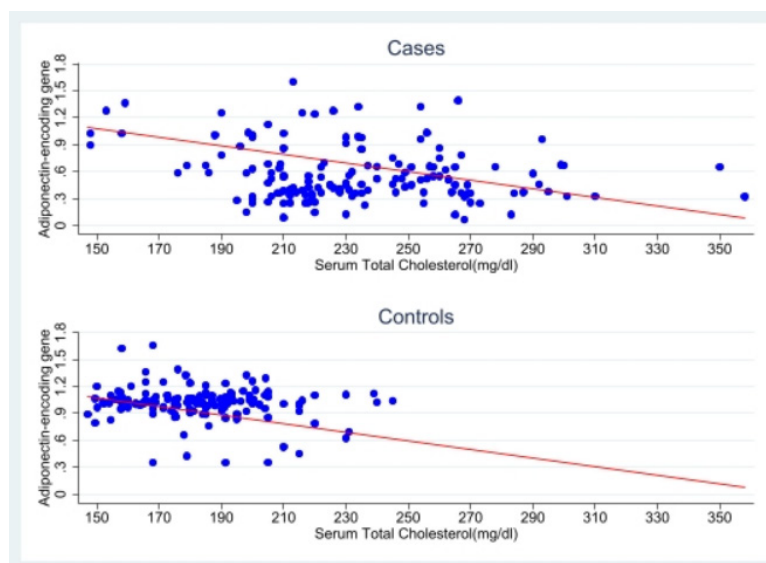


Figure 4. Scatterplot of adiponectin-encoding gene expression and serum total cholesterol in cases and controls

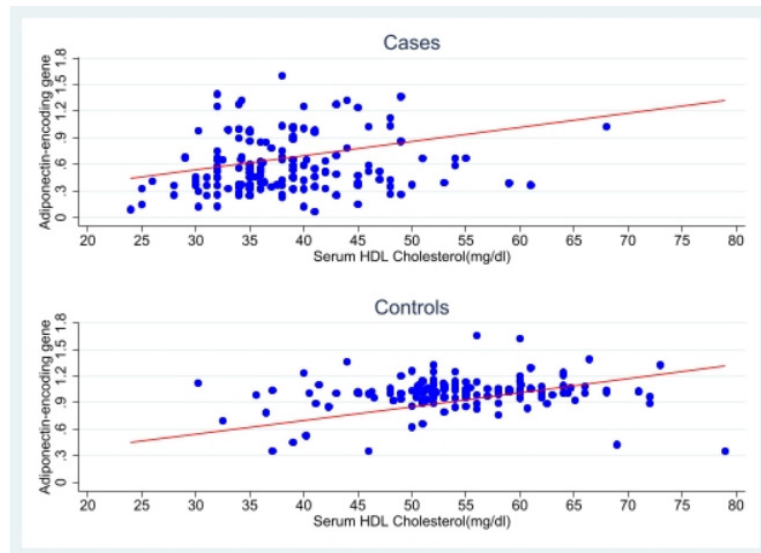


Figure 5. Scatterplot of Adiponectin-encoding gene expression and serum HDL cholesterol in cases and controls

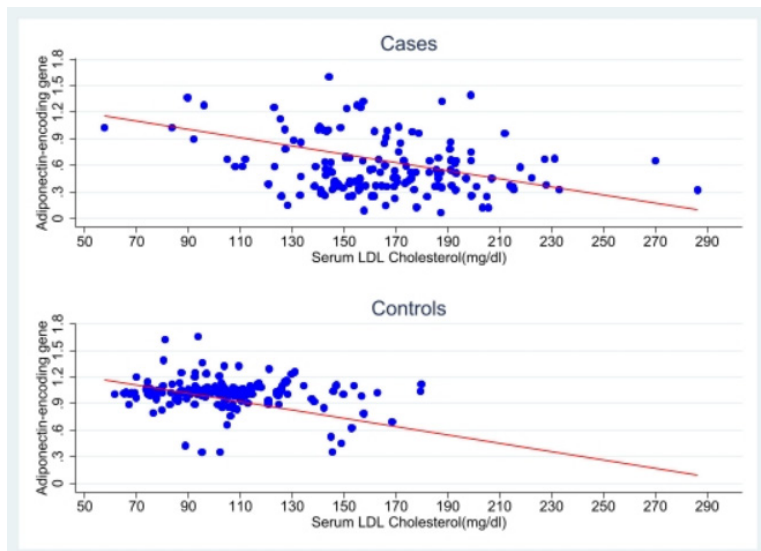


Figure 6. Scatterplot of adiponectin-encoding gene expression and serum LDL cholesterol in cases and controls

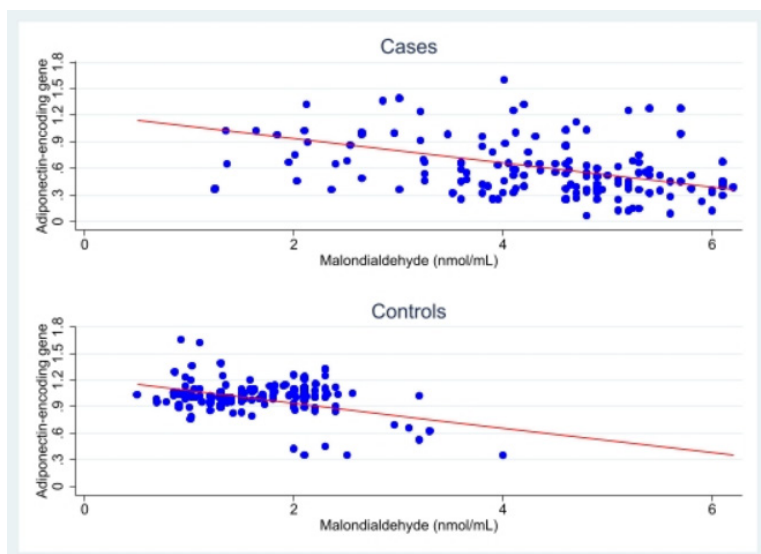


Figure 7. Scatterplot of adiponectin-encoding gene expression and malondialdehyde in cases and controls

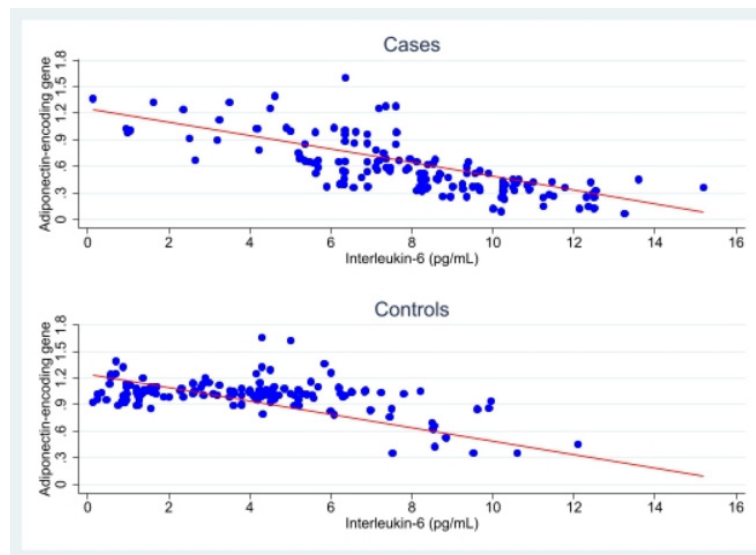


Figure 8. Scatterplot of adiponectin-encoding gene expression and interleukin 6 in cases and controls

Table 4. Comparative analysis of clinical investigation parameters between cases and controls

Variable	Model 1 OR [95%CI]	Model 2 OR [95%CI]
Adiponectin-encoding gene (<i>ADIPOQ</i>)	0.0034 [0.0010-0.0114]***	0.0220 [0.00004-11.6542]
Fasting blood sugar (mg/dL)	1.0995 [1.0781-1.1214]***	1.2089 [1.0667-1.3701]***
Serum triglycerides (mg/dL)	1.0473 [1.0358-1.0590]***	1.0185 [0.9769-1.0618]
Serum HDL cholesterol (mg/dL)	0.7861 [0.7480-0.8263]***	0.7674 [0.6369-0.9248]***
Serum LDL cholesterol (mg/dL)	1.0712 [1.0558-1.0868]***	1.0002 [0.9619-1.0401]
Malondialdehyde (MDA) (nmol/mL)	11.5491 [6.4634-20.6362]***	29.1660 [3.5853-237.2580]***
Interleukin-6 (pg/mL)	1.6907 [1.5034-1.9014]***	0.2859 [0.1122-0.7284]***

Model 1: Unadjusted logistic regression; Model 2: Adjusted for relevant covariates.

Significance indicators: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. All biochemical variables were presented as mean $\pm$ SD in descriptive analyses.

OR, odds ratio, 95%CI, 95% confidence interval; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde

Logistic regression models

Table 4 summarizes binary logistic regression results. In unadjusted models, *ADIPOQ* expression showed a strong inverse association with disease status (OR = 0.0034), but this lost statistical significance after adjustment (OR = 0.0220). FBS remained an independent predictor in both models (OR = 1.0995 unadjusted; OR = 1.2089 adjusted). HDL cholesterol consistently exhibited a protective effect (OR = 0.7861 and 0.7674). TG and LDL cholesterol were significant in unadjusted models but lost significance after adjustment, suggesting confounding.

Notably, MDA was a powerful independent predictor, with odds ratios of 11.5491 (unadjusted) and 29.1660 (adjusted), both highly significant. Interestingly, IL-6 showed a positive association in the unadjusted model (OR = 1.6907) but reversed

to a significant inverse association after adjustment

(OR = 0.2859), indicating complex interactions with other predictors.

DISCUSSION

CVDs continue to be a significant global health concern, contributing significantly to morbidity and mortality rates across populations. In recent years, adiponectin, an adipocyte-derived cytokine encoded by the *ADIPOQ* gene, has garnered attention for its pivotal role in regulating metabolic homeostasis, insulin sensitivity, and inflammatory responses, all of which are crucial to the pathogenesis of both CVD and metabolic disorders. In parallel, NAFLD has emerged as a prevalent liver condition, particularly among younger individuals, driven by increasing rates of obesity, sedentary

lifestyles, and insulin resistance. NAFLD is not only a hepatic disorder but also a significant risk factor for cardiovascular complications, given the shared pathophysiological mechanisms, including systemic inflammation, oxidative stress, and dysregulated lipid metabolism.

This study was designed to explore the differential expression of the *ADIPOQ* gene and its correlation with biochemical and clinical parameters in individuals affected by NAFLD and/or CVD. By focusing on young adults, this research highlights an at-risk population where early genetic and biochemical profiling could inform targeted preventive strategies.

Previous literature has reported associations between alterations in the *ADIPOQ* gene and specific metabolic diseases. For example, Saki et al. and Gruzdeva et al. evaluated *ADIPOQ* gene expression in the context of either liver-related or cardiovascular conditions, demonstrating that altered adiponectin levels are linked to disease severity and progression (8, 9). However, integrated studies examining *ADIPOQ* expression in individuals simultaneously exhibiting both NAFLD and CVD remain scarce.

Błażejewska et al. contributed to this field by investigating the epigenetic regulation of *ADIPOQ* via DNA methylation in patients with NAFLD, revealing its role in suppressing adiponectin expression and promoting liver fat accumulation (10). De et al. identified a genetic protective factor, the G allele of rs17300539, in the *ADIPOQ* gene, which was associated with reduced risk of NAFLD complications, likely due to its influence on adiponectin transcriptional activity (11). Furthermore, the rs1501299 polymorphism, another well-studied variant in the *ADIPOQ* gene, has been consistently associated with an increased risk of NAFLD, suggesting a genetic predisposition to disease susceptibility.

Together, these findings underscore the need for comprehensive studies examining the expression and regulation of the *ADIPOQ* gene across overlapping metabolic conditions, such as NAFLD and CVD. The current research aims to bridge this gap by offering insights into how *ADIPOQ* expression interacts with inflammatory and metabolic biomarkers in dual-pathology cases, potentially guiding future diagnostic and therapeutic approaches.

The present study revealed significant differences in biochemical and molecular parameters between cases and healthy controls, emphasizing the metabolic and inflammatory derangements associated with cardiovascular and hepatic dysfunction. FBS levels were markedly elevated in cases, with a mean of 129.1 mg/dL compared to 90.3 mg/dL in controls. The broader range observed among cases (75.0–261.0 mg/dL) indicates a higher prevalence of hyperglycemia, a known contributor to endothelial dysfunction and the development of atherosclerosis (Table 2). This finding aligns with previous studies that have identified impaired glucose regulation as a significant metabolic abnormality in individuals with cardiovascular pathology as well as its role in hepatic steatosis by promoting insulin resistance (12).

Serum TG levels were considerably higher in cases, with a mean of 147.1 mg/dL compared to 114.1 mg/dL in controls. The broader range observed in cases (90.6–220.0 mg/dL) reflects increased variability and the presence of dyslipidemia, a well-established risk factor for both cardiovascular complications and fatty liver disease (Table 2). Elevated TG has been linked to impaired hepatic lipid metabolism and increased risk of atherosclerosis, supporting the hypothesis of overlapping mechanisms in early cardiohepatic dysfunction (13).

TC was significantly elevated in cases (mean: 232.8 mg/dL) compared to controls (182.2 mg/dL), with values in cases reaching as high as 358.0 mg/dL (Table 2). Such elevated levels are indicative of systemic lipid imbalance and contribute to the formation of plaques and liver fat accumulation (14). In contrast, serum HDL cholesterol levels, which offer protective effects against both vascular and hepatic injury, were considerably lower in cases (38.2 mg/dL) compared to controls (54.3 mg/dL). The reduced HDL levels further emphasize a loss of protective lipid regulation in individuals at risk (Table 2).

LDL cholesterol levels were also significantly increased in cases, with a mean of 165.1 mg/dL versus 105.1 mg/dL in controls. The upper limit of LDL in cases reached 286.4 mg/dL, reinforcing its critical role in atherogenesis and possibly in hepatic inflammation through lipotoxicity (Table 2). This dyslipidemic profile, in conjunction with elevated glucose levels, suggests a synergistic

burden on both the cardiovascular and hepatic systems.

Oxidative stress and inflammation—two central processes in the pathogenesis of both CVD and liver dysfunction—were reflected by elevated levels of MDA and IL-6 in cases. MDA levels averaged 4.3 mg/L in cases compared to 1.7 mg/L in controls, indicating enhanced lipid peroxidation and oxidative damage (Table 2). Similarly, IL-6 levels were significantly higher in cases (7.9 pg/mL) compared to controls (3.8 pg/mL), indicating persistent low-grade systemic inflammation (Table 2). These biochemical perturbations are consistent with previous findings that link oxidative and inflammatory stress to endothelial damage and hepatocellular injury (15, 16).

Significantly, the expression of the *ADIPOQ* gene, which encodes adiponectin, a key regulator of metabolic balance and anti-inflammatory protection, was significantly reduced in the case group (mean: 0.59 µg/mL) relative to controls (1.01 µg/mL) (Table 2). Adiponectin deficiency has been strongly associated with insulin resistance, dyslipidemia, and inflammatory conditions affecting both the heart and liver. The inverse relationship observed between adiponectin levels and other metabolic and inflammatory markers in this study supports its role as a protective factor in cardiohepatic health (17, 18). Its reduced expression in cases highlights a potential early genetic signal of metabolic distress.

Collectively, these results underscore the interrelated nature of metabolic dysfunction, systemic inflammation, oxidative stress, and genetic regulation via *ADIPOQ* expression in the development of early cardiovascular and hepatic diseases. These findings not only validate adiponectin as a crucial biomarker of dual-organ dysfunction but also underscore the importance of integrating biochemical and molecular parameters to predict disease onset and progression.

The comparative analysis of clinical investigation parameters (Table 3) revealed significant biochemical and molecular alterations between cases and controls, underscoring their relevance in the early stages of cardiovascular and hepatic dysfunction. FBS levels were significantly higher in cases ($p < 0.01$), reinforcing the association between hyperglycemia and disease risk. Similarly, serum TG, TC, and LDL cholesterol were markedly

elevated (all $p < 0.01$). In contrast, HDL cholesterol levels were significantly reduced ($p < 0.01$), reflecting atherogenic dyslipidemia commonly linked to cardiovascular and hepatic pathologies. Metabolic indicators such as oxidative stress, as evidenced by increased MDA levels ($p < 0.01$), further highlight the pathophysiological burden in affected individuals.

Elevated IL-6 levels in cases ($p < 0.01$) confirmed heightened systemic inflammation. Most notably, *ADIPOQ* gene expression was significantly downregulated in cases ($p < 0.01$), emphasizing the diminished protective role of adiponectin in metabolic homeostasis and its potential as a key molecular marker for early cardiohepatic dysfunction.

The present study introduces a novel diagnostic perspective by evaluating the ROC performance of biochemical and genetic markers (Figure 1) in distinguishing young adults with NAFLD and/or CVD. MDA showed the highest accuracy (AUC = 0.9605), followed by LDL, HDL, TC, and FBS (all AUC > 0.90), indicating excellent diagnostic potential. Notably, *ADIPOQ* gene expression (AUC = 0.8654) and IL-6 (AUC = 0.8589) also demonstrated strong discriminatory power, highlighting their roles in metabolic and inflammatory pathways. These findings underscore the novelty of integrating oxidative, metabolic, and genetic markers for early risk identification.

In the present study, we observed a consistent and statistically significant negative correlation between adiponectin-encoding gene (*ADIPOQ*) expression and various metabolic, oxidative, and inflammatory markers, including FBS, serum TG, TC, LDL cholesterol, MDA, and IL-6. In contrast, a positive correlation was noted with HDL cholesterol levels. These findings, as illustrated in Figures 2–8, suggest that diminished *ADIPOQ* expression is associated with worsening metabolic and inflammatory profiles in both cases and controls, with a more pronounced pattern observed in affected individuals. The inverse relationship between *ADIPOQ* and FBS, TG, and LDL supports the role of adiponectin in enhancing insulin sensitivity and regulating lipid metabolism, consistent with prior evidence indicating its protective effect against metabolic syndrome and atherosclerosis (19). Moreover, the observed decline in *ADIPOQ* expression with increasing MDA and IL-6 levels

emphasizes its antioxidative and anti-inflammatory functions, which may be compromised in individuals at higher cardiometabolic risk.

The logistic regression analysis highlights key biochemical predictors of disease status (Table 4). *ADIPOQ* expression showed a strong inverse association in the unadjusted model, although it lost significance after adjustment. FBS remained a significant independent risk factor, while HDL cholesterol consistently demonstrated a protective effect. TG and LDL were significant only before adjustment, suggesting potential confounding. Notably, MDA emerged as a strong independent predictor, emphasizing the role of oxidative stress. Interestingly, IL-6 exhibited a reversed association after adjustment, indicating complex interactions with other variables. These findings underscore the multifactorial nature of disease risk, involving metabolic, oxidative, and inflammatory pathways.

The novelty of our findings lies in the comprehensive gene-level analysis of *ADIPOQ* in relation to a broad panel of clinical parameters among young adults at risk for CVD and NAFLD. While previous studies have focused on circulating adiponectin levels, only limited research has explored its gene expression profile in this demographic, particularly in the context of combined metabolic and inflammatory dysregulation. Our study is among the first to highlight the potential of *ADIPOQ* gene expression as a molecular biomarker reflecting early changes in glucose metabolism, lipid imbalance, oxidative stress, and inflammation in a high-risk population. The observed associations reinforce the hypothesis that reduced adiponectin activity may be a central mechanism linking metabolic dysfunction and systemic inflammation to early cardiovascular and hepatic pathogenesis. These insights underscore the relevance of *ADIPOQ* expression not only as a marker of disease presence but also as a potential target for early intervention strategies aimed at mitigating long-term cardiometabolic complications.

CONCLUSIONS

This study highlights significant biochemical and genetic differences between individuals with NAFLD and/or cardiovascular risk and healthy controls in a young adult population. Elevated levels of fasting blood sugar, TG, TC, LDL, MDA,

and IL-6, alongside reduced HDL and adiponectin gene (*ADIPOQ*) expression, underscore the metabolic and inflammatory disturbances commonly associated with these conditions, notably, the downregulation of *ADIPOQ* gene expression in cases suggests a diminished protective role of adiponectin, which may contribute to the development and progression of metabolic and inflammatory pathologies. The study emphasizes the relevance of integrating biochemical and molecular profiling for early identification and risk stratification in young adults at risk for NAFLD and/or cardiovascular diseases.

This study has certain limitations that should be acknowledged. First, its cross-sectional design restricts the ability to establish causal relationships between *ADIPOQ* expression, biochemical parameters, and disease outcomes. Second, the relatively modest sample size, although adequately powered for the primary analyses, may limit the generalizability of findings to broader or more diverse populations. Third, as the study population was restricted to young adults aged 18–40, the results may not fully capture the effects of *ADIPOQ* dysregulation across different age groups. Additionally, unmeasured confounding factors, such as dietary intake, physical activity, and genetic variations beyond *ADIPOQ*, could have influenced the observed associations. Future longitudinal and multi-center studies incorporating larger, ethnically diverse cohorts and broader genetic profiling are warranted to validate and extend these findings.

Future research should explore longitudinal designs to validate adiponectin as a predictive biomarker and investigate potential therapeutic interventions aimed at restoring its expression and function. Early screening and targeted lifestyle modifications may prove pivotal in mitigating long-term complications in this vulnerable population.

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CONFLICTS OF INTEREST

There was no conflict of interest in this manuscript.

AUTHOR CONTRIBUTIONS

R.R.: conceptualization, study design, data collection, experimental work, statistical analysis, interpretation of results, and manuscript drafting; A.J.: Supervision, guidance in study design and methodology, critical revision of the manuscript, and corresponding author responsibilities; V.S.: Support in experimental design, validation of laboratory techniques, and manuscript review; P.K.: Contributed to quality control measures; V.M.G., S.k.K.S.: participant recruitment, clinical data collection, and data curation; S.S., M.K, S. J.P., J.P.J., R.K.S., S.P.: technical support in sample processing, laboratory work, and data management; D.R.D.: senior supervision, statistical validation, manuscript refinement, and corresponding author responsibilities.

All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request. All datasets have been anonymized to ensure participant privacy and confidentiality.

INSTITUTIONAL REVIEW BOARD STATEMENT,

Ethics approval (09/2022/IECG) was secured from the Institutional Ethics Committee of Genetika.

INFORMED CONSENT STATEMENT

All participants were informed about the purpose and procedures of the study in detail. They were told about any possible risks and benefits before taking part. Each participant gave written consent voluntarily after understanding the information provided. Their personal details and data were kept confidential and used only for research purposes.

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Prevalence of Alcohol and Drugs in Fatal Road Traffic Injuries (RTIs) in a Thai Population Between 2018 and 2023

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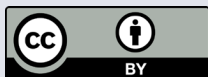
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ABSTRACT

OBJECTIVE To determine and compare the prevalence of alcohol, drugs of abuse and medicines in fatal road traffic injuries (RTIs) in Thailand from 2018 through 2023.

METHODS A retrospective study was conducted of fatal RTI cases sent for autopsy at the Department of Forensic Medicine, Siriraj Hospital, Mahidol University from 2018 through 2023. Subject data, including sex, age, type of RTI case, blood alcohol concentration (BAC), and urine drug profile, were collected. Descriptive statistics, Chi-square tests, Mann-Whitney U tests, and Kruskal-Wallis H tests were performed as appropriate.

RESULTS From 2018 through 2023 a total of 1,979 fatal RTI cases were included in this study. Of those cases, 1,649 were male (83.3%) and the mean subject age was 37.3 years. The yearly prevalence of driving under the influence (DUI) of alcohol (BAC > 50 mg/dL) during the study period was between 45.80% and 53.40% with the exception of 2022, which showed a significant increase at 57.10% ($p < 0.05$). The prevalence of drugs in 2023 was 21.00%, a significant difference compared with the prevalence in 2018 of 13.70% ($p < 0.05$). The prevalence of cannabis use showed an increasing trend, with a statistically significant increase in 2023 ($p < 0.05$). The prevalence of multiple drug use also significantly increased over the study period, particularly in 2023 ($p < 0.05$).

CONCLUSIONS The prevalence of drugs of abuse and medication in fatal RTI cases markedly increased over the study period, particularly in 2023. There was a significant increase in cannabis use and poly-drug use in fatal RTI cases over the period, especially in 2023.

KEYWORDS road traffic injury, alcohol, drugs, Thai, postmortem

INTRODUCTION

Road traffic injuries (RTIs) are among the leading causes of death worldwide. In Thailand, RTIs were reported to be among the top five causes of death (1). The annual number of RTI deaths from 2011 through 2021 ranged from 18.7 to 34.3 per 100,000 per year, with an average number of RTI deaths of 30.3 per 100,000 population per year (1). Driving under the influence (DUI) of alcohol and

drugs has been well-studied as one of the major causes of RTI, other than roadway geometric features, light, and weather conditions (2). A previous study suggested that an increased blood alcohol concentration (BAC) was associated with a higher risk of fatal RTIs, and the odds ratio (OR) for a BAC of 20 and 80 mg/dL was estimated to be 3.64 and 13.0, respectively (3). A meta-analysis study indicated that a BAC above 20 mg/dL was

associated with impairment of driving performance when assessed on a simulated driving platform (4). Among DUIs involving drugs, alcohol-drug combinations were found to be strongly correlated with severe RTIs (adjusted OR = 39.15), followed by drug-drug combinations (adjusted OR = 7.02) (5).

The prevalence of DUI of alcohol and drugs and the drug types in the RTI population can be different in each country. In Thailand, Sribanditmongkol et al. reported that the alcohol-positive rate in autopsied fatal RTI cases during the period 2007–2019 was 48.47% and the median BAC among those alcohol-positive blood samples was 192 mg/dL (6). Another study in Thailand found that the prevalence of psychoactive drugs present in urine samples from drivers in Thailand tested between 2005 and 2006 was 9.70%, and the most common drugs found were antihistamines, amphetamine, and cannabis (7). These figures differ from those reported in some European countries. For example, in Italy, it was reported that the average prevalence of BAC > 50 mg/dL for all driver types from 2011 to 2018 was 16.2% (13.80%–17.30%) (8). Similarly, the overall prevalence of BAC > 50 mg/dL in Norway between 2011 and 2020 was found to be 14.40% (9). For cases of DUI of drugs, the prevalence of drug-positive injured drivers ranged from 16.80%–24.10% in Italy (8), while the overall prevalence of drug-positive drivers and illicit drug-positive fatal driver cases in Norway were 15.80% and 10.70%, respectively (9).

Based on published accounts and other available data, it is noteworthy that there has seemingly been a critical transformation in the world in recent years, and especially in Thailand, the focus of this study, since the COVID-19 pandemic outbreak in 2020 and since the implementation of new drug policies in Thailand. Specifically, the Thai government-initiated endorsement of a legalized medical cannabis policy in 2019 and subsequently enacted legislation that allowed cannabis and mitragynine to be used legally by the general population beginning in 2020 and 2021, respectively (10). It has been suggested that the effects of the COVID-19 pandemic on changing Thai people's behaviors and the decriminalization of cannabis and mitragynine as official Thai government policy may have had an impact on alcohol and drug use

trends in RTI cases in Thailand. The objective of this study was to determine the prevalence of alcohol, drugs of abuse, and medicines in fatal RTI cases in Thailand from 2018 through 2023 to test this hypothesis. Our findings provide essential insights which are important for achieving greater awareness of current alcohol and drug use trends in fatal RTI cases in Thailand for forensic pathologists which will be fundamental for informed consideration of Thailand's public policies on drugs and traffic accident prevention.

METHODS

A retrospective study was conducted on fatal RTI cases sent for autopsy at the Department of Forensic Medicine, Faculty of Medicine, Siriraj Hospital, between 2018 and 2023. The inclusion criteria were Thai people who were 15 years old or over at death, and who had died within 3 hours of sustaining an RTI. The exclusion criteria were cadavers that had signs of decomposition and RTI cases of vehicles hitting pedestrians. Femoral blood samples were obtained for analysis of the BAC following the Thai legislation Ministerial Regulations B.E. 2567 (2024) by virtue of the provisions of Section 5 of the Road Traffic Act B.E. 2522 (1979). In addition, urine samples were collected for analysis of illicit drugs and medications based on the relevant statement in the Thai legislation in Ministerial Regulations B.E. 2548 (2005) for urine analysis in RTI cases.

The subjects' data, including sex, age, type of RTI case, BAC, and urine drug profile, were collected by year of death from 2018 through 2023 for assessing annual statistics. Analysis of the BAC was performed using headspace gas chromatography with a flame ionization detector (HS-GC-FID). The method used for alcohol analysis was adapted from previous studies performed elsewhere (11). The limit of detection (LOD) and lower limit of quantitation (LLOQ) for blood ethanol analysis were 2.5 mg/dL and 10 mg/dL, respectively. The definition of alcohol-positive cases (following the new Thai legislation) applies to subjects with a BAC $\geq$ 20 mg/dL. The urine drug profiles were obtained by a drug screening panel using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF/MS) and gas chromatography triple quadrupole tandem mass spectrometry (GC-MS/MS). Urinary drug

profiles obtained from LC-QTOF/MS analysis had a LOD and a LLOQ of 10 ng/mL and 20 ng/mL, respectively. Urinary cannabis profiles were analyzed by GC-MS/MS and LOD and LLOQ of delta 9-tetrahydrocannabinol (THC), 11-hydroxy-delta 9-tetrahydrocannabinol (11-OH-THC) and 11-Nor-9-carboxy- delta 9-tetrahydrocannabinol (THC-COOH) set at 0.1 ng/mL and 0.2 ng/mL, respectively (12). All analytical methods used in the present study were validated using the Standard Guidelines in Forensic Toxicology (13).

Statistical analysis was conducted using IBM SPSS® Statistics for Windows version 25. Descriptive statistics were used for the descriptive data, including mean, median, and standard deviation (SD). Comparisons between the prevalence of DUI of alcohol and drug use were performed using the chi-square test. Comparisons of the BAC over the six-year study period were performed using the Mann-Whitney U test and Kruskal-Wallis H test.

RESULTS

From 2018 to 2023, the total number of fatal RTIs sent for autopsy at the Department of Forensic Medicine, Faculty of Medicine, Siriraj Hospital and were recruited into this study was 1,979, divided into study years as follows: 348 (2018), 366 (2019), 316 (2020), 325 (2021), 314 (2022), and 310 (2023). Among the total cases, 330 were female (16.70%) and 1,649 were male (83.30%). The average ages of the female and male subjects at death were 42.12 and 36.37 years old, respectively. The most frequent category of RTI subject was motorcycle riders (72.06%), followed by pedestrians (13.44%), and car drivers (6.01%). The three types of RTI cases classified by each year are provided in Table 1.

Table 1. The three main types of RTI cases (motorcycle riders, car drivers, and pedestrians) classified by year

Year	Motorcycle rider	Car driver	Pedestrian
2018	238 (68.39)	33 (9.48)	50 (14.37)
2019	256 (69.95)	20 (5.46)	52 (14.21)
2020	229 (72.45)	9 (2.85)	51 (16.14)
2021	243 (74.77)	18 (5.54)	44 (13.54)
2022	227 (72.29)	20 (6.37)	39 (12.42)
2023	233 (75.16)	19 (6.13)	30 (9.68)
Total	1426 (72.06)	119 (6.01)	266 (13.44)

RTI, road traffic injury

The average BAC levels for all RTI cases and for the driver-only cases (motorcycle riders and car drivers) in each of the years of the study period (2018–2023) are shown in Figure 1. The annual mean BAC in both the total RTI cases and rider/driver-only cases did not show any significant difference, except in 2022 ($p = 0.008$). The overall results for the alcohol-positive cases are shown in Table 2. The majority of the DUI of alcohol cases were men and most were motorcycle riders. Overall, the male subjects had a significantly higher BAC than the female subjects ($p < 0.001$). When considering BAC > 50 mg/dL, however, the BAC of the male subjects was not significantly different from that of the female subjects ($p = 0.344$). Overall, pedestrians had a significantly lower BAC than motorcycle riders and car drivers ($p = 0.031$). However, when considering cases with BAC > 50 mg/dL, the pedestrians had a significantly higher BAC than both the motorcycle riders and car drivers ($p < 0.001$). Comparing cases of only alcohol use and cases with a combination of alcohol and drug use, subjects who were positive only for alcohol had a significantly higher BAC than subjects who

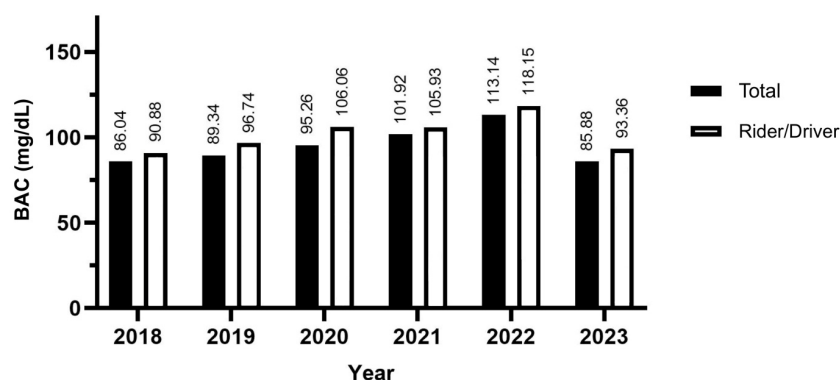


Figure 1. Mean blood alcohol concentration levels of all road traffic injury cases and rider/driver cases over the study period 2018–2023

Table 2. Prevalence of alcohol-positive cases and BAC by sex, type of RTI case, and patterns of drugs found in subjects

Parameters	Alcohol positive N (%)	Alcohol > 50 mg/dL N (%)	BAC Mean, SD	BAC > 50 mg/dL Mean, SD
Total (N = 1979)	994 (50.23)	907 (45.83)	95.01, 114.01	204.35, 78.69
Sex				
Male	920 (92.56)	848 (93.50)	106.77*, 115.89	205.03, 78.43
Female	74 (7.44)	59 (6.50)	36.20, 81.95	194.50, 82.34
Types of RTI case				
motorcycle rider	780 (78.47)	715 (78.83)	101.53, 111.78	198.50, 75.56
Car driver	68 (6.84)	59 (6.50)	102.08, 108.59	200.66, 64.93
Pedestrian	90 (9.05)	85 (9.37)	83.02*, 130.76	258.73*, 89.47
Pattern				
Alcohol only	897 (90.24)	822 (90.63)	102.24*, 89.14	207.14*, 77.84
Alcohol and drugs	97 (9.76)	85 (9.37)	53.05, 90.98	177.32, 82.14

BAC, blood alcohol concentration; RTI, road traffic injury

*at each parameter indicated that the parameter was significantly different from the other parameter(s) in the other row(s) at $p < 0.05$

had used alcohol with drugs in both the alcohol-positive cases ($p < 0.001$) and BAC > 50 mg/dL cases ($p = 0.002$).

The most frequent drug substance found in the RTI cases was methamphetamine (4.80%), followed by cannabis (3.80%), and benzodiazepines (3.50%). Diazepam (2.90%) was the dominant drug in the benzodiazepine group. Mitragynine (3.10%) was another drug frequently found in this study. Tramadol and antihistamine were found at similar rates (2.50%). Chlorpheniramine (0.90%) and diphenhydramine (0.80%) were frequently found drugs in the antihistamine group. The other drugs that were detected, albeit at a relatively low rate, were ketamine (1.30%), heroin (0.90%), antidepressants (0.70%), and 3,4 methylenedioxy-N-methamphetamine (MDMA) (0.40%).

In total, 1,188 cases (60.03%) were positive for alcohol, drugs of abuse and/or medicines, of which 1,010 (51.04%) were motorcycle riders or car drivers. The positive RTI cases were predominantly males, with 1,096 cases (92.26%). The prevalence of alcohol (BAC over the legal limit of 50 mg/dL) and drugs in the total RTI cases and in the rider/driver cases during the study period, 2018–2023, are summarized in Figure 2. The number of RTI cases and DUI cases who had a BAC greater than 50 mg/dL was significantly higher in 2022 than in the other five years ($p = 0.033$), whereas the prevalence of RTI and DUI cases that had a BAC greater than 50 mg/dL in the other five years did not show any statistically significant difference. Urine analysis revealed that

14.70% of the samples were positive for drugs of abuse or medication. The trend of RTI subjects who tested positive for drugs of abuse or medication escalated from 2018 to 2023, with a significant increase noted in 2023 ($p = 0.023$). Similarly, the riders and drivers cases also showed a similar increasing trend of drug use, with a significantly higher number observed in 2023 ($p = 0.017$). Figure 2A and 2B shows the trends in the prevalence of alcohol and drugs found in all RTI cases and for driver cases, both of which showed a similar trend over the study period.

Methamphetamine and benzodiazepine in fatal RTI cases did not show significant differences over the six-year study period. The average positive rates (range) for methamphetamine and benzodiazepines were 4.84% (2.53–6.69%) and 3.50% (2.46–4.75%), respectively. Cannabis use gradually increased over the same period, with statistical significance only in 2023 ($p = 0.012$), as shown in Figure 3A. Mitragynine, tramadol, and ketamine use also gradually increased, although the figures were still not significantly different over the six years (Figure 3A and 3B). The number of antihistamine and 3,4 methylenedioxy-N-methamphetamine (MDMA) users significantly increased, particularly in 2022 and 2023 ($p = 0.002$) (Figure 3A and 3B).

The most common pattern of drug use in the fatal RTI cases remained single drug use (9.30%), a pattern which was relatively stable over the six-year study period. However, use of more than one drug was also reported in many cases, with

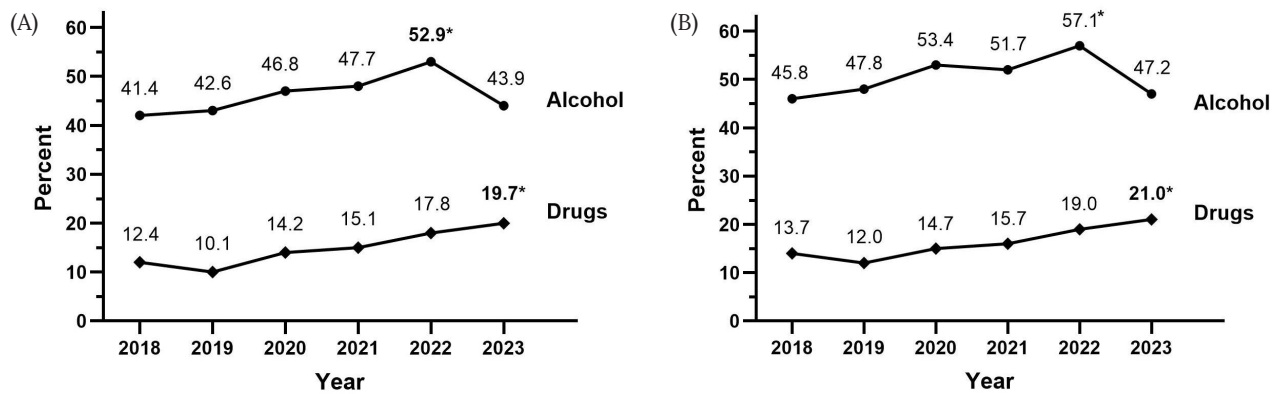


Figure 2. Prevalence of alcohol and drug use in fatal road traffic injury (RTI) cases over the study period 2018–2023 (Figure 2A: all RTI cases, Figure 2B: riders/drivers) *significant at $p < 0.05$

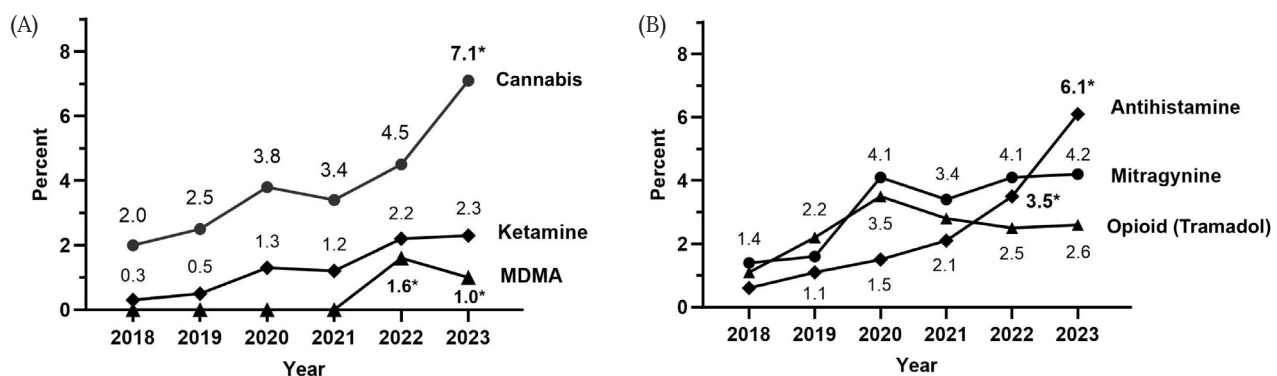


Figure 3. Prevalence of specific drugs of abuse and medication in the fatal rider/driver cases over the study period 2018–2023 (Figure 3A: cannabis, ketamine, and MDMA; Figure 3B: mitragynine, antihistamine, and tramadol) *significant at $p < 0.05$

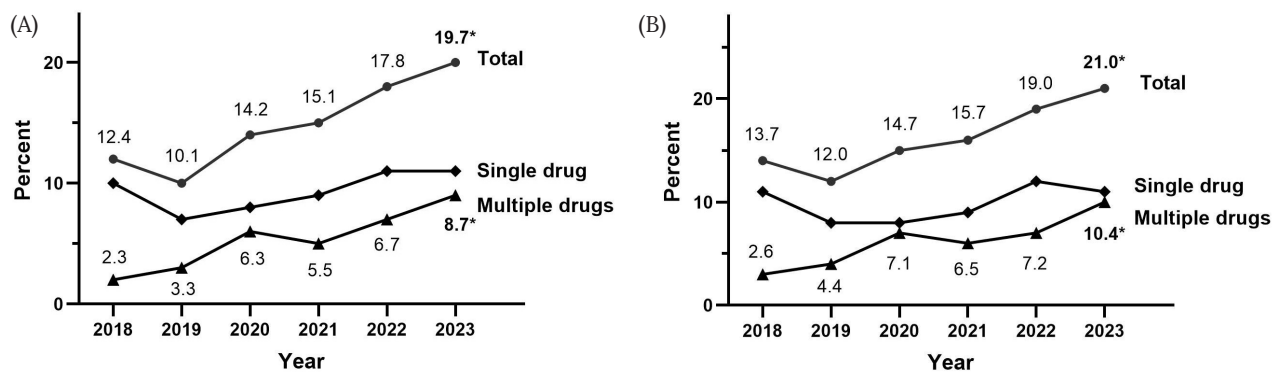


Figure 4. Prevalence of single and multiple drug use in fatal road traffic injury (RTI) cases over the study period 2018–2023 (Figure 4A: all RTI cases, Figure 4B: riders/drivers) *significant at $p < 0.05$

combinations of two or more drugs becoming increasingly prevalent. The maximum number of drugs found per case was nine in 2020; however, the number of cases with multiple drug use (two or more drugs) significantly increased, particularly in 2023 ($p = 0.027$), as shown in Figure 4A. A similar trend of multiple drug use was also found in riders and drivers, and it also significantly increased in 2023 ($p = 0.013$) (Figure 4B).

DISCUSSION

This study investigated the prevalence of alcohol, drugs of abuse, and medicines in fatal RTI cases corresponding to a study period covering the COVID-19 pandemic outbreak and the recent legalization of certain drugs in Thailand. The first positive COVID-19 case in Thailand was reported in January 2020, resulting in the government declaring a state of emergency in March 2020 and

an intense lockdown. This period coincided with a decrease in the number of fatal road traffic injuries from 348 (2018) and 366 (2019) prior to the COVID-19 outbreak to 316 (2020) during the COVID-19 lockdown period. Interestingly, the number remained relatively constant from 2020 through 2023. However, looking at the finer details, it was found that the number of motorcycle rider RTI fatalities, which were the majority of our subjects, remained relatively constant throughout the study period of 2018–2023 (Table 1). This suggests that the government measures did not have an obvious impact on motorcycle use in Thailand.

The prevalence of BAC > 50 mg/dL cases among the total fatal RTI cases and rider/driver cases from 2018 to 2023 ranged from 41.70% to 52.90% and from 45.80% to 57.10%, respectively. These figures are consistent with the findings of Sribanditmongkol et al., who studied Thai medico-legal cases and reported an overall prevalence of alcohol-positive RTI cases of 48.47% (6). In addition, a previous study reported a similar prevalence of DUI of alcohol in Brazil of 43.00%–48.00% (14). However, our figures differ from trends of DUI of alcohol in European countries, Australia, and USA, which have reported prevalences ranging from 2.90% to 19.30% (14). Moreover, most of the trends of DUI of alcohol in the aforementioned developed countries have shown a decline in recent years (14), whereas the trend in Thailand for the period 2018–2023 was relatively stable with the exception of 2022. A spike we observed in DUI of alcohol in 2022 after the COVID-19 lockdown in Thailand which corresponds with the findings of Mehranbod et al., who reported an increasing proportion of DUI of alcohol cases after lockdown (15). Sribanditmongkol et al. reported that the median BAC in alcohol-positive cases in RTI cases was 192 mg/dL (6). Walters et al. also reported mean BAC levels in DUI of alcohol cases (BAC cut-off > 80 mg/dL) ranging from 151 to 199 mg/dL over the period 2019–2021 (16). These figures were consistent with our study, where the mean BAC levels among motorcycle riders and car drivers who had BAC > 50 mg/dL were 198.41 and 200.66 mg/dL, respectively. These findings show that although the BAC levels in those subjects were much higher than the legal limit in Thailand, they were not significantly different from the levels found in other studies.

However, the proportion of cases that had a BAC greater than the legal limit in Thailand was significantly higher than in other developed countries. Thus, further law enforcement might be required to reduce the fatal RTI cases associated with DUI of alcohol.

Regarding the prevalence of drug use in RTI cases in Thailand, a previous study stated that the proportion of drug-positive urine samples in drivers in Thailand was 9.70% in 2005–2006 (7). This figure was slightly lower than the prevalence of drug-positive urine samples in drivers in 2018 and 2019 found in this study (13.70% and 12.00%, respectively). However, we did find an increasing trend from 2019 to 2023, with a significant increase to 21.0% in 2023. The prevalence of drug-impaired drivers in fatal RTI cases in Norway, Australia, and USA were reported to be 15.80%, 26.70%, and 27.70%, respectively (9, 17, 18). However, care should be exercised when comparing the figures related to DUI of drugs as our study concentrated on the detection of drugs in urine, whereas in most previous studies the figures were based on the detection of drugs in blood (9, 17, 18). Our results thus only suggest that the prevalence of drug-positive drivers in Thailand has shown an increasing trend recently compared with the trend observed in 2005–2006 (7). Although the latest recommendation for the toxicological investigation of DUI cases suggests blood or oral fluid collection as specimens of choice (19), the current Thai legislation still requires the use of urine samples for the detection of drugs in DUI cases, especially for methamphetamine. Thus, further study should be conducted related to the detection of drugs in blood or oral fluids in DUI cases to bridge this gap.

A previous study stated that the most common drugs found in Thai drivers were antihistamine, amphetamine, and cannabis (7). However, the present study found that the most common drugs detected in riders/drivers in Thai post-mortem cases were methamphetamine, cannabis, and benzodiazepine. Undoubtedly, the drug use trends in Thailand are different from in other countries in Europe, Australia, and USA, because the common drugs found in those countries are relatively similar and consist mostly of cannabis, stimulants (methamphetamine and cocaine), and benzodiazepine (9, 14, 17, 18, 20). However,

the most common drugs found in DUI of drugs in Italy have been reported to be cocaine, opiates/opioids, and cannabis in descending order (8). Meanwhile in Hong Kong, cocaine, ketamine, and methamphetamine (in that order) were found to be the most prevalent drugs in DUI of drugs cases (21). In contrast, our study did not identify cocaine use in any of the RTI cases. This finding suggests that the common drugs found in RTI cases depend on the geographical area, local drug prevalence and drug availability.

An increasing trend of cannabis use was observed in Thai riders/drivers in this study, and this trend is consistent with the initiation of cannabis legalization in Thailand in 2019, with a significant surge of 7.1% seen in 2023, a subject which needs further investigation. This pattern is consistent with a study in the USA that showed an increased prevalence of cannabis use in fatally injured drivers in 12 states in the USA after the implementation of legalized medical marijuana laws in those states (22). These findings suggest that legalization might result in an increase in the prevalence of cannabis use in the future, as well as in both RTI cases and other causes of death. This trend should be closely observed as the current Thai government plans to re-consider changing the cannabis law under pressure from the public sector. In addition, an increasing trend of multiple drug use in fatal RTI cases was found in this study, even though single drug use was still the most common pattern found in RTI cases. Poly-drug use in DUI drug cases is more common in Europe, Australia, and USA, with reported figures of around 20.00%-40.00% of DUI of drugs cases (9, 14, 18, 23, 24).

Although multiple drug use in our study was lower, at 10.4%, a continuously increasing trend was observed. This trend should be closely monitored because drug-drug combinations can have a more significant impact on driving ability (5).

The main limitation of this study is related to the area where the RTI cases were recruited in this study, because the Department of Forensic Medicine, Siriraj Hospital is responsible for medico-legal cases only in the western area of Bangkok (western side of the Chao Phraya river). The trends observed in this area may not reflect the trends for the whole or other parts of Thai-

land as the prevalence of the use of some drugs may differ in other areas, leading to potentially different laboratory results. In addition, there were only a small number of female subjects (16.70%), so gender-related findings should be carefully interpreted. Therefore, we recommend that further study should be conducted in multi-centered settings and should include more female subjects to supplement our findings.

CONCLUSIONS

This study showed there was a relatively steady trend in the prevalence of DUI of alcohol over the study period (2018-2023) with the exception of a significantly higher prevalence in 2022. Additionally, the prevalence of drugs of abuse and medication in fatal RTI cases significantly increased over the study period, especially in 2023. In particular, a significant increase in cannabis use was observed which coincides with the recent legalization of cannabis in Thailand. In addition, the prevalence of multiple drug use in fatal RTI cases also significantly increased in 2023.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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AUTHOR CONTRIBUTIONS

P.C.: conceptualization, literature review, methodology, data curation, data analysis, writing—original draft preparation; P.P.: conceptualization, literature review, methodology, review & editing, supervision.

All authors read and approved the final version of this manuscript that was submitted for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital with COA no. Si 184/2024 (SIRB Protocol No. 131/2567 (IRB2), approval date March 3, 2024).

INFORMED CONSENT STATEMENT

Informed consent was waived due to the retrospective nature of the study and the analysis used anonymous data.

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Multimodal Evaluation of Pulmonary Rehabilitation in Newly Diagnosed Tuberculosis Patients Using Respiratory Culture-Based Microbiome Profiling and Inflammatory Biomarkers

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ABSTRACT

OBJECTIVE Pulmonary rehabilitation (PR) plays a vital role in enhancing lung function and exercise capacity in individuals with pulmonary conditions. This study evaluated the clinical effectiveness of an 8-week PR program in newly diagnosed pulmonary tuberculosis (TB) patients through the assessment of lung function, exercise capacity, inflammatory biomarkers, and respiratory microbiome changes.

METHODS Sixty newly diagnosed TB patients were enrolled, with 30 completing the 8-week PR program. Pulmonary function (FEV₁, FVC, FEV₁/FVC) and exercise capacity (6-minute walk test, 6MWT) were measured before and after PR. Inflammatory biomarkers (CRP, IL-6, TNF-α) and sputum microbiome profiles were assessed. ROC curves and multiple regression models identified biomarkers that were predictive of functional improvement.

RESULTS PR led to significant improvements: FEV₁ increased from 2.50 ± 0.60 L to 3.10 ± 0.50 L ($p < 0.001$), FVC from 3.20 ± 0.70 L to 3.70 ± 0.60 L ($p = 0.002$), and 6MWT distance from 350.00 ± 40.00 m to 400.00 ± 50.00 m ($p = 0.003$). Oxygen saturation improved from 93.00 ± 2.00% to 95.00 ± 1.00% ($p = 0.022$). Inflammatory markers decreased significantly: CRP 18.00 ± 7.00 to 10.00 ± 5.00 mg/L ($p < 0.001$), IL-6 25.00 ± 8.00 to 15.00 ± 6.00 pg/mL ($p < 0.001$), and TNF-α 50.00 ± 20.00 to 32.00 ± 15.00 pg/mL ($p = 0.002$). ROC analysis identified CRP as the strongest predictor of improvement (AUC = 0.84). Multiple regression confirmed CRP as an independent predictor of lung function and exercise capacity gains.

CONCLUSIONS An 8-week PR program significantly improved lung function, exercise capacity, and reduced systemic inflammation in TB patients, with CRP emerging as a key biomarker for predicting rehabilitation outcomes.

KEYWORDS pulmonary rehabilitation, tuberculosis, inflammatory biomarkers, microbiome, lung function, exercise capacity

INTRODUCTION

Tuberculosis (TB) remains a significant global health concern, especially in low- and middle-income nations such as India. The World Health Organization (WHO) reports that India accounts for about 25% of the global TB burden, making it the leading contributor globally (1). Despite successful elimination of the pathogen with anti-tuberculosis therapy (ATT), many patients develop persistent pulmonary complications such as fibrosis, bronchiectasis, restrictive lung disease, and airflow obstruction (2). These post-TB sequelae significantly impact respiratory function and quality of life and remain inadequately addressed by current therapeutic approaches (3).

Pulmonary rehabilitation (PR) is a comprehensive intervention designed to enhance physical fitness, alleviate dyspnoea, and improve health-related quality of life in individuals with chronic respiratory conditions. PR programs typically combine supervised exercise training, patient education, nutritional advice, and psychological support. While substantial evidence supports the efficacy of PR in diseases such as chronic obstructive pulmonary disease (COPD) and post-COVID-19 lung dysfunction, its role in TB survivors remains underexplored. Emerging studies suggest that PR may help mitigate functional limitations and enhance recovery in patients recovering from pulmonary TB (4).

In resource-constrained settings, the implementation of practical, cost-effective assessment methods is essential. A comprehensive, non-invasive assessment approach integrating sputum culture analysis to monitor microbial colonization and respiratory microbiome alterations, along with inflammatory biomarkers including C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) to evaluate systemic inflammation (5), offers a pragmatic framework to objectively monitor PR outcomes and guide long-term care in TB survivors.

Study objective

This study aimed to evaluate the clinical effectiveness of an 8-week PR program in newly diagnosed TB patients through assessment of lung function, exercise capacity, inflammatory biomarkers, and respiratory microbiome changes during standard anti-tuberculosis treatment.

METHODS

A prospective, interventional cohort study was conducted at PSG Hospital, Coimbatore, Tamil Nadu, India. Sixty individuals were recruited over the first 12 months of the 24-month study period. Newly diagnosed smear-positive or CBNAAT-positive pulmonary TB patients, aged between 18 and 60 years, with no history of previous TB treatment and capable of providing informed consent and adhering to study procedures were included. Patients with co-existing chronic lung diseases (COPD, ILD), active human immunodeficiency virus infection, multidrug-resistant or extensively drug-resistant TB, and those who were pregnant or lactating were excluded.

The study protocol was approved by the Institutional Ethics Committee (ECN: 1183/ICE/2023) and written informed consent was obtained from all participants.

All eligible patients were identified at the TB treatment clinic and invited to participate. Sixty patients were initially enrolled; however, only 30 patients completed the full 8-week PR program due to logistical constraints, transportation difficulties, and treatment adherence issues.

PR program

The PR program was initiated two weeks after commencing ATT to ensure patient stability. The structured 8-week program consisted of:

- Frequency: three supervised sessions per week
- Duration: 60 minutes per session
- Setting: hospital-based rehabilitation center
- Personnel: qualified physiotherapist and respiratory therapist
- Components:
 - Aerobic exercises: Walking, stationary cycling, and treadmill exercises (20-30 minutes), with intensity gradually increased based on individual tolerance using the Borg scale (6)
 - Breathing exercises: Diaphragmatic breathing, pursed-lip breathing, and inspiratory muscle training (15 minutes) to enhance lung expansion and respiratory control (7)
 - Strengthening exercises: Upper and lower limb resistance training using elastic bands and light weights (15 minutes) to improve overall physical endurance (8)

- Patient education: Weekly 30-minute sessions focusing on disease management, medication adherence, lifestyle modifications, and smoking cessation counselling

Assessment protocol

Assessments were conducted at two time points: baseline (pre-PR) and post-intervention (8 weeks).

Pulmonary Function Testing: Spirometry was performed using a calibrated digital spirometer (Model: Spirobank II, Mobile Industrial Robots (MIR), Rome, Italy) following American Thoracic Society guidelines (9). Measurements included forced expiratory volume in one second (FEV₁), forced vital capacity (FVC), and the FEV₁/FVC ratio. Three reproducible measurements were obtained, with the best values recorded.

Exercise capacity: the Six-Minute Walk Test (6MWT) was conducted following standardized protocols in a 30-meter corridor (10). Distance walked and oxygen saturation levels using pulse oximetry were recorded.

Quality of life: evaluation was performed using the St. George's Respiratory Questionnaire (SGRQ).

Inflammatory biomarkers: CRP, IL-6, and TNF- α were analyzed using validated enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) with established reference ranges. Blood samples were collected after 12-hour fasting and processed within 2 hours (11). These markers were selected based on their established roles in TB pathophysiology and systemic inflammation.

Sputum microbiological analysis: sputum samples were collected using standardized techniques and were processed within 4 hours. Aerobic and anaerobic bacterial cultures were performed using standard microbiological methods on blood agar and MacConkey agar plates, with identification confirmed by biochemical testing (12).

Statistical analysis

Data analysis was performed using SPSS version 25.0. Continuous variables are presented as mean $\pm$ standard deviation (SD). The paired t-test was used to compare pre- and post-PR outcomes for normally distributed continuous variables. The Chi-square test was utilized for categorical data comparisons. Pearson's correlation coefficient

was calculated to examine relationships between inflammatory biomarkers and changes in pulmonary function parameters. Receiver operating characteristic (ROC) curves were constructed to evaluate the predictive value of inflammatory biomarkers for clinical improvement, defined as $\geq 10\%$ increase in FEV₁ (13). The area under the curve (AUC) was calculated to assess diagnostic accuracy. Multiple linear regression analysis was performed to identify independent factors associated with improvements in lung function (FEV₁, FVC) and exercise capacity (6MWT). A p -value < 0.05 was considered statistically significant.

RESULTS

The study enrolled 60 patients with newly diagnosed pulmonary TB, of whom 30 completed the 8-week PR program. The demographic characteristics showed no significant differences between baseline and post-intervention assessments within the study cohort. The mean age of participants was 40.00 ± 12.00 years, with 18 males and 12 females. The body mass index (BMI) remained stable: 22.40 ± 2.10 kg/m² at baseline and 22.50 ± 2.00 kg/m² post-PR ($p = 0.927$). Smoking history was present in 10 patients, and showed no change during the study period ($p = 1.000$). TB severity distribution remained consistent, with 5 mild, 15 moderate, and 10 severe cases (Table 1).

The study demonstrated significant improvements in pulmonary function and exercise capacity following the 8-week PR intervention. FEV₁ increased from 2.50 ± 0.60 L pre-PR to 3.10 ± 0.50 L post-PR ($p < 0.001$), and FVC improved from 3.20 ± 0.70 L pre-PR to 3.70 ± 0.60 L post-PR ($p = 0.002$). Additionally, the FEV₁/FVC ratio improved from $78.00 \pm 5.00\%$ pre-PR to $84.00 \pm 3.00\%$ post-PR ($p = 0.001$). The 6MWT was used to evaluate exercise tolerance prior to and following the PR program. The distance walked in 6 minutes significantly increased from 350.00 ± 40.00 meters pre-PR to 400.00 ± 50.00 meters post-PR ($p = 0.003$). Oxygen saturation also improved, increasing from $93.00 \pm 2.00\%$ pre-PR to $95.00 \pm 1.00\%$ post-PR ($p = 0.022$) (Table 2).

Inflammatory biomarkers showed significant reductions following PR intervention. CRP levels diminished from 18.00 ± 7.00 mg/L before PR to 10.00 ± 5.00 mg/L after PR ($p < 0.001$), IL-6 levels reduced from 25.00 ± 8.00 pg/mL to 15.00 ± 6.00

Table 1. Demographics and baseline characteristics

Characteristic	Baseline (n = 30)	Post-PR (n = 30)	p-value
Age (years)	40.00±12.00	40.00±12.00	0.789
Gender (male/female)	18/12	18/12	1.000
BMI (kg/m ²)	22.40±2.10	22.50±2.00	0.927
Smoking history (yes/no)	10/20	10/20	1.000
TB severity (mild/moderate/severe)	5/15/10	5/15/10	1.000

Values are expressed as mean ± SD

BMI, body mass index; PR, pulmonary rehabilitation; TB, tuberculosis

Table 2. Pulmonary function and exercise parameters

Parameter	Pre-PR (n = 30)	Post-PR (n = 30)	p-value
FEV ₁ (L)	2.50±0.60	3.10±0.50	< 0.001*
FVC (L)	3.20±0.70	3.70±0.60	0.002*
FEV ₁ /FVC (%)	78.00±5.00	84.00±3.00	0.001*
6MWT distance (m)	350.00±40.00	400.00±50.00	0.003*
Oxygen saturation (%)	93.00±2.00	95.00±1.00	0.022*

Values are expressed as mean ± SD

FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; 6MWT, six-minute walk test; *p < 0.05 indicates statistical significance

Table 3. Inflammatory biomarkers and microbiome changes

Parameter	Pre-PR (n = 30)	Post-PR (n = 30)	p-value
Inflammatory biomarkers			
CRP (mg/L)	18.00±7.00	10.00±5.00	< 0.001*
IL-6 (pg/mL)	25.00±8.00	15.00±6.00	< 0.001*
TNF-α (pg/mL)	50.00±20.00	32.00±15.00	0.002*
Sputum microbial culture			
<i>Streptococcus pneumoniae</i>	10 (33.3)	3 (10.0)	0.034*
<i>Pseudomonas aeruginosa</i>	8 (26.7)	2 (6.7)	0.042*
<i>Fusobacterium</i> species	6 (20.0)	1 (3.3)	0.023*
<i>Bacteroides fragilis</i>	5 (16.7)	0 (0.0)	0.011*

Values are expressed as mean ± SD for continuous variables and n (%) for categorical variables.

*p < 0.05 indicates statistical significance

CRP, C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha

pg/mL ($p < 0.001$), and TNF-α levels declined from 50.00 ± 20.00 pg/mL to 32.00 ± 15.00 pg/mL ($p = 0.002$). Analysis of sputum microbiological profiles revealed favorable changes in respiratory microbiome composition. The presence of *Streptococcus pneumoniae* decreased from 33.3% pre-PR to 10% post-PR ($p = 0.034$), and *Pseudomonas aeruginosa* colonization reduced from 26.7% pre-PR to 6.7% post-PR ($p = 0.042$). Additionally, anaerobic pathogens such as *Fusobacterium* and *Bacteroides fragilis* also showed significant reductions, with *Fusobacterium* decreasing from 20% pre-PR to 3.3% post-PR ($p = 0.023$) and *Bacteroides fragilis* from 16.7% to 0% ($p = 0.011$)

(Table 3).

Correlation analysis revealed strong negative associations between inflammatory biomarkers and functional improvements. Negative correlations were observed between the levels of CRP, IL-6, TNF-α, and improvements in FEV₁, FVC, and the 6MWT distance. Specifically, improvements in FEV₁ ($r = -0.63$), FVC ($r = -0.59$), and 6MWT distance ($r = -0.71$) were all statistically significantly negatively correlated with reductions in CRP (Table 4).

ROC analysis and multiple regression modeling identified inflammatory biomarkers as significant predictors of rehabilitation outcomes

Table 4. Correlation analysis between inflammatory biomarkers and functional parameters

Parameter	CRP	IL-6	TNF- α
FEV ₁ (L)	-0.63*	-0.58*	-0.52*
FVC (L)	-0.59*	-0.54*	-0.49*
6MWT Distance (m)	-0.71*	-0.69*	-0.62*

Strong negative correlations were observed according to Cohen's guidelines ($r > 0.50$) (14), * $p < 0.05$ indicates statistical significance

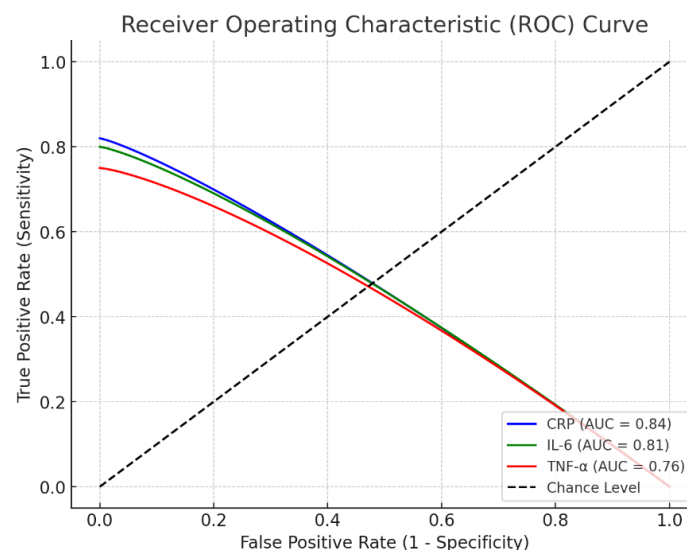
FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity, 6MWT, Six-Minute Walk Test

(Figure 1). CRP had the highest AUC of 0.84 (95%CI: 0.74–0.94), demonstrating a sensitivity of 82% and a specificity of 78% ($p < 0.001$). IL-6 and TNF- α had statistically significant predictive value, with AUCs of 0.81 (95%CI: 0.70–0.92) and 0.76 (95%CI: 0.64–0.88), respectively. In terms of

FEV₁ enhancement, elevated baseline concentrations of CRP ($\beta = -0.35$, $p = 0.001$), IL-6 ($\beta = -0.28$, $p = 0.004$), and TNF- α ($\beta = -0.22$, $p = 0.009$) were substantially statistically correlated with less improvement. Likewise, for the 6MWT distance, heightened baseline levels of CRP ($\beta = -0.41$, $p < 0.001$), IL-6 ($\beta = -0.33$, $p = 0.002$), and TNF- α ($\beta = -0.30$, $p = 0.004$) were autonomous predictors of diminished improvements in exercise ability (Table 5).

DISCUSSION

This study demonstrates the beneficial effects of PR in enhancing pulmonary function, exercise capacity, and reducing systemic inflammation in newly diagnosed TB patients. These findings align with previous studies demonstrating significant improvements in lung function and overall health

**Figure 1.** Receiver operating characteristic (ROC) curves for CRP, IL-6, and TNF- α in predicting clinical outcome**Table 5.** ROC analysis and regression predictors

Analysis type	Biomarker	AUC (95% CI)	Sensitivity (%)	Specificity (%)	β Coefficient	p-value
ROC analysis	CRP	0.84 (0.74–0.94)	82.0	78.0	–	< 0.001*
	IL-6	0.81 (0.70–0.92)	80.0	75.0	–	< 0.001*
	TNF- α	0.76 (0.64–0.88)	75.0	72.0	–	0.002*
FEV ₁ predictors	CRP	–	–	–	-0.35	0.001*
	IL-6	–	–	–	-0.28	0.004*
	TNF- α	–	–	–	-0.22	0.009*
6MWT predictors	CRP	–	–	–	-0.41	< 0.001*
	IL-6	–	–	–	-0.33	0.002*
	TNF- α	–	–	–	-0.30	0.004*

* $p < 0.05$ indicates statistical significance

ROC, receiver operating characteristic; FEV₁, forced expiratory volume in 1 second; 6MWT, Six-Minute Walk Test; AUC, area under curve; CI, confidence interval

following rehabilitation in patients with chronic respiratory diseases (15).

The mechanisms underlying improved lung function may involve several pathways. Enhanced respiratory muscle strength through targeted breathing exercises likely contributed to increased lung volumes and improved ventilation patterns. The observed improvements in FEV₁ and FVC suggest enhanced airway function and lung compliance, potentially resulting from reduced airway inflammation and improved respiratory mechanics (16). The structured aerobic training component may have enhanced cardiopulmonary efficiency, contributing to improved oxygen utilization and reduced ventilatory limitations during physical activity.

The significant reduction in inflammatory biomarkers (CRP, IL-6, TNF- α) following PR reflects the anti-inflammatory effects of appropriate regular exercise training. Physical exercise is known to reduce systemic inflammation through multiple mechanisms, including decreased production of pro-inflammatory cytokines and enhanced anti-inflammatory mediator release. In TB patients, this anti-inflammatory response may be particularly beneficial, as persistent inflammation contributes to long-term pulmonary complications including fibrosis and functional impairment (17).

The improvements in exercise capacity, as evidenced by enhanced 6MWT performance and oxygen saturation, likely resulted from combined cardiopulmonary adaptations. Enhanced oxygen delivery and utilization, improved respiratory muscle endurance, and reduced dyspnea perception contribute to better exercise tolerance. These physiological adaptations are particularly important for TB patients, who often experience significant functional limitations during recovery (18).

The observed beneficial changes in the respiratory microbiome, including reduced pathogenic bacterial colonization, suggest that PR may help restore microbial homeostasis. Regular physical activity has been associated with improved immune function and reduced susceptibility to respiratory infections (19). This finding is clinically relevant for TB patients, who remain at increased risk for secondary respiratory infections during treatment and recovery.

The strong correlations between inflammatory markers and functional improvements, along with the predictive value of these biomarkers, support their potential clinical utility. CRP emerged as

the strongest predictor of rehabilitation success, which may facilitate personalized treatment approaches and outcome monitoring in TB patients undergoing PR (20).

Study limitations include the relatively small sample size and absence of a control group, which limits causal inference. The completion rate of 50% (30 of 60 enrolled) highlights implementation challenges in resource-limited settings. Future studies should include larger, randomized controlled trials with extended follow-up to assess long-term benefits and optimal PR protocols for TB patients.

CONCLUSION

An 8-week structured PR program significantly improved lung function, exercise capacity, and reduced systemic inflammation in newly diagnosed TB patients. CRP demonstrated the strongest predictive value for rehabilitation outcomes, suggesting its potential utility as a biomarker for monitoring treatment response. These findings support the integration of PR into standard TB care protocols to help optimize patient recovery and reduce long-term complications.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

L.V.: conceptualization, investigation, writing—original draft preparation; S.R.: conceptualization, methodology, supervision, project administration, funding acquisition, writing—review & editing; V.M.: methodology, validation, resources, investigation, writing—review & editing; D.M.: software, validation, formal analysis, data curation, writing—

review & editing; R.S.: formal analysis, data curation, visualization, writing—review & editing; M.K.I.: investigation, writing—original draft preparation, visualization.

All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy and ethical restrictions related to patient confidentiality.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee (ECN: 1183/ICE/2023).

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study. All participants received detailed information about the study procedures, potential risks, and benefits before providing written consent to participate in this research.

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The Effect of Dohsa-hou on Reducing Attention Deficit and Hyperactivity in 6- to 12-Year-Old Children and Adolescents

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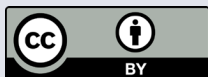
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ABSTRACT

OBJECTIVE To investigate the effect of Dohsa-hou relaxation on reducing attention deficit and hyperactivity.

METHODS Eighty children with attention-deficit/hyperactivity disorder (ADHD) aged 6-12 years were selected by the block randomization method and assigned to either an experimental group or a control group. The data collection tools included a demographic questionnaire, Conners' parent rating scale and Conners' (CPRS-RS) child attention deficit index.

RESULTS The mean score of the children's hyperactivity and attention deficit decreased after the intervention, which was statistically significant ($p < 0.001$). The children's hyperactivity scores were significantly different before and after the intervention in the two groups: a greater reduction was observed in the intervention group than in the control group. Although hyperactivity decreased in both groups, it was more pronounced in the intervention group ($p < 0.001$). The comparison of children's attention deficit scores before and after the intervention in the two groups also showed that Dohsa-hou treatment led to a more significant decrease in the scores of the intervention group ($p < 0.001$).

CONCLUSION Dohsa-hou exercises may lead to reduced hyperactivity and attention deficit in most children with ADHD.

KEYWORDS Dohsa-hou, hyperactivity, attention deficit, complementary treatment

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a neurobehavioral disorder affecting school-age children around the world. More than 50% of referrals to child and adolescent psychiatric clinics are attributed to ADHD, and 3-5% of school-age children are affected by this disorder (1, 2). This neurodevelopmental disorder is known to affect 8-12% of children worldwide (3). A study in the United States found that 10% of children had

ADHD and that 7% used ADHD medications (4). One study in Iran showed that 18% of students had ADHD (5). Other studies of ADHD in Iran have reported the prevalence of this disorder among school-age children to be 9%-15% (7). It is well-established that ADHD is often associated with other mental disorders. Research shows that 54-84% of children and adolescents with ADHD may also experience other disorders, and that some of these disorders may exacerbate their

behavioral and emotional problems (6).

This disorder has been discussed in numerous studies, as it has a variety of consequences for children and imposes heavy costs on the family and the community (8).

Although the rate of hyperactivity decreases with age, at least half of children with this disorder continue to exhibit symptoms in adulthood (9). Today, one of the most common treatments for ADHD is the use of medications, including the central nervous system stimulants (10); however, due to the heterogeneity of this disorder in children, medications may not be similarly effective for all children (11). Therefore, the use of medications alone cannot meet the therapeutic needs of all children with ADHD, and must be accompanied by other interventions (12).

Nervous tics are among complications of ADHD in children after taking medications. They may appear unexpectedly while the child is performing their school activities. Although these tics may seem worrying and frustrating, they do not physically damage the brain. Impacts of ADHD may also appear in other forms, such as excessive blinking, verbal tics (e.g., repeating words), clearing the throat, burping, and making unusual sounds. Illusions (e.g., seeing snakes, insects, and worms that do not really exist) are other complications of using ADHD medications (13).

Additionally, some children with ADHD experience severe behavioral changes or become frustrated or anxious. These complications are not usually dangerous, but they can impair the child's emotional stability. Long-term complications, on the other hand, threaten the health and normal life of a child with ADHD (14).

Dohsa-hou is a Japanese psychological rehabilitation method which was first applied by Naruse (1966) to improve motor problems in children with cerebral palsy. Naruse believed that disabilities associated with cerebral palsy were associated with physiological disorders and were also affected by mental activities (15). He explained that elements of cognitive processing are involved in motor control. In Dohsa-hou a solidarity between the physiological and psychological process of movement is achieved when the client desires to move a body part, strives toward that goal, and realizes the movement they intended (16). Exercise alone is not adequate for achieving the desired

mental health in the patient. Dohsa-hou is a client-centered method in which the therapist's role is facilitating the movements of clients which can help them experience positive changes. Overall, the role of the therapist is to take the necessary measures to help the patient achieve successful results (17). Dohsa-hou has two patterns of intervention approaches: relaxation (with the goal of easing tension in the body) and standing (with the goal of improving posture and movement) (18).

In the Dohsa-hou method, physical activity with the aim of learning and developing perceptual, sensory-motor and cognitive skills is performed. Dohsa-hou has also been enlisted in the treatment of many psychological problems such as cerebral palsy (19), depressive symptoms (15), Parkinson's disease (17), anxiety disorders (20), multiple sclerosis (21) and schizophrenia (22).

Ohno and Dadkhah (1999) conducted a study with three children with ADHD in Japan. Based on their final evaluation, communication, attention, concentration, and body awareness problems improved in these children after Dohsa-hou treatment (23). Naoki showed that Dohsa-hou helped ADHD patients to recognize their emotion and mood states and made them calm and stable (24). Also, with regulation of emotional arousal and expression, these patients were better able to establish good relationships with others (25). However, there are few reports of the effectiveness of the Dohsa-hou technique with children. Due to the importance of ADHD and the demonstrated effectiveness of Dohsa-hou technique, this study aimed to investigate the effect of this treatment on reducing ADHD in 6- to 12-year-old children and adolescents referred to a child psychiatric subspecialty clinic.

METHODS

Participants

This study was conducted using the block randomization method with an experimental and a control group in two stages: before and after the intervention.

The study population consisted of children and adolescents with ADHD who had been referred to a psychiatric subspecialty clinic in 2020. With a minimum reduction of 3 units in the hyperactivity score, a confidence factor of 95% and a test power of 80%, using G*Power software it

was determined that a sample of 38 people per group was required for the study. A total of 40

participants was recruited in each group.

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 \times [\sigma_1^2 + \sigma_2^2]}{[d]^2} = \frac{(1.96 + 0.84)^2 \times (4.17^2 + 5.14^2)}{(3)^2} \cong 38$$

Inclusion criteria included age 6-12 years, parental consent to participate in the research, a diagnosis of ADHD based on the DSM5 criteria and the approval of a child and adolescent psychiatry subspecialist. The exclusion criteria included lack of interest in continuing to participate in the research, presence of obvious physical or movement disorders, a history of seizures and use of seizure-related drugs. Participants were selected by simple random sampling and assigned to one of two groups using the block randomization method (Figure 1).

Instruments

The data collection tools used in this study included three questionnaires: a demographic and medical information questionnaire, Conners' hyperactivity index, and Conners' child attention deficit index. The demographic information questionnaire included information such as birth order, parents' educational level, parents' occupation, duration of illness, types and amounts of medications used, economic status, family history of ADHD, and ADHD medications used.

The 48-item Conners' parent rating scale (CPRS-48) was used in this study. This scale ex-

amines the child's behavior on a four-point Likert scale (from "not at all" to "extremely") in six areas: conduct problems, psychosomatic problems, learning problems, hyperactivity/impulsivity problems, anxiety problems, and hyperactivity index. In the CPRS-48, Conners does not suggest a specific cutoff point for identifying clinically significant problems; however, scores above 60 were considered to be "above average". In this study a cutoff point of 60 was used for screening hyperactive children as used in a study by Khooshabi and colleagues (2006). In that study, an alpha coefficient of 0.91 was reported for the questionnaire (26).

Children's attention deficit or inattention was assessed using the revised Conners' 26-item parent rating scale. This scale examines parents' rating of a child's behavior using a four-point Likert scale (from "not at all true" to "absolutely true"). The total score range is from 26 to 104. If a child obtains a score above 34, it indicates an attention deficit: the higher the score, the greater the level of the child's disorder. Alizadeh et al. reported the reliability of this scale to be 0.90. The reliability of the questionnaire was estimated to be 0.85 (27).

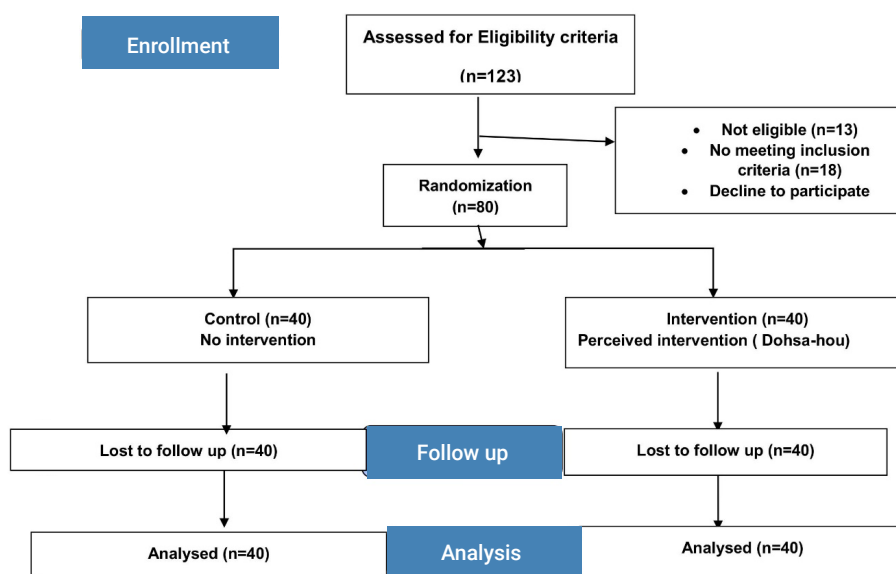


Figure 1. Consort diagram of Dohsa-hou treatment

Intervention (Dohsa-hou technique)

Patients in the experimental group underwent Dohsa-hou psychomotor relaxation therapy for eleven 45-minute weekly sessions (Table 1). In each session, the first 15 minutes of counseling focused on establishing a relationship with the patient. The next 25 minutes of the session were devoted to Dohsa-hou psychomotor relaxation exercises, and the final 5 minutes was allocated to relaxation. The basic steps (28) of this technique are as follows: (a) Relaxation (relaxing the body and the mind). This step is performed based on “Kukan-no-hineri” techniques; (b) Movements of arms, hands, and legs. This step helps the patient perform basic movements of arms, hands, and legs using a variety of techniques, including the “Ude-age” technique; and (c) The “Tate-kei” technique. “Tate-kei” refers to placing the body in a straight vertical line. By aligning the vertical axis of the body, different parts of the body are placed in a straight line perpendicular to the ground. The Dohsa-hou relaxation was performed in cross-legged sitting, kneeling, half-kneeling, standing, and walking positions.

Before starting treatment, the researcher provided a full explanation of the training to the patients and their families. After the questionnaires were completed by the patients' families

(both experimental and control groups), the intervention group received training for 11 sessions. The control group received the standard treatment. At the end of the sessions, the questionnaires were completed again by the patients' families, and the results were compared with the previous results. Dohsa-hou was provided for the control group during a follow-up period.

Ethical considerations

Patients participated in this study after their parents or legal guardians had voluntarily signed a written informed consent form. It was explained to them that they could withdraw from the study at any time. Additionally, they were assured of the confidentiality of their information and the accuracy and reliability of the information obtained during data collection and analysis. After the explanation of the research objectives and methods, the patients were invited to participate in the study. They were also informed that their participation in the study did not prevent them from receiving routine hospital services. The present study was ethically approved (code: ir.QUMS.REC.1399.048).

Data was analyzed using SPSS version 24. Frequencies, percentages, means, and standard deviations (SD) were used to describe the data.

Table 1. Summary of the Dohsa-hou relaxation sessions

Sessions	Techniques	Duration of movements
Session 1-3	1. Moving the hands up and down in a lying position (Ude-age)	7 min
	2. Moving the shoulders up and down (Kata-age)	7 min
	3. Moving the shoulders and the upper body back and forth in a lying position (Kukan-no-hineri)	7 min
	4. Pulling the shoulders back (Se-so-ra-se)	7 min
Session 4-6	1. Moving the shoulders and the upper body back and forth in a lying position (Kukan-no-hineri)	7 min
	2. Pulling the shoulders back (Se-so-ra-se)	7 min
	3. Moving the shoulders up and down (Kata-age)	7 min
	4. Moving the hands up and down in a lying position (Ude-age)	7 min
Session 7-9	1. Moving the shoulders and the upper body back and forth in a lying position (Kukan-no-hineri)	7 min
	2. Standing on two knees and moving the hips (Hizatachi)	7 min
	3. Standing on one knee and moving the hips (Kata-hizatachi)	7 min
Session 10-11	4. Opening and closing the chest and shoulders (Mune-hiraki)	7 min
	1. Pulling the upper body forward, with the sides in a sitting position (Zai)	7 min
	2. Kneeling and moving the hips (Hizatachi)	7 min
	3. Taking a knee and moving the hips (Kata-hizatachi)	7 min
	4. Opening and closing the chest and shoulders (Mune-hiraki)	7 min

Data were analyzed using the Mann-Whitney U test, Fisher's exact test, the independent t-test, the paired t-test, the chi-square test, and ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS

Out of 123 children who were initially contacted, 80 children agreed to participate in the study and were assigned to one of two groups, either intervention or control. The patients were examined regarding quantitative and qualitative demographic variables, including age, gender, living status, place of residence, medication use, family history of mental illness, duration of hospitalization, frequency of hospitalization, and type of disease. Some variables such as marital status, education level, occupational status, and income level referred to the parents. (Table 2). There were no significant differences between the two groups in terms of these variables ($p > 0.05$).

Children's hyperactivity decreased statistically significantly more in the intervention group than in the control group, and this difference was

statistically significant, also the results showed demonstrating that the Dohsa-hou method resulted in a significant decrease in the attention disorder of the children in the intervention group (Table 3).

DISCUSSION

The findings of the present study regarding the effect of Dohsa-hou treatment on hyperactivity and attention deficit in 6- to 12-year-old children and adolescents who had been referred to a clinic are consistent with the findings of a study by Yazdkhasti and Shabazi, which investigated the effect of Dohsa-hou treatment on reducing ADHD symptoms and increasing social skills in 6- to 12-year-old students with ADHD (29). The present study results demonstrated that using mental rehabilitation and building will-power in children with ADHD can decrease their hyperactivity and attention-deficit symptoms. These findings are comparable to a study by Adachi that showed Dohsa-hou decreases situational anxiety and improves cognitive task per-

Table 2. Demographic characteristics of children in the study

	Control (n = 40) n (%)	Intervention (n = 40) n (%)	Total (N = 80) N (%)	p-value
Age (years), mean±SD	8.68±2.28	9.10±2.07	8.88±2.17	0.386
ADHD diagnosis time (year), mean±SD	3.4±1.83	4.6±2.59	4±2.31	0.019
Gender				0.809
Male	12 (30.0)	13 (32.5)	25 (31.3)	
Female	28 (70.0)	27 (67.5)	55 (68.8)	
Grade				0.292
Pre-school	8 (20.0)	5 (12.5)	13 (16.3)	
Grade 1	8 (20.0)	5 (12.5)	13 (16.3)	
Grade 2	5 (12.5)	7 (17.5)	12 (15.0)	
Grade 3	5 (12.5)	4 (10.0)	9 (11.3)	
Grade 4	3 (7.5)	10 (25.0)	13 (16.3)	
Grade 5	3 (7.5)	5 (12.5)	8 (10.0)	
Grade 6	8 (20.0)	4 (10.0)	12 (15.0)	
Family birth order				0.335
1	20 (50.0)	27 (67.5)	47 (58.8)	
2	15 (37.5)	10 (25.0)	25 (31.3)	
3	4 (10.0)	3 (7.5)	7 (8.8)	
4	1 (2.5)	0 (0.0)	1 (1.3)	
Family history of hyperactivity				0.809
No	27 (67.5)	28 (70.0)	55 (68.8)	
Yes	13 (32.5)	12 (30.0)	25 (31.3)	
Medication use				0.060
No	9 (22.5)	3 (7.5)	12 (15.0)	
Yes	31 (77.5)	37 (92.5)	68 (85.0)	

ADHD, Attention-deficit/hyperactivity disorder

Table 3. Comparison of mean attention deficit and hyperactivity disorder between the intervention and control groups

	Control (n = 40) Mean±SD	Intervention (n = 40) Mean±SD	p-value ^a	p-value ^b
Children's mean hyperactivity score before the intervention	73.18±27.62	90.15±21.75	0.003	< 0.001
Children's mean hyperactivity score after the intervention	51.35±27.31	18.58±16.87	< 0.001	
Children's attention deficit score before the intervention	76.78±18.28	87.45±15.77	0.007	< 0.001
Children's attention deficit score after the intervention	64.43±18.83	38.35±14.68	> 0.001	

^aIndependent t-test, ^bRepeated measurement

formance (30). Although more studies are needed in this area, families and special education experts are recommended to use this method to improve the social interactions and hyperactivity symptoms of these children. In terms of subject matter, both the Adachi study and the present study investigated the effect of the Dohsa-hou technique on ADHD in students. In both studies, psycho-physical tension was minimized by Dohsa-hou, especially the relaxation techniques. This demonstrates that self-control over the movement of one's body is directly related to one's self-control over the mind (28).

Dohsa-hou helped patients recognize their emotions and moods and made them calm and stable. By learning to control their emotional arousal and expression, patients were able to communicate with others (24).

Dehkordi et al. explained that changes were evident in body posture and in efforts to use and control the body (19). Based on the Ohno and Dadkhah assessment, communication, attention, concentration, and body awareness problems improved in children after treatment (23). Dohsa-hou promotes emotional stability and increases social activity. When we want to move our body, our physical perception takes place according to our intention and will. This process of mental movement is aimed at matching the movement pattern of the body with the movement pattern of the will. This is the process of self-regulation of a person's movements by himself (31).

After Dohsa-hou training, parents' knowledge of ADHD and about their child's disorder and behavioral problems significantly increased (32); in other words, the parents' increased under-

standing of ADHD lead to changes in their attitude. Through Dohsa-hou training, the parents learned to associate their children's behaviors to ADHD and not consider them to be intentional.

Additionally, Nejatifar and Babarabi investigated the effect of the Dohsa-hou technique and showed that the technique, as a psychological rehabilitation treatment, was effective in improving the quality of life of children with cerebral palsy (33). Using this method, the child increases their ability to control their movements, and, as a result, their self-confidence also increases. Reducing impulsivity and increasing attention leads to an increase in law-abidingness, a decrease in aggression, an increase in the desire to communicate as well as improved social skills and social adequacy.

Based on current findings, it can be stated that the Dohsa-hou technique, a Japanese psychological rehabilitation method, is highly effective for the treatment of hyperactivity. In spite of various sociocultural characteristics specific to Iranian society, this method can be applied to motivate researchers to perform clinical activities on different disorders, as it has no cultural bias. This technique can reduce sympathetic activities and stress, which subsequently activates the parasympathetic system in individuals with ADHD (34).

Recently, the Dohsa-hou technique has been applied as a complementary approach, because it improves the attention and effort of ADHD patients to relax their body, making them more conscious of their body and helping them understand it better. With Dohsa-hou exercises, a person does purposeful activities and performs specific movement patterns. During exercises, ADHD patients begin to understand their body

and feel their body movements. These exercises focus on the person's feeling of actual movement, their mental image of their body, their experience of the environment and the outside world, and their feeling of presence and being, all of which are essential in psychotherapy (35).

Based on the results of this study, our research hypothesis was confirmed and the effects of Dohsa-hou technique on the target variables were found to be significant. The Dohsa-hou technique can lead to significantly increased attention in children with ADHD. Dohsa-hou training increases the person's attention control and consciousness of the body's senses. The therapist, through verbal encouragement that complements the Dohsa-hou treatment process, constantly gives feedback to children and encourages and motivates them to persevere and perform their activities, increasing their concentration on the activities.

Konno believed that the Dohsa-hou technique stabilizes patients emotionally and increases their social activities (36). In these exercises, physical contact and modeling improve the patients' concentration, eye contact, attention, and body awareness, change their behavioral patterns, build willpower, increase effort and activity in line with the process (effort, willpower, and movement), improve self-awareness and social relationships, and help the patients cope with different situations. The Dohsa-hou technique can also significantly decrease hyperactivity in children with ADHD.

The effect of the Dohsa-hou technique on hyperactivity has not yet been adequately investigated in children. However, it can be argued that when a person aims to move his/her body, the physical perception depends on his/her intention and willpower; if the effort made is adequate for moving the body, the desired movement is perceived and takes place. It is a purposeful mental process which matches a known motor pattern in the body with a voluntary motor pattern; this refers to the self-regulation of body movements (self-governance). As a result of self-regulation of body movements, the person develops the ability to control sudden motor movements and stop emotional outbursts (37). In the Dohsa-hou technique, change of breathing pattern into a calmer and more controlled one can neutralize

the attention deficit response by creating a relaxed state in the individual. It also allows the person to focus on the body and breathing rather than thinking negative thoughts (21).

The Dohsa-hou technique influences the level of effort in individuals, and to complete the learning process, their motivation needs to increase. Self-management skills help children with disabilities to establish social interactions because with the Dohsa-hou technique children feel in control of their body and physical activities, as a result of which their confidence and social relationships increase. Moreover, increased confidence due to changes in the child's body image increases their tendency to communicate with others. In other words, by decreasing impulsivity and increasing attention through Dohsa-hou exercises, the child's agreeability is increased, while their aggression is decreased due to increased relaxation. In addition, the children with ADHD gain an increased tendency to communicate with others, express emotions, and control impulses. Dohsa-hou exercises increase their peer communication and improve their social competence. Dohsa-hou as a form of psychological rehabilitation can be used as a complementary approach alongside other therapeutic methods. Therefore, application of this useful and non-pharmacological method is recommended for children with ADHD; however, additional studies are recommended to determine its effectiveness in different age groups and patients with other disorders.

A limitation of this study was the limited age range of patients due to hospital conditions which influenced the generalizability of our findings. Therefore, further research is suggested on a larger sample size and without any age restrictions. It is also suggested to consider long-term follow-ups in subsequent studies, as that can help understanding of the long-term effects of treatment in patients.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M.A.: conceptualization, formal analysis, investigation, methodology, resources, writing—original draft; R.Z.: conceptualization, formal analysis, funding acquisition, Investigation, methodology, project administration, resources, supervision, validation, writing—original draft, writing—review & editing; B.R.: conceptualization, methodology, resources, validation; A.J.: conceptualization, data curation, formal analysis, methodology, resources, software, supervision, validation.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT,

The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. All procedures performed in studies involving human participants were approved by QUMS ethic committee (ir.QUMS.REC.1399.048).

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study

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Antimutagenic Potential of *Piper sarmentosum* Leaf Extracts: Insights from Culinary Preparation

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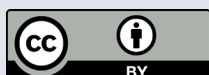
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ABSTRACT

OBJECTIVE *Piper sarmentosum* Roxb., a traditional vegetable in Lanna and Thai cuisine, is valued for its flavor but its health-promoting properties after cooking remain unclear. This study investigated the phytochemical profiles, antioxidant capacity, and mutagenicity of its leaf extracts prepared to mimic typical culinary practices.

METHODS Three *P. sarmentosum* extracts were prepared: an ethanolic extract of uncooked leaves (PEE), a hot water extract simulating curry broth (PHWE), and an ethanolic extract of boiled leaf residues representing the cooked leaves in curry (PREE). Their chemical composition, antioxidant activity, and antimutagenic effects were evaluated.

RESULTS The extracts showed distinct phytochemical patterns. PEE and PREE were rich in phenolics and flavonoids, yielding strong antioxidant activity in ABTS and FRAP assays. PHWE contained higher levels of condensed tannins, reflecting the extraction of water-soluble compounds. None of the extracts showed mutagenicity in the Salmonella assay. Notably, PEE and PREE displayed significant antimutagenic activity against aflatoxin B₁ (AFB₁) in *Salmonella typhimurium* strains TA98 and TA100 with metabolic activation.

CONCLUSIONS *P. sarmentosum* leaves retain considerable antioxidant and antimutagenic potential after cooking, highlighting their value as a safe and functional food ingredient with potential health benefits.

KEYWORDS *P. sarmentosum*, wild betel, Salmonella mutation assay, antioxidant, antimutagenicity

INTRODUCTION

Piper sarmentosum Roxb., commonly known as “wild betel” or “cha-plu” in Thai, is a perennial creeping herb native to Southeast Asia. It is valued both as a traditional medicinal plant and as a culinary ingredient, particularly in Thai and Lanna cuisine (1). The fresh leaves are often consumed raw in traditional snacks such as “Miang

Kham” or incorporated into cooked dishes such as “Kaeng Khae”, a northern Thai curry (Figure 1).

Phytochemical studies have shown that *P. sarmentosum* contains diverse bioactive constituents, including phenolic acids, flavonoids, tannins, xanthophylls, and essential oils (2). These compounds have been associated with a wide range of biological activities, including antimicrobial,

anti-inflammatory, antioxidant, and anticancer properties (3-5). The leaves, in particular, are a rich source of natural antioxidants owing to their high levels of flavonoids and related phenolic compounds (6, 7). Notable flavonoids such as quercetin, naringenin, myricetin, and apigenin (5) are well documented for their anticancer potential through modulation of multiple molecular pathways, including apoptosis, autophagy, NF- κ B, JAK/STAT, and tumor suppressor signaling (8).

Beyond their antioxidant potential, extracts of *P. sarmentosum* leaves have demonstrated therapeutic properties such as anti-inflammatory, antimicrobial, and cardioprotective effects (4, 9-11). For example, methanolic extracts have been shown to protect against stress-induced gastric ulcers via antioxidant and anti-inflammatory mechanisms (12), while aqueous extracts exhibit *in-vivo* anti-nociceptive and anti-inflammatory effects (13) and *in-vitro* cytoprotective activity against oxidative stress (14). Importantly, aqueous extracts have also been reported to be non-mutagenic, supporting the safety of *P. sarmentosum*

for dietary and medicinal use (15).

Despite increasing interest in natural antioxidants and functional foods, limited information is available on the phytochemical composition and biological activities of *P. sarmentosum* following traditional cooking processes. In everyday cuisine, the leaves are often boiled at 100 °C for 15-30 minutes when prepared in soups or curries. To address this knowledge gap, the present study simulated culinary preparation to obtain three types of extracts: PEE (ethanolic extract of uncooked leaves), PHWE (hot water extract simulating curry broth), and PREE (ethanolic extract of boiled leaf residues representing the cooked leaves in curry). These extracts were analyzed for their phytochemical composition, antioxidant activity, mutagenicity, and antimutagenicity, providing new insights into the functional properties of *P. sarmentosum* and its potential applications in food and health product development. A summarized workflow diagram illustrating the experimental process is presented in Figure 2.



Figure 1. Photographs of *P. sarmentosum* leaves (A), the northern Thai snack Miang Kham (B), and the northern Thai curry Kaeng Khae (C).

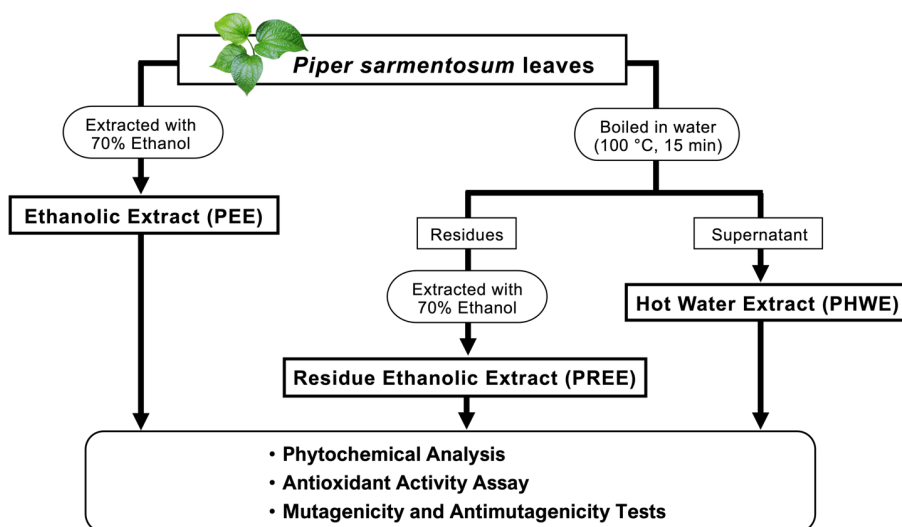


Figure 2. Schematic overview of the experimental workflow in this study

METHODS

Preparation of *P. sarmentosum* Roxb. extracts

Fresh *P. sarmentosum* leaves were collected from Terdwalai Farm, Chiang Mai, Thailand, and deposited at the Faculty of Pharmacy, Chiang Mai University (voucher specimen no. 0023333). The leaves were washed, chopped, and dried at 60°C in a hot-air oven, then ground into powder. This powder was extracted with 70% ethanol (1 g : 10 mL), filtered, concentrated, and freeze-dried to obtain the ethanolic extract (PEE).

To mimic traditional cooking, fresh leaves were boiled in an open-lid stainless-steel vessel with boiling water for 15 min and the temperature was maintained using a thermostatically controlled hot plate. The decoction was filtered and freeze-dried to obtain the hot-water extract (PHWE). The boiled leaf residues were then oven-dried at 60°C, ground into powder, and extracted with 70% ethanol (1 g : 10 mL). The extract was concentrated and freeze-dried to obtain the residue ethanolic extract (PREE). All extracts (PEE, PHWE, and PREE), collectively referred to as *P. sarmentosum* leaf extracts (PSLEs), were stored at -20°C until use.

Total phenolic content

Total phenolic content of PSLEs was determined using a modified Folin-Ciocalteu colorimetric assay (16). Extracts were mixed with Folin-Ciocalteu reagent and incubated at room temperature for 10 min. Sodium carbonate was then added, and the mixture was incubated at 45°C for 15 min. Absorbance was measured at 765 nm, and phenolic content was calculated from a gallic acid calibration curve, expressed as mg gallic acid equivalents per gram of extract (mg GAE/g extract).

Total flavonoid content

Total flavonoid content of PSLEs was determined using a modified aluminum chloride colorimetric assay (16). Extracts were mixed with 5% sodium nitrite and 10% aluminum chloride, incubated at room temperature for 20 min, followed by sodium hydroxide addition and 5 min further incubation. Absorbance was measured at 532 nm, and flavonoid content was calculated from a catechin standard curve, expressed as mg catechin equivalents per gram of extract (mg CE/g extract).

Total chlorophyll and carotenoid determination

Total chlorophyll and carotenoids in PSLEs were determined using a modified acetone extraction method (17). Extracts were homogenized in 80% acetone and incubated at 4°C for 3 h, followed by centrifugation at 9,000 rpm and 4°C for 5 min. Absorbance of the supernatants was measured at 480, 510, 645, and 663 nm. Concentrations were calculated using following equations and expressed as mg per gram of extract (mg/g extract).

$$\text{Total chlorophyll (mg/L)} = 20.2(A_{645}) + 8.02(A_{663})$$

$$\text{Total carotenoids (mg/L)} = 7.6(A_{480}) - 1.49(A_{510})$$

Tannins determination

Hydrolysable tannins were quantified by acid hydrolysis. PSLEs were mixed with sulfuric acid and incubated at 85°C for 20 h. After centrifugation (3,000 × g, 5 min), the supernatant was adjusted to pH 5.5 with ethanolamine and ammonium acetate, incubated at 4°C for 48 h, then reacted with potassium iodate at 30°C for 50 min. Absorbance was measured at 525 nm, and results were expressed as mg methyl gallate equivalents per gram of extract (mg MGE/g extract).

Condensed tannins were determined using a modified acidified vanillin assay (18). PSLEs were mixed with vanillin reagent (vanillin in methanol with concentrated HCl), incubated at 30°C for 30 min, and absorbance was recorded at 500 nm. Values were expressed as mg catechin equivalents per gram of extract (mg CE/g extract).

High-performance liquid chromatography (HPLC) analysis

HPLC profiling of PSLEs was performed on an Agilent system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax Eclipse Plus C18 column (4.6 × 250 mm, 5 µm) and a photodiode array detector (19). For phenolics, the mobile phase comprised 3% acetic acid in water (A) and methanol (B). A gradient from 90% to 10% A was applied over 50 min, followed by 10 min re-equilibration at 90% A/10% B. The injection volume was 10 µL, flow rate 1 mL/min, and detection at 280 nm. Compounds were identified by retention time and spectral matching, confirmed by co-injection (spiking) with authentic standards, and quantified against calibration curves.

For flavonoids, the mobile phase was 1% acetic acid in water (A) and 1% acetic acid in methanol (B). Elution was run from 90% to 0% A over 40 min, followed by 5 min re-equilibration (90% A/10% B). Detection was at 280 and 329 nm, with peak identification and quantification performed as described above.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

Antioxidant capacity of PSLEs was determined by the ABTS radical scavenging assay. Extracts were mixed with ABTS radical working solution and incubated at room temperature in the dark for 6 min. Absorbance was measured at 734 nm, with Trolox as the standard. Results are expressed as inhibitory concentration at 50% (IC₅₀) values.

Ferric reducing antioxidant power (FRAP) assay

FRAP was measured using a reagent containing 2,4,6-tripyridyl-s-triazine and 20 mM ferric chloride in sodium acetate buffer (pH 3.6). Extracts were incubated with the reagent at room temperature for 4 min, and absorbance was read at 593 nm. Ferrous sulfate was used to generate a standard curve, and antioxidant activity was expressed as mg of ferrous sulfate equivalents per milliliter (mg FeSO₄/mL), with Trolox as a positive control.

Salmonella mutation assay

The mutagenic potential of PSLEs was evaluated using the *Salmonella typhimurium* strains TA98 and TA100, with and without metabolic activation ($\pm$ S9). Test extracts were mixed with either the S9 mix (+S9, liver fraction and cofactors) or sodium phosphate buffer (–S9), followed by addition of the bacterial culture. After 20 min incubation at 37°C with shaking, the mixture was combined with top agar containing L-histidine and D-biotin and overlaid on minimal glucose agar plates. Plates were incubated at 37°C for 48 h, and revertant colonies were counted. DMSO served as the negative control for PEE and PREE, while distilled water was used for PHWE. Positive controls were 2-aminoanthracene (2-AA) for +S9 and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) for –S9. The mutagenic index (MI) was calculated. Extracts with MI higher than 2 were considered potentially mutagenic.

Antimutagenicity was assessed against aflatoxin B₁ (AFB₁; 25 ng/plate) in the presence of S9. For –S9 conditions, AF-2 (50 ng/plate) was used with TA98 and sodium azide (NaN₃; 1000 ng/plate) with TA100. PSLEs were mixed with the mutagens, followed by the addition of bacteria and either S9 mix (+S9) or buffer (–S9). After 20 min incubation at 37°C, the mixtures were plated as above. Revertant colonies were counted after 48 h, and antimutagenic activity was expressed as the percentage inhibition relative to the mutagen control. The degree of antimutagenicity was classified according to the criteria shown in Table 1.

Statistical analysis

The data are reported as mean $\pm$ SD, except for the results obtained from mutagenicity in the *Salmonella* mutation assay, which was expressed as mean $\pm$ SEM values and performed in three independent experiments. The statistical significance of differences between groups was analyzed by one-way analysis of variance (ANOVA) and Tukey's HSD in GraphPad Prism software version 9.5.1, and $p < 0.05$ was considered statistically significant.

RESULTS

Appearance, yield, and phytochemical composition of *P. sarmentosum* leaf extracts

The characteristics of the PEE, PHWE, and PREE are summarized in Table 2. PEE and PREE were obtained as dark brown powders, whereas PHWE was black, reflecting differences in solvent polarity and compound solubility. Among the extracts, PEE and PHWE showed higher yields compared to PREE, consistent with the sequential extraction process in which water removed a portion of soluble phytochemicals before subsequent ethanol extraction of the residue.

Phytochemical profiling revealed that PEE contained the highest total phenolic content

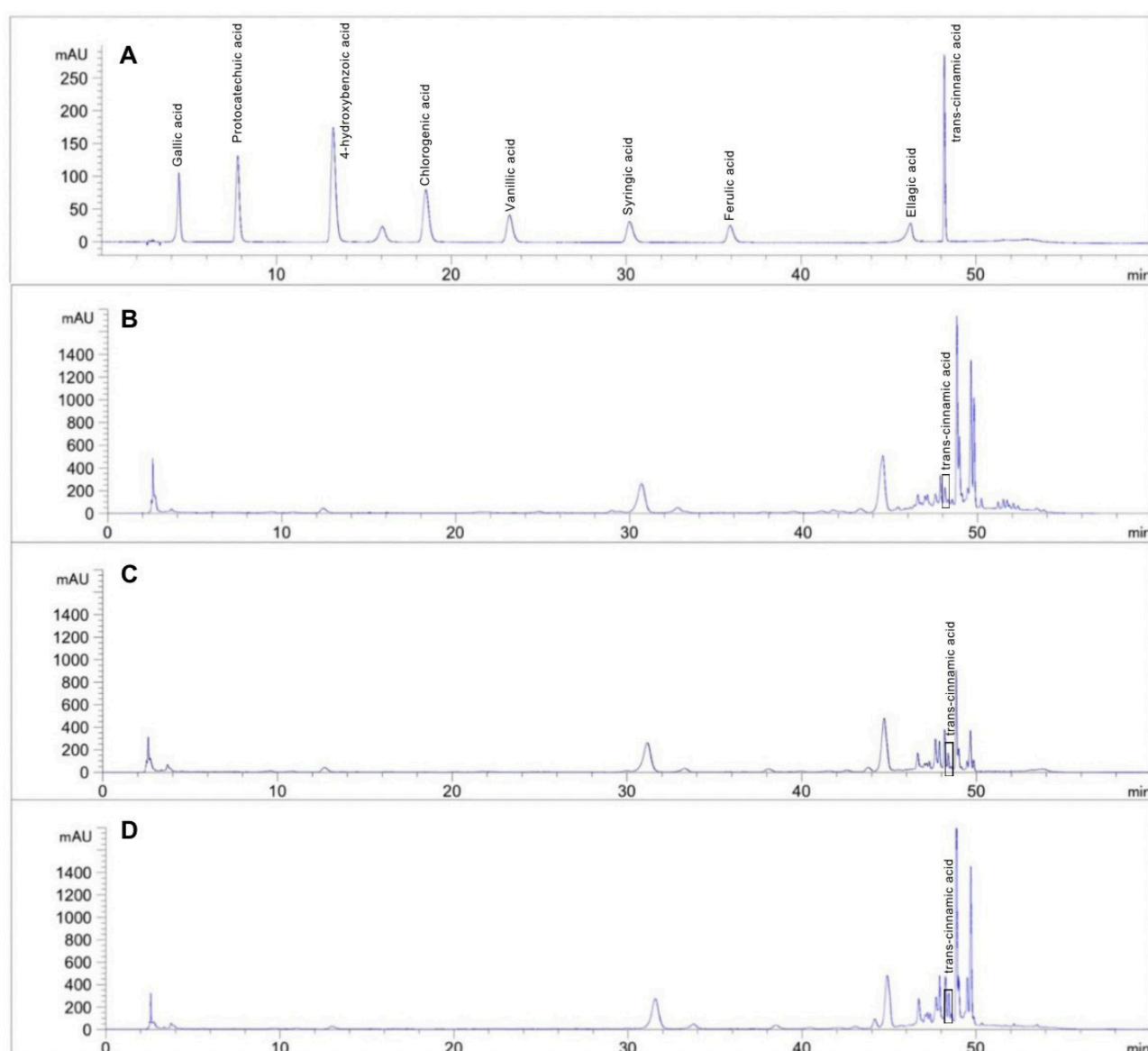
Table 1. Classification of antimutagenicity efficacy

% inhibition	Antimutagenicity
More than 90%	Very strong antimutagenicity
71-90%	Strong antimutagenicity
51-70%	Moderate antimutagenicity
31-50%	Mild antimutagenicity
30-0%	No antimutagenicity
Less than -30%	Co-mutagenicity

Table 2. Appearance, yield and phytochemical composition of PSLEs

Parameter	PEE	PHWE	PREE
Color of extract powder	Dark brown	Black	Dark brown
Yield (%w/w of fresh leaves)	3.77±1.00	3.26±0.70	1.76±0.20
Total phenolic content (mg GAE/g extract)	51.51±15.67	41.14±7.55	48.12±6.24
Total flavonoid content (mg CE/g extract)	14.25±2.20	4.10±0.44	12.35±2.11
Chlorophylls (mg/g extract)	7.86±1.69	0.25±0.03	11.27±0.73
Carotenoids (mg/g extract)	2.44±0.63	0.17±0.02	3.58±0.26
Hydrolysable tannins (mg MGE/g extract)	1.50±0.52	0.48±0.10	1.64±0.17
Condensed tannins (mg CE/g extract)	23.16±1.93	133.12±8.24	41.52±2.09
Trans-cinnamic acid (mg/g extract)	0.32±0.00	0.62±0.00	0.72±0.00
Apigenin (mg/g extract)	3.59±0.14	0.45±0.02	0.22±0.01

GAE, gallic acid equivalent; CE, catechin equivalent; MGE, methyl gallate equivalent

**Figure 3.** HPLC chromatograms of reference standard phenolic acids (A), PEE (B), PHWE (C), and PREE (D). The trans-cinnamic acid peak is observed at a retention time of 48.3 min.

(TPC) and total flavonoid content (TFC), followed by PREE, while PHWE exhibited the lowest values. PREE was particularly rich in chlorophylls and carotenoids, whereas PHWE showed the greatest amount of condensed tannins, consistent with the water solubility of these compounds.

HPLC analysis of phenolic acids (Figure 3) revealed compositional differences among the extracts. Common peaks were observed at retention times of approximately 31 and 45 min across all samples, suggesting the presence of shared hydrophilic constituents. However, peaks at approximately 49–50 min were more prominent in PEE and PREE, indicating that these extracts contained higher levels of hydrophobic compounds. Similarly, HPLC analysis of flavonoids (Figure 4) showed compositional differences among the

extracts, with common peaks observed at retention times of approximately 24, 30, and 38 min across all samples. Peaks at approximately 38–39 min were more prominent in PEE and PREE. Further quantitative analysis revealed that PREE contained the highest trans-cinnamic acid content (0.72 ± 0.00 mg/g extract), whereas PEE was the richest source of apigenin (3.59 ± 0.14 mg/g extract), far exceeding levels in PHWE and PREE.

Antioxidant activities of *P. sarmentosum* leaf extracts

The antioxidant capacities of PSLEs were assessed using ABTS radical scavenging and FRAP assays, with Trolox as a reference standard (Table 3). Among the extracts, PEE exhibited the strongest ABTS scavenging activity ($IC_{50} = 31.9 \pm 4.4$ mg/mL),

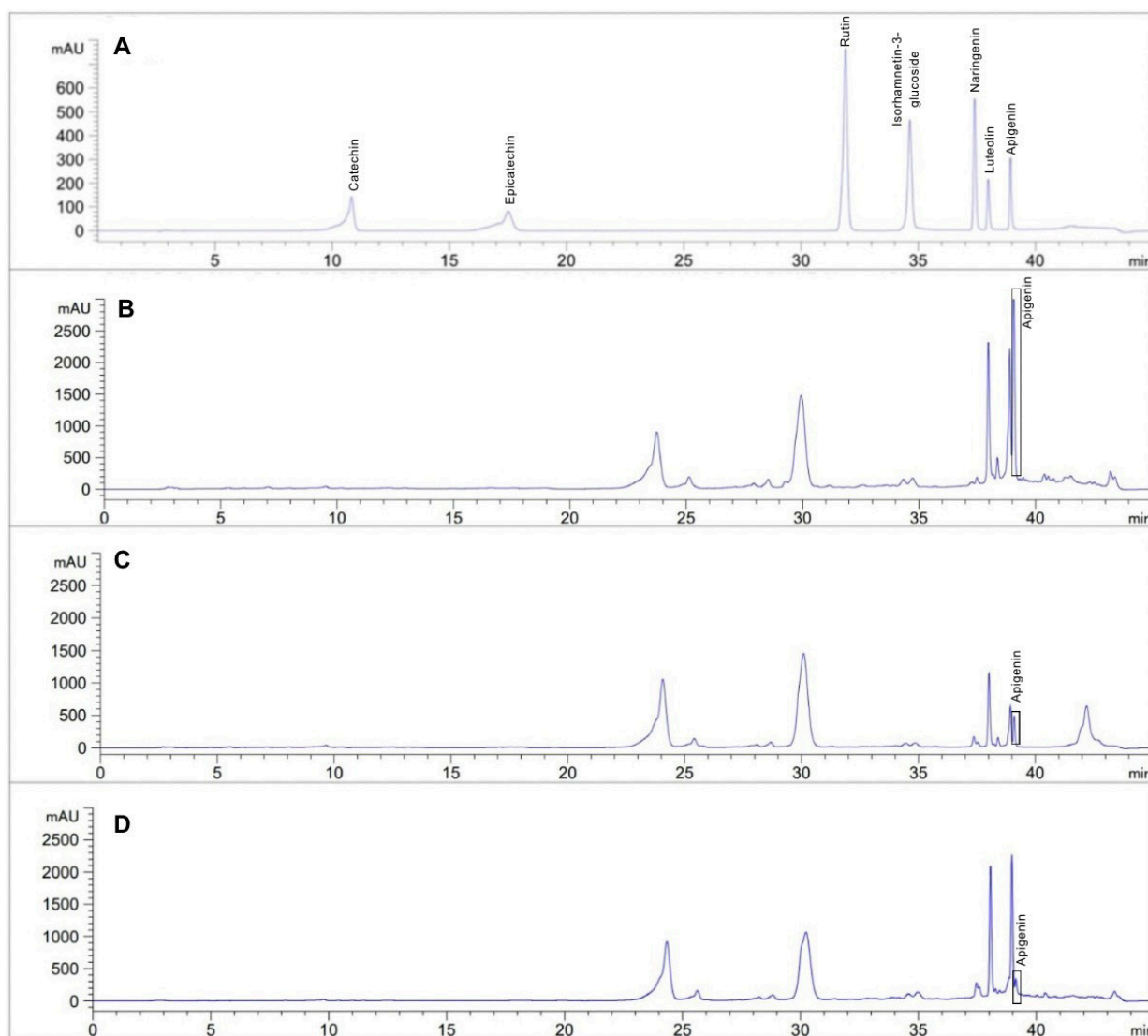


Figure 4. HPLC chromatograms of reference standard flavonoids (A), PEE (B), PHWE (C), and PREE (D). The apigenin peak is observed at a retention time of 38.9 min.

whereas PHWE showed the weakest effect. This trend was consistent with the phytochemical profiles, as extracts with higher phenolic and flavonoid contents (PEE and PREE) displayed stronger antioxidant activities.

In agreement, FRAP analysis revealed that PEE possessed the highest reducing power (0.12 ± 0.03 mg FeSO₄/mL), followed by PREE and PHWE. These findings suggest that phenolic and flavonoid constituents contribute significantly to the antioxidant potential of PSLEs.

Mutagenicity and antimutagenicity of *P. sarmentosum* leaf extracts

The mutagenic potential of PSLEs was assessed using the Salmonella mutation assay (Figure 5). None of the extracts (PEE, PHWE, PREE) produced a significant increase in revertant colonies in strains TA98 or TA100, either in the presence (+S9) or absence (-S9) of metabolic activation. All mutagenic indices remained below 2.0, indicating no detectable mutagenic activity. The larger standard deviation observed in the mutagenicity results for TA98 (Figures 5A and 5B) but not for

Table 3. Antioxidant capacities of PSLEs

Extract	ABTS	FRAP*
	IC ₅₀ (mg/mL)	(mg FeSO ₄ /mL)
Trolox	0.35±0.01	0.14±0.03
PEE	31.90±4.36	0.12±0.03
PHWE	44.67±4.67	0.08±0.02
PREE	34.70±10.30	0.10±0.03

*FRAP values represent activity equivalent to 0.05 mg/mL Trolox and 1 mg/mL PSLEs.

TA100 (Figures 5C and 5D) can be attributed to the lower baseline revertant counts typical of TA98 compared to TA100, resulting in greater relative variability.

Antimutagenicity testing against AFB₁-induced mutagenesis in the presence of metabolic activation (+S9) showed that both PEE and PREE significantly suppressed revertant colony formation in a clear dose-dependent manner, with inhibition consistently above 30% at all tested concentrations (Figure 6). At concentrations of 1,000–5,000 µg/plate, both extracts exhibited marked antimutagenic activity exceeding 70%, indicating strong protective potential. At the

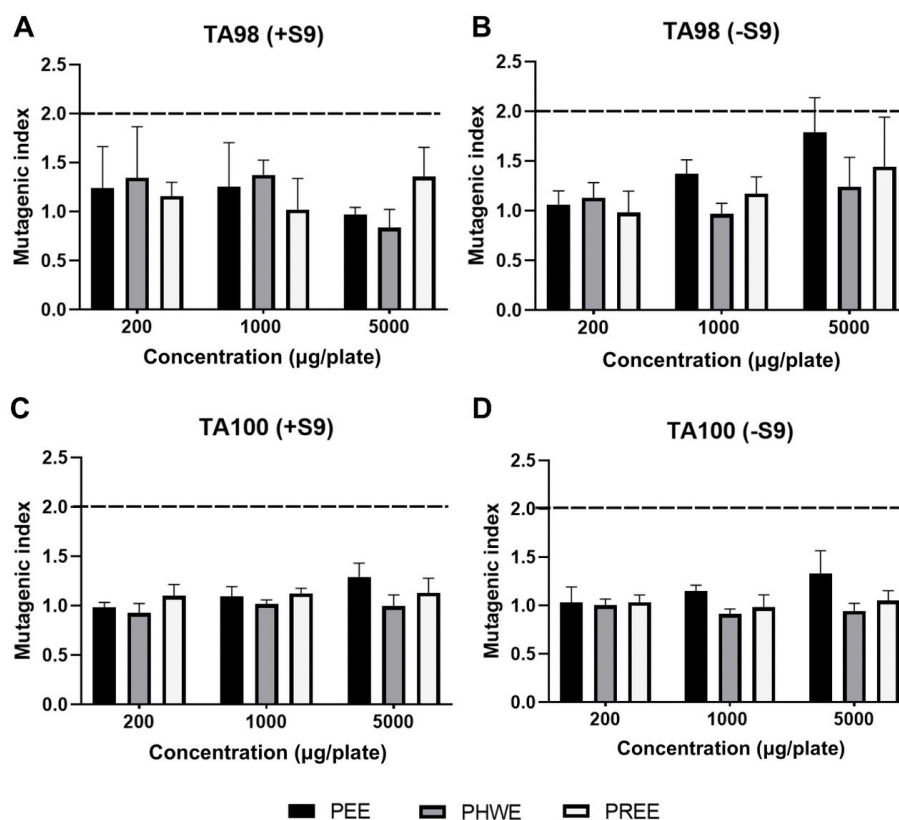


Figure 5. Mutagenicity of PSLEs in *S. typhimurium* strains TA98 with (A) and without S9 (B) and TA100 with (C) and without S9 (D). Dashed lines at 2 indicate thresholds for mutagenicity.

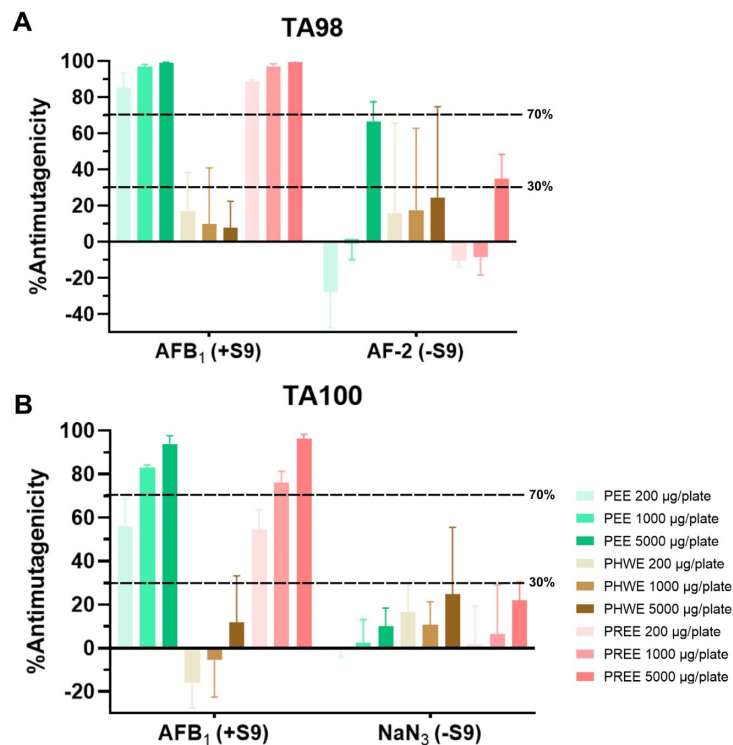


Figure 6. Antimutagenicity of PSLEs in *S. typhimurium* against mutagens: AFB₁ with S9 and AF-2 without S9 in TA98 strain (A) and AFB₁ with S9 and NaN₃ without S9 in TA100 strain (B) dashed lines at 30% and 70% indicate thresholds for anti-mutagenicity and strong anti-mutagenicity, respectively.

highest concentration (5,000 µg/plate), PEE and PREE achieved inhibition greater than 90%, demonstrating very strong protective effects in both TA98 and TA100 strains. In contrast, PHWE exhibited no detectable antimutagenic activity under the same experimental conditions.

In the absence of metabolic activation (–S9), none of the extracts achieved more than 30% inhibition of NaN₃-induced (TA100) or AF-2-induced (TA98) mutagenesis at 200–1,000 µg/plate. However, at 5,000 µg/plate, both PEE and PREE suppressed AF-2-induced mutagenesis in TA98 by more than 30%. Under these conditions, PEE achieved an antimutagenicity of 66%, while PREE reached 34%, indicating moderate and mild protective effects, respectively. The larger standard deviation observed for PHWE antimutagenicity in Figures 6A and 6B may reflect the extract's matrix properties, including potential for partial precipitation, which can affect plating efficiency and interactions with the S9 metabolic activation system, thereby increasing plate-to-plate variability in addition to the inherent biological variability of the Salmonella assay.

DISCUSSION

Piper sarmentosum Roxb. (Cha-plu) is an aromatic herb commonly consumed in Lanna and Thai cuisine. This study investigated the phytochemical composition, antioxidant, and anti-mutagenic activities of *P. sarmentosum* leaf extracts prepared to reflect traditional culinary forms: PEE (ethanolic extract of uncooked leaves), PHWE (hot water extract simulating curry broth), and PREE (ethanolic extract of boiled leaf residues representing the cooked leaves in curry).

Ethanol, with both polar and non-polar properties, enabled extraction of a broad spectrum of bioactive compounds, while hot water primarily extracted hydrophilic molecules. Consistently, PEE and PREE showed higher TPC and TFC, whereas PHWE was enriched in condensed tannins. HPLC profiling identified trans-cinnamic acid and apigenin as major phenolic and flavonoid constituents, respectively, both recognized for antioxidant, anti-inflammatory, antimutagenic, and anticancer activities (20–22). These findings are consistent with previous reports showing that ethanolic extracts of *P. sarmentosum* are rich in phenolic acids and flavonoids, particularly cinnamic acid derivatives and apigenin (23).

PREE was further distinguished by high chlorophyll and carotenoid contents, pigments with well-documented antioxidant and disease-preventive properties (24, 25). These hydrophobic compounds are efficiently extracted in ethanol, explaining their prevalence in PEE and PREE.

The strong antioxidant activities observed can be attributed to the abundance of phenolics and flavonoids, which act via hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms. The ABTS assay reflects both HAT and SET, while FRAP captures SET alone (26). The higher phenolic and flavonoid contents along with hydrophobic pigments (chlorophylls/carotenoids) in PEE and PREE corresponded with stronger ABTS and FRAP activities, whereas PHWE, which contained higher condensed tannins and lower flavonoid levels explained its weaker radical scavenging and reducing power. PEE and PREE consistently exhibited superior activity, likely due to their higher levels of trans-cinnamic acid, apigenin, chlorophylls, and carotenoids. These results align with previous studies reporting potent antioxidant effects of *P. sarmentosum*, driven by its diverse phytochemical profile (6).

The mutagenicity assessment using the Salmonella mutation assay (TA98 and TA100) demonstrated that none of the extracts induced an increase in revertant colonies, with or without S9 activation. As these strains represent frameshift (TA98) and base-pair substitution (TA100) mutations in the histidine operon (27), the results confirm the absence of mutagenic activity, supporting the safety of *P. sarmentosum* for consumption.

Notably, under metabolic activation, both PEE and PREE strongly inhibited AFB₁-induced mutagenesis, achieving more than 90% inhibition at 5,000 µg/plate, indicating very strong antimutagenic potential. In contrast, their activity against direct-acting mutagens (AF-2 and NaN₃) without S9 was limited, with only mild to moderate inhibition at the highest dose. This selectivity suggests that the protective effects are preferentially exerted through modulation of metabolic activation pathways rather than direct interaction with mutagens. The protective effects are likely mediated by trans-cinnamic acid, apigenin, and carotenoids, all of which have been reported to suppress mutagen-induced mutagenesis (28–31). These compounds may interfere with mu-

tagen bioactivation pathways or enhance DNA protection mechanisms. Phenolic acids and flavonoids are also known to counteract environmental mutagens such as AFB₁ (32). The superior antimutagenicity of PEE and PREE compared to PHWE can be explained by not only higher total phenolic content but also the presence of specific flavonoids (e.g., apigenin) and hydrophobic antioxidants; water extraction for PHWE preferentially enriches highly polymerized tannins that may exhibit limited antimutagenic efficacy in the Salmonella system. Although regression analysis of individual constituent compounds was not conducted in the present study, comparative evidence from the literature suggests that these phytochemicals are likely responsible for the strong antimutagenic activity observed in both PEE and PREE. However, definitive confirmation of their causal roles will require bioassay-guided fractionation and detailed mechanistic investigations.

The observed differences in antimutagenic potency among the three extracts, despite their similar phytochemical profiles, can be attributed to variations in constituent concentrations and their synergistic interactions. PEE and PREE consistently demonstrated stronger antioxidant and antimutagenic effects, suggesting that not only the presence but also the ratios and synergistic interactions of bioactive compounds determine functional efficacy. This agrees with earlier findings that combinations of phenolics and flavonoids can exert enhanced bioactivity (33). From a culinary perspective, the boiled leaf residue (PREE) retained substantial phytochemicals and bioactivities, suggesting that brief cooking, as in soups or curries, preserves functional compounds at levels comparable to the uncooked leaves.

CONCLUSIONS

In summary, *P. sarmentosum* leaf extracts prepared to represent common culinary forms exhibited distinct phytochemical characteristics and bioactivities. The PEE and PREE extracts contained higher levels of total phenolics, flavonoids, and hydrophobic pigments (chlorophylls and carotenoids), corresponding with stronger antioxidant capacities in both ABTS and FRAP assays. In contrast, PHWE was enriched in condensed tannins but showed comparatively lower antioxi-

dant activity. None of the extracts demonstrated mutagenicity in the Salmonella assay. Notably, PEE and PREE markedly inhibited AFB₁-induced mutagenesis under metabolic activation, likely due to the combined influence of elevated phenolic and flavonoid contents and the presence of specific compounds such as trans-cinnamic acid, apigenin, and other hydrophobic antioxidants. These findings indicate that *P. sarmentosum* retains substantial functional potential following brief culinary processing, and that specific phenolic and flavonoid constituents may play key roles in its antimutagenic effects.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

N.S.: investigation, data curation, visualization, writing – original draft preparation; B.L.: investigation, data curation; S.T.: data curation, validation, supervision; T.C.: IBC approval, supervision; R.W.: conceptualization, methodology, supervision; O.K.: conceptualization, methodology, data curation, validation, supervision, writing – review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable.

INFORMED CONSENT STATEMENT

Not applicable.

DECLARATION OF GENERATIVE AI IN SCIENTIFIC WRITING

The authors used ChatGPT to assist with improving the readability and language of this manuscript. All content was subsequently reviewed, revised, and approved by the authors, who take full responsibility for the final version.

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Living Will Completion During Palliative Care Consultations and Factors Influencing Completion: A Retrospective Study in a University Hospital

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ABSTRACT

OBJECTIVE To determine the proportion of palliative care patients completing a living will and to identify factors associated with completion.

METHODS This retrospective study analyzed electronic medical records from Maharaj Nakorn Chiang Mai Hospital, a university hospital in Thailand. Patients who had their initial palliative care consultations between November 1, 2021 and April 30, 2022 were included. Descriptive statistics and multivariable logistic regression were used.

RESULTS Of the 389 patients in the study, 205 (52.7%) were female, and the median age was 68 (IQR: 59–79). Most had cancer (67.6%) and poor performance status (PPS 10–30: 69.9%). Decision-making capacity was present in 177 patients (45.5%). A living will was introduced to 83 patients (21.3%) and was completed by 26 (6.7%). Higher PPS (70–100) was associated with being introduced to a living will. The most common reason for not introducing patients to a living will was poor patient symptom control. No factors were significantly associated with living will completion among those who were introduced to a living will.

CONCLUSIONS Most patients presented to palliative care with advanced disease and poor performance status, limiting opportunities for living will discussions and emphasizing the need for early consultation. Strengthening symptom management may support earlier and more frequent introduction of a living will, potentially improving completion rates.

KEYWORDS living will, completion, palliative care, university hospital

INTRODUCTION

Healthcare providers play a crucial role in delivering appropriate palliative care to patients with incurable illnesses (1). The World Health Organization (WHO) defines palliative care as an approach that improves the quality of life of patients facing life-threatening illness and their families (2). A living will (LW) is an important tool in palliative care, allowing patients to express their

end-of-life treatment preferences to healthcare providers and family members. A LW is a written document specifying an individual's wishes for medical treatment in circumstances where they are no longer able to provide informed consent (3). It empowers patients to refuse unnecessary or burdensome interventions, thereby preserving their right to a dignified and peaceful death. Beyond medical decisions, a LW also facilitates emotional

and spiritual preparation for death, offering patients the opportunity to reflect on life, say good-bye to loved ones, and accept the impermanence of human existence (4). Legally, everyone has the right to their own living will, and from a clinical perspective, it is recommended that ideally all palliative patients complete one (5, 6).

In Thailand, the National Health Act of 2007 legally supports end-of-life decision-making, allowing individuals to record a LW in which they can refuse life-prolonging or suffering-relieving treatments. Implementation follows ministerial regulations, and healthcare providers complying with a valid LW are exempt from legal liability (7).

In clinical practice in Thailand, however, most patients referred to the palliative care team do not have a LW (8). Supporting patients in preparing for their inevitable death and facilitating the completion of their own LW is usually a responsibility of the palliative care team. This facilitation process generally involves three sequential steps: (1) the attending healthcare provider evaluates whether the patient has the decision-making capacity to complete a LW; (2) the attending provider introduces the LW to the patient; and (3) the provider offers additional information the patient may require to make informed decisions regarding making a LW. The process is carried out in accordance with the patient's individual pace and often requires more than one encounter. Completion of a LW at the end of consultations is not mandatory, though it is preferred.

This study aimed to examine the proportion of LW completions during first palliative care consultations in a university hospital in Thailand and to address the lack of quantitative data on LW implementation in Thai university hospitals as well as the need to explore physician- and system-related barriers. These findings could help inform palliative care teams regarding how to systematically improve the LW facilitation process in the future.

To operationalize this aim, the study focused on the following objectives, building on the LW facilitation process described above.

1. Examine the proportion of patients progressing through each step of the facilitation process, specifically:

- a) Patients assessed to have decision-making capacity for LW completion

- b) Patients to whom the palliative care team introduced a LW

- c) Patients who ultimately completed a LW

2. Identify clinical and demographic factors associated with:

- a) The decision by the palliative care team to introduce a LW to patients with decision-making capacity

- b) The completion of a LW by patients to whom LW was introduced

3. Explore reasons why with some patients who were assessed as having decision-making capacity the palliative care team chose not to introduce a LW to the patient.

METHODS

Study design

This is a retrospective observational study. All data were extracted from electronic medical records (EMR) of Maharaj Nakorn Chiang Mai Hospital, Thailand.

Setting

All data were obtained from records of in-patient department consultations. All physicians' notes were recorded within the EMR system. Regarding the LW, after the physician introduced the concept including an explanation of its purpose and the steps involved in completing it:

- a) If the patient wished to proceed, the LW was completed using the hospital's standardized note template and documented in the EMR.

- b) If the patient was interested but not ready to make treatment decisions during the current admission, the palliative care team provided LW information to appropriate hospital staff for review and arranged follow-up either at the out-patient clinic or by coordinating referral to a nearby hospital.

- c) If the patient declined to make a LW, no further LW discussions were initiated during that admission.

Sample calculations

The sample size was estimated using the formula for a single proportion, based on the following assumptions: an expected proportion of LW completion of 30.0% among first palliative consultations (9), a 95% confidence level, and a 5% margin of error. The minimum required sample size was 323 patients.

Ethics approval

This study was approved by Research Ethics Committee, Faculty of Medicine, Chiang Mai University (Study Code: FAM-2564-08503/28th October 2021).

Study population

Inclusion criteria

All patients who were referred for consultation with the palliative care team between November 1, 2021, and April 30, 2022.

Exclusion criteria

1. Patients who had previously been consulted by the palliative care team
2. Patients younger than 18 years
3. Patients who already had a LW prior to the consultation.

Data collection

Patient health information was extracted from the EMR. Variables included sex, age, type of health insurance, type of primary diagnosis (cancer vs. non-cancer), comorbid conditions (chronic non-life-threatening diseases), Palliative Performance Status (PPS) (10, 11), a validated score reflecting an individual's ability to perform daily activities, fulfill normal roles, and maintain health and well-being, Pain Numeric Rating Scale (12), Dyspnea Numeric Rating Scale (13), history of receiving interventions including endotracheal intubation, cardiopulmonary resuscitation, hemodialysis, and central venous catheter insertion, and data on whether advance care planning (ACP) (14)—defined as decisions regarding general treatment direction but not limited to LW—which had been documented by the consulting physician prior to referral to the palliative care team were also extracted from the EMR.

Outcome variables included

1. Whether the attending palliative care team deemed the patient to have decision-making capacity for LW completion
2. Whether the concept of LW was introduced to the patient
3. Whether the patient completed a LW

Decision-making capacity was defined as the ability to: (1) understand and retain relevant information, (2) appreciate the situation and its likely consequences, (3) reason and weigh options ra-

tionally, and (4) communicate a choice (15). These assessments were made solely by the attending team based on clinical judgment guided by this definition.

Reasons for not introducing a LW to eligible patients were also collected from the EMR. At the time of the study, the palliative care team required physicians to document reasons for not introducing a LW to patients.

Statistical analysis

Analyses were conducted in three stages:

1. Descriptive statistics (means, medians, frequencies, and proportions, as appropriate) were used to summarize patient characteristics and outcomes in each group of patients throughout the LW facilitation process (had decision-making capacity, LW was introduced, LW completion). Proportions of patients progressing through each step were reported. Among patients with decision-making capacity, demographic and clinical factors were compared between those who were and were not introduced to LW using t-tests, chi-square tests, or Fisher's exact tests, depending on data type. Similarly, among patients introduced to LW, comparisons were made between those who completed and those who did not complete a LW.

2. Univariable and multivariable logistic regression analyses were used to identify demographic and clinical factors associated with the introduction of LW among patients with decision-making capacity, and LW completion by those to whom LW was introduced. Both unadjusted and adjusted associations were examined.

3. Reasons documented by the palliative care team for not introducing LW to eligible patients were summarized descriptively.

RESULTS

Between 1 November 2021 and 30 April 2022, the palliative care team received a total of 429 patients. Of those patients, 177 had decision-making capacity. LW was introduced to 83 of those patients, of whom 26 completed a LW during their hospital stay (Figure 1).

Among 389 new patients referred to the palliative care team at Maharaj Nakorn Chiang Mai Hospital during the six-month period of this study, 205 were female (52.7%). The median age

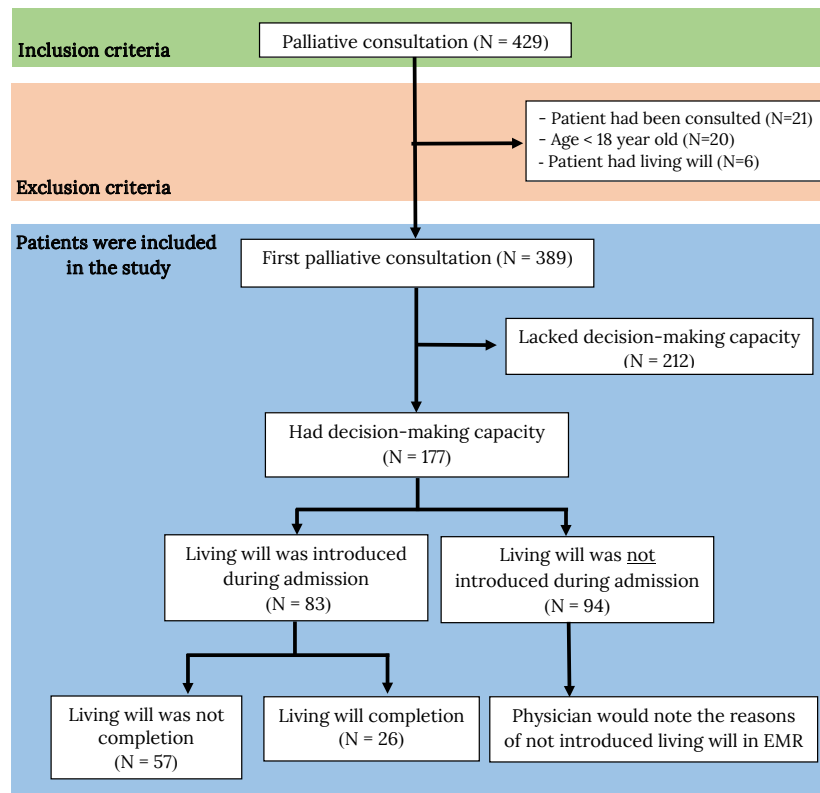


Figure 1. Patient recruitment flow diagram

was 68 years. A total of 182 patients were covered by the Civil Servant Medical Benefit Scheme (46.8%), 263 had cancer (67.6%), and 240 had at least one co-morbidity disease (61.7%). ACP was documented in 262 patients (67.4%). Most patients (272, 69.9%) had PPS of 10-30. Pain was absent in 249 patients (64.0%), and dyspnea was absent in 160 patients (41.1%). A total of 177 patients (45.5%) were assessed as having decision-making capacity. Of these, 93 were female (52.5%), with a median age of 64 years. Eighty-eight patients (49.7%) were covered under the Universal Health Coverage scheme (UHC), 162 had cancer (91.5%), and 98 had at least one co-morbidity disease (55.4%). ACP was documented in 92 patients (52.0%). Most patients (95, 53.7%) had PPS of 40-60. Pain was absent in 65 patients (36.7%), and dyspnea was absent in 82 patients (46.3%).

Eighty-three patients (21.3% of all included patients, or 46.9% of those with decision-making capacity) were invited to complete a LW. This group included 46 females (55.4%) and 43 patients (51.8%) younger than 65 years. The majority were covered under UHC (40 patients, 48.2%), and most had cancer (79 patients, 95.2%). Forty-three patients (51.8%) had at least one co-morbidity.

More than half (47 patients, 56.6%) had had no discussions of ACP. Most had PPS of 40-60 (50 patients, 60.2%), experienced moderate pain (28 patients, 33.7%), and reported no dyspnea (43 patients, 51.8%).

Twenty-six patients (6.7% of all included patients, or 31.3% of those with decision-making capacity) completed a LW. This group included 15 females (57.7%) and 15 patients younger than 65 years (57.7%). Nearly half were covered by the Civil Servant Medical Benefit Scheme (12 patients, 46.2%), and all had cancer (100.0%). Fourteen patients (53.8%) had at least one co-morbidity. More than half (15 patients, 57.7%) had not discussed ACP. Most had PPS of 40-60 (17 patients, 65.4%), experienced moderate pain (11 patients, 42.4%), and reported no dyspnea (13 patients, 50.0%) (Table 1).

Among the 177 patients, factors associated with being introduced to a LW were examined using logistic regression. In the univariable analysis, ACP was associated with lower odds of a LW introduction (OR = 0.52, 95%CI: 0.29-0.95, $p = 0.032$), while higher PPS was strongly associated with increased odds of an introduction. Compared with patients with PPS 10-30, those with

Table 1. Participant characteristics

	First consultation	Had decision-making capacity	LW introduced	LW completed
N (%)	389 (100.0)	177 (45.5)	83 (21.3)	26 (6.7)
Variables				
Female, n (%)	205 (52.7)	93 (52.5)	46 (55.4)	15 (57.7)
Age, median [Q1, Q3]	68.0 [59.0, 79.0]	64.0 [56.0, 70.0]	64.0 [57.0, 68.0]	63.5 [57.0, 67.0]
Age group ≥ 65 years old, n (%)	230 (59.1)	81 (45.8)	40 (48.2)	11 (42.3)
Insurance, n (%)				
Civil servant	182 (46.8)	70 (39.5)	35 (42.2)	12 (46.2)
UHC	153 (39.4)	88 (49.7)	40 (48.2)	10 (38.5)
Social security	27 (6.9)	15 (8.5)	6 (7.2)	3 (11.5)
Other	27 (6.9)	4 (2.3)	2 (2.4)	1 (3.8)
Cancer as primary diagnosis, n (%)	263 (67.6)	162 (91.5)	79 (95.2)	26 (100.0)
Had at least one co-morbidity disease, n (%)	240 (61.7)	98 (55.4)	43 (51.8)	14 (53.8)
Had ACP discussed, n (%)	262 (67.4)	92 (52.0)	36 (43.4)	11 (42.3)
PPS, n (%)				
10-30	272 (69.9)	68 (38.4)	20 (24.1)	5 (19.2)
40-60	102 (26.2)	95 (53.7)	50 (60.2)	17 (65.4)
70-100	15 (3.9)	14 (7.9)	13 (15.7)	4 (15.4)
Pain, n (%)				
None	249 (64.0)	65 (36.7)	26 (31.3)	5 (19.2)
Mild	31 (8.0)	23 (13.0)	13 (15.7)	5 (19.2)
Moderate	63 (16.2)	50 (28.3)	28 (33.7)	11 (42.4)
Severe	46 (11.8)	39 (22.0)	16 (19.3)	5 (19.2)
Dyspnea, n (%)				
None	160 (41.1)	82 (46.3)	43 (51.8)	13 (50.0)
Mild	58 (14.9)	29 (16.4)	13 (15.7)	5 (19.2)
Moderate	74 (19.1)	37 (20.9)	16 (19.3)	3 (11.6)
Severe	97 (24.9)	29 (16.4)	11 (13.2)	5 (19.2)

UHC, The Universal Health Coverage scheme; ACP, advance care planning; LW, living will

Table 2. Factor associated with introducing LW in patients who had decision-making capacity

Characteristics (n = 177)	Univariable			Multivariable		
	OR	95% CIs	p-value	OR	95% CIs	p-value
Female	1.24	0.69, 2.25	0.471	1.34	0.68, 2.65	0.396
Age group ≥ 65	1.20	0.67, 2.18	0.542	1.78	0.84, 3.77	0.136
Non-civil servant	0.81	0.45, 1.49	0.503	0.55	0.27, 1.13	0.104
Had at least one co-morbidity disease	0.76	0.42, 1.38	0.371	0.43	0.21, 0.90	0.025
Had ACP discussed	0.52	0.29, 0.95	0.032	0.58	0.28, 1.19	0.139
PPS						
10-30	Ref.					
40-60	2.67	1.38, 5.15	0.004	3.38	1.60, 7.11	0.001
70-100	31.20	3.82, 254.73	0.001	49.77	5.40, 459.05	0.001
Pain						
None	Ref.					
Mild	1.95	0.75, 5.10	0.174	1.57	0.53, 4.63	0.415
Moderate	1.91	0.90, 4.03	0.090	2.20	0.92, 5.28	0.077
Severe	1.04	0.47, 2.34	0.918	0.91	0.36, 2.31	0.837
Dyspnea						
None	Ref.					
Mild	0.74	0.32, 1.73	0.482	0.86	0.33, 2.28	0.763
Moderate	0.69	0.32, 1.51	0.354	1.14	0.47, 2.81	0.768
Severe	0.55	0.23, 1.32	0.182	0.86	0.31, 2.36	0.766

ACP, advance care planning; PPS, palliative care performance status

Table 3. Factors associated with LW completed among patients who were introduced for LW

Characteristics (n = 83)	Univariable			Multivariable		
	OR	95% CIs	p-value	OR	95% CIs	p-value
Female	1.14	0.45, 2.92	0.779	1.37	0.48, 3.93	0.563
Age group ≥ 65	0.71	0.28, 1.80	0.469	0.87	0.28, 2.68	0.807
Non-civil servant	0.79	0.31, 2.01	0.620	0.72	0.22, 2.34	0.584
Had at least one co-morbidity disease	1.13	0.45, 2.85	0.802	1.46	0.46, 4.60	0.519
Had ACP discussed	0.94	0.37, 2.40	0.895	1.57	0.44, 5.63	0.491
PPS						
10-30	Ref.					
40-60	1.55	0.48, 4.98	0.466	1.84	0.49, 6.97	0.368
70-100	1.33	0.28, 6.30	0.717	1.88	0.32, 11.14	0.485
Pain						
None	Ref.					
Mild	2.63	0.60, 11.57	0.202	3.39	0.67, 17.19	0.140
Moderate	2.72	0.79, 9.35	0.113	4.05	0.93, 17.62	0.063
Severe	1.91	0.45, 8.04	0.378	1.98	0.37, 10.60	0.424
Dyspnea						
None	Ref.					
Mild	1.44	0.40, 5.26	0.579	1.25	0.31, 5.02	0.750
Moderate	0.53	0.13, 2.19	0.382	0.38	0.07, 1.95	0.246
Severe	1.92	0.50, 7.44	0.344	2.24	0.47, 10.61	0.311
History interventions	0.84	0.28, 2.49	0.753	1.09	0.30, 3.95	0.892

LW, living will; ACP, advance care planning; PPS: palliative care performance status

PPS 40-60 had over twofold higher odds (OR = 2.67, 95%CI: 1.38-5.15, $p = 0.004$), and those with PPS 70-100 had markedly higher odds (OR = 31.20, 95%CI: 3.82-254.73, $p = 0.001$) of an introduction to a LW.

In the multivariable analysis, PPS remained the only significant predictor of introduction to a LW. Patients with PPS 40-60 (OR = 3.38, 95%CI: 1.60-7.11, $p = 0.001$) and PPS 70-100 (OR = 49.77, 95%CI: 5.40-459.05, $p < 0.001$) were substantially more likely to be introduced LW than those with PPS 10-30. Conversely, having at least one co-morbidity disease was independently associated with lower odds of LW introduction (OR = 0.43, 95%CI: 0.21-0.90, $p = 0.025$). Other factors, including sex, age ≥ 65 years, having health insurance, having discussed ACP, and pain, and dyspnea, were not significantly associated with LW introduction (Table 2). The characteristics of patients who had decision-making capacity are provided in [Supplementary material: Table 1](#).

Among the 83 patients, no demographic or clinical characteristics were statistically significantly associated with LW completion. In the multivariable analysis, moderate pain demonstrated a trend toward higher odds of LW completion

(OR = 4.05, 95%CI: 0.93-17.62, $p = 0.063$), although this did not reach statistical significance. Other factors, e.g., female gender (OR = 1.37, $p = 0.563$), age ≥ 65 years (OR = 0.87, $p = 0.807$), PPS 40-60 (OR = 1.84, $p = 0.368$), PPS 70-100 (OR = 1.88, $p = 0.485$), and dyspnea (OR range: 0.38-2.24, all $p > 0.24$), were likewise not statistically significantly associated with LW completion (Table 3). The characteristics of participating patients who were introduced to LW are provided in [Supplementary material: Table 2](#).

The predominant reason for not introducing a LW was that 67 physicians (69.1%) perceived the patient was experiencing severe distress symptoms, which the physician considered likely to interfere with their decision-making capacity. Additionally, 6 (6.2%) reported that the patient was not informed of their diagnosis due to a conspiracy of silence; in the case of 5 patients (5.1%) although there had been a plan to propose completion of a LW, the patient had been discharged from the hospital before this could take place. These findings represent responses from 94 physicians who were able to provide more than one reason (Table 4).

Table 4. Reasons for not introducing a LW (N = 94)

Reasons	N (%)
1. The patient was not informed of their diagnosis due to a conspiracy of silence	6 (6.2)
2. The patient was not informed of their diagnosis, as they expressed a preference not to know	2 (2.1)
3. The patient's family declined discussions on the matter, despite the patient being aware of their diagnosis	2 (2.1)
4. Although the patient was aware of their diagnosis, they chose not to participate in treatment planning, instead delegating treatment decisions to their family	4 (4.1)
5. The patient was experiencing severe distressing symptoms, which the physician considered likely to interfere with their decision-making capacity	67 (69.1)
6. A plan had been made to propose the completion of a living will; however, the patient was discharged from the hospital before this could take place	5 (5.1)
7. The physician was not comfortable proposing the completion of a living will to this patient	1 (1.0)
8. Others	10 (10.3)
Total	97 (100.0)

Responses from 94 physicians. One physician could give multiple responses

The reasons of others included factors such as the physician not having enough time to talk, the patient still needing more information before making a treatment decision, and the setting being unsuitable for such a discussion, among others.

DISCUSSION

In this study of 389 patients who had received a first palliative care consultation, the majority were older adults with cancer, had low PPS, and lacked decision-making capacity. A LW was introduced to 21.3% of eligible patients, but only 6.7% of all patients completed one during admission. Among patients who had decision-making capacity and who were introduced to a LW, the completion rate was 31.3% which is comparable to findings in previous studies that reported completion rates of around 20.0–55.0% (3, 9, 16–19). The rate varied depending on the population and the context being studied.

Based on these results, more than half the patients lacked decision-making capacity, indicating that palliative care is often initiated too late, particularly in the case of patients with non-cancer terminal illnesses, where palliative care typically begins when the patient is already in the final stages (20). If these patients and their families were engaged in palliative care discussions and planning from the early stages, it could allow more time for ACP and decision-making (21, 22). This finding aligns with previous studies suggesting that if individuals have adequate time to prepare for the end of life, they may engage in a process of achieving a sense of completion or closure (4) and that to reduce inequalities in end-of-life care, timely actions to promote ACP are essential (3).

Among the 177 patients who had sufficient decision-making capacity to complete a LW, logistic regression showed that those with higher

PPS (70–100) were significantly more likely to be introduced to LW by healthcare professionals, whereas the presence of at least one co-morbidity disease was associated with a lower odds ratio after adjustment. These findings highlight the importance of initiating palliative care consultations early, before functional decline and multiple comorbidities develop, in order to increase the rate of LW completion. Consistent with Steinhäuser et al. (4), many physicians hesitate to discuss end-of-life planning due to concerns about causing distress, perceptions of medical failure, or fear of diminishing hope; however, patients often wish to plan their funerals and to know the expected timing of death. For example, the use of tools such as Supportive & Palliative Care Indicators Tool (SPICT) (23) can support timely initiation of palliative care. The study by Huang et al. (24) demonstrated that SPICT facilitates clinical teams in initiating palliative care consultations, engaging in goals-of-care discussions, and making appropriate referrals for patients in routine practice.

The present study found that factors such as sex, age, health insurance, having at least one co-morbidity disease, having ACP discussed, PPS, pain, dyspnea, or a history of receiving interventions were not statistically significantly associated with the decision to complete a LW. This finding contrasts with previous studies (3, 9, 16, 25–28). For example, Andreassen et al. (3) studied 10,178 older adults receiving long-term care in Finland and found that female sex ($p < 0.001$), having a

proxy decision-maker ($p = 0.001$), higher age ($p = 0.003$), reduced functional capacity (activities of daily living hierarchy, $p < 0.001$), lower Cognitive Performance Scale scores ($p < 0.001$), unstable health status (changes in health, end-stage disease and signs and symptoms, $p < 0.001$), and proximity to death ($p < 0.001$) were significantly associated with having a LW. Similarly, Muneeno et al. (16) examined 180 cancer patients attending tertiary-hospital outpatient clinics in Bangkok and reported that older age (≥ 60 years), advanced cancer stage, symptom severity, and knowledge of LW increased the likelihood of LW completion (OR = 2.63, 7.59, 1.05, and 1.79, respectively; $p < 0.05$). These results may be due to the population context and the number of patients included in the study.

Among the 94 patients who had decision-making capacity but who were not introduced to LW by physicians, the most common reason was that the patients were experiencing severe distress symptoms that physicians believed could interfere with decision-making capacity (69.1%). The second most common reason was that patients were unaware of their diagnosis due to a “conspiracy of silence” (6.2%). The conspiracy of silence is common in Thai culture, often driven by familial desire to protect the patient from distress or bad news (29–31). This highlights that, apart from the patients’ clinical condition, one of the key factors influencing the opportunity to complete a LW is the physician’s assessment of the patient’s readiness. If physicians are able to more appropriately assess symptoms and evaluate readiness for ACP, patients experiencing distressing symptoms may still be able to participate in discussions and complete a LW. This aligns with the findings of Saimmai et al. (31), who examined the challenges faced by Thai general practitioners and family physicians in discussing advanced care planning. They reported barriers including limited knowledge of palliative care, difficulties in symptom management, and insufficient communication skills. This aligns with previous studies by Omondi et al. (27) which stated that discussions about death and dying are considered taboo in most African societies, and the study by Steinhäuser et al. (4) which noted that preparation for the end of life, particularly in terms of predicting timing, is often hindered by discomfort, skill deficits, and ambiguity regarding the definition of end of life. Furthermore, initiating conversations about

death and dying can evoke discomfort among clinicians due to feelings of failure or fear of removing hope.

In reality, many patients may wish to discuss these matters but are simply waiting for physicians to initiate the conversation (4, 32) because patients wish to make their own healthcare decisions, which is consistent with previous studies by Mezey et al., (26) that the patients desire to make independent decisions regarding their own care (92.0%), the belief that clarifying their wishes would benefit their family (89.0%), and the wish to avoid being sustained by invasive measures such as tubes, wires, or prolonged coma care (81.0%). Therefore, early identification and referral criteria (e.g., using SPICt or symptom-based triggers), along with training programs for physicians and nurses to enhance their communication skills and confidence in initiating ACP or LW discussions, should be implemented.

Lastly, if the government plays a more active role in promoting public awareness and implementing policies regarding ACP and palliative care, along with encouraging community participation, it could significantly enhance public understanding and increase the completion rates of LW (9, 27, 32).

CONCLUSIONS

Most patients presented to palliative care with advanced disease and lacked decision-making capacity. Among patients with decision-making capacity, one key factor associated with physicians not introducing a LW is low PPS, further highlighting the importance of earlier referral to palliative care, which may lead to an increased LW completion rate. Strengthening physicians’ skills in symptom management could also increase the proportion of patients introduced to LW, thereby improving the proportion of LW completions.

Limitations of the study

As this is a retrospective EMR-based study, there is an inherent risk of documentation bias. However, there was no missing data because a standardized data entry protocol was implemented. All reported variables were mandatory for assessment and documentation in the EMR. In addition, physicians had been trained and provided with standardized recording guidelines to minimize bias, although some residual bias may still exist.

Collecting data solely from patients who have been referred to the palliative care team at Maharaj Nakorn Chiang Mai Hospital may be insufficient for identifying factors influencing the completion of a LW. Expanding the survey to other units to allow patients earlier access to LW discussions may increase the amount of data collected regarding physical, psychological, and other factors.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

T.R.: conceptualization, methodology, data collection, analysis, writing—review and editing; T.U.: conceptualization, methodology, data collection, analysis; N.B.: supervision, writing—review and editing; W.J.: supervision, writing—review and editing; N.D.: supervision, writing—review and editing; N.V.: data collection, analysis; T.C.: data collection; A.B.: data collection; C.A.: conceptualization, methodology, data collection, analysis, writing—review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of Faculty of Medicine, Chiang Mai University (Study Code: FAM-2564-08503/28th October 2021).

INFORMED CONSENT STATEMENT

Patient consent was waived because the study involved no more than minimal risk.

DECLARATION OF GENERATIVE AI IN SCIENTIFIC WRITING

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors used ChatGPT 5 to enhance the clarity and language of the manuscript during its preparation. All content was subsequently reviewed and edited by the authors, who take full responsibility for the final published version.

SUPPLEMENTARY MATERIALS

The following supporting information can be downloaded at: [Supplementary file](#)

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Supplementary materials

Table 1. Participant characteristics : Patients who had decision-making capacity

Characteristics	Having decision-making capacity to complete LW			
	Total 177 (100)	LW not introduced 94 (53.1)	LW introduced 83 (46.9)	P-value
Sex, n (%)				
Male	84 (47.5)	47 (50.0)	37 (44.6)	0.569
Female	93 (52.5)	47 (50.0)	46 (55.4)	
Age Median (IQR)	64 (56 – 70)	63.5 (55 -72)	64 (57 - 68)	0.960
Age, n (%)				
< 65	96 (54.2)	53 (56.4)	43 (51.8)	0.647
≥ 65	81 (45.8)	41 (43.6)	40 (48.2)	
Insurance, n (%)				
Civil servant	70 (39.5)	35 (37.2)	35 (42.2)	0.886
UHC	88 (49.7)	48 (51.1)	40 (48.2)	
Social security	15 (8.5)	9 (9.6)	6 (7.2)	
Other	4 (2.3)	2 (2.1)	2 (2.4)	
Cancer as primary diagnosis, n (%)				
Cancer	162 (91.5)	83 (88.3)	79 (95.2)	0.171
Non-cancer	15 (8.5)	11 (11.7)	4 (4.8)	
Had at least one co-morbidity disease, n (%)				
Yes	98 (55.4)	55 (58.5)	43 (51.8)	0.457
No	79 (44.6)	39 (41.5)	40 (48.2)	
Had ACP discussed, n (%)				
Yes	92 (52.0)	56 (59.6)	36 (43.4)	0.045
No	85 (48.0)	38 (40.4)	47 (56.6)	

Characteristics	Having decision-making capacity to complete LW			
	Total 177 (100)	LW not introduced 94 (53.1)	LW introduced 83 (46.9)	P-value
PPS, n (%)				
10-30	68 (38.4)	48 (51.0)	20 (24.1)	< 0.001
40-60	95 (53.7)	45 (47.9)	50 (60.2)	
70-100	14 (7.9)	1 (1.1)	13 (15.7)	
Pain, n (%)				
None	65 (36.7)	39 (41.5)	26 (31.3)	0.231
Mild	23 (13.0)	10 (10.6)	13 (15.7)	
Moderate	50 (28.3)	22 (23.4)	28 (33.7)	
Severe	39 (22.0)	23 (24.5)	16 (19.3)	
Dyspnea, n (%)				
None	82 (46.3)	39 (41.5)	43 (51.8)	0.533
Mild	29 (16.4)	16 (17.0)	13 (15.7)	
Moderate	37 (20.9)	21 (22.3)	16 (19.3)	
Severe	29 (16.4)	18 (19.2)	11 (13.2)	

Table 2. Participant characteristics : Patients who were introduced for LW

Characteristics	Introduced LW			
	Total 83 (100)	LW not completed 57 (68.7)	LW completed 26 (31.3)	P-value
Sex, n (%)				
Male	37 (44.6)	26 (45.6)	11 (42.3)	0.966
Female	46 (55.4)	31 (54.4)	15 (57.7)	
Age Median (IQR)	64 (57-68)	66 (57 - 70)	63.5 (57 - 67)	0.520
Age, n (%)				
<65	43 (51.8)	28 (49.1)	15 (57.7)	0.626
>=65	40 (48.2)	29 (50.9)	11 (42.3)	
Insurance, n (%)				
Civil servant	35 (42.2)	23 (40.3)	12 (46.2)	0.535
UHC	40 (48.2)	30 (52.6)	10 (38.5)	
Social security	6 (7.2)	3 (5.3)	3 (11.5)	
Other	2 (2.4)	1 (1.8)	1 (3.8)	
Cancer as primary diagnosis, n (%)				
Cancer	79 (95.2)	53 (93.0)	26 (100)	0.304
Non-cancer	4 (4.8)	4 (7.0)	0 (0.0)	
Had at least one co-morbidity disease, n (%)				
Yes	43 (51.8)	29 (50.9)	14 (53.8)	0.989
No	40 (48.2)	28 (49.1)	12 (46.2)	
Had ACP discussed, n (%)				
Yes	36 (43.4)	25 (43.9)	11 (42.3)	1.000
No	47 (56.6)	32 (56.1)	15 (57.7)	

Characteristics	Introduced LW			P-value
	Total 83 (100)	LW not completed 57 (68.7)	LW completed 26 (31.3)	
PPS, n (%)				
10-30	20 (24.1)	15 (26.3)	5 (19.2)	0.763
40-60	50 (60.2)	33 (57.9)	17 (65.4)	
70-100	13 (15.7)	9 (15.8)	4 (15.4)	
Pain, n (%)				
None	26 (31.3)	21 (36.9)	5 (19.2)	0.407
Mild	13 (15.7)	8 (14.0)	5 (19.2)	
Moderate	28 (33.7)	17 (29.8)	11 (42.4)	
Severe	16 (19.3)	11 (19.3)	5 (19.2)	
Dyspnea, n (%)				
None	43 (51.8)	30 (52.7)	13 (50.0)	0.470
Mild	13 (15.7)	8 (14.0)	5 (19.2)	
Moderate	16 (19.3)	13 (22.8)	3 (11.6)	
Severe	11 (13.2)	6 (10.5)	5 (19.2)	

In vitro Antioxidant, Acetylcholinesterase, and Amyloid-beta Inhibitory Effects of Captopril, An Angiotensin-Converting Enzyme (ACE) Inhibitor

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ABSTRACT

OBJECTIVE This study aims to investigate the potential of captopril, an angiotensin-converting enzyme (ACE) inhibitor, to reduce reactive oxygen species (ROS) production and address critical factors associated with Alzheimer's disease (AD) pathology, including acetylcholinesterase (AChE) activity and amyloid-beta (A β) peptide aggregation. The broader goal was to explore captopril's neuroprotective properties and its potential as a therapeutic agent for AD.

METHODS The antioxidant effects of captopril were evaluated using DPPH and ABTS assays, as well as xanthine oxidase inhibition tests. The impact of captopril on AChE activity and A β aggregation was assessed through *in-vitro* experiments. Molecular docking studies were conducted to analyze captopril's binding affinity with essential AD-related proteins.

RESULTS Captopril demonstrated notable antioxidant activity with IC₅₀ values of 71.99 $\pm$ 0.40 μ M (DPPH) and 106.3 $\pm$ 0.28 μ M (ABTS), comparable to ascorbic acid (92.32 $\pm$ 0.26 μ M and 110.1 $\pm$ 0.24 μ M). Significant inhibition of xanthine oxidase activity was observed; however, the IC₅₀ value exceeded the maximum tested concentration of 320 μ M for both captopril and ascorbic acid. Captopril showed AChE inhibitory activity with an IC₅₀ of 208.2 $\pm$ 0.36 μ M, whereas donepezil exhibited an IC₅₀ of 79.05 $\pm$ 0.25 μ g/mL. In the A β aggregation assay, captopril had an IC₅₀ of 87.69 $\pm$ 0.25 μ M, while donepezil also displayed significant inhibition, with an IC₅₀ above 320 μ M. Molecular docking analyses revealed favourable binding interactions of captopril with AChE and A β -related targets, supporting its multi-target mechanism.

CONCLUSIONS Captopril demonstrated notable neuroprotective effects, including antioxidant activity and the inhibition of AChE activity and A β aggregation. These findings indicate that captopril holds promise as a therapeutic agent for AD, warranting further research and clinical trials to evaluate its efficacy in combating neurodegeneration.

KEYWORDS health, diseases, antioxidant, neuroprotection, captopril, acetylcholinesterase, amyloid peptide

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INTRODUCTION

The renin-angiotensin system (RAS) is essential for maintaining circulatory homeostasis in the periphery, but its role in brain function has only recently been recognized (1). This localised brain RAS is expressed separately and has a substantial impact on numerous brain activities, as well as contributing to neurological illnesses such as Alzheimer's disease (AD), dementia, Parkinson's disease, and Huntington's disease (2). Furthermore, angiotensin-converting enzyme (ACE) and other RAS components are present in the brain, and elevated ACE levels contribute to the development of AD (3). Elevated ACE activity has been discovered in the cerebrospinal fluid of both Alzheimer's and Parkinson's patients, which could be a sign of brain inflammation. Angiotensin II (Ang II) binds to AT1 receptors, leading to oxidative stress, inflammation, amyloid- β accumulation, and tau protein phosphorylation. This, in turn, leads to cognitive impairment and the onset of AD (4).

According to studies, angiotensin-converting enzyme inhibitors (ACEIs) may be neuroprotective, lowering the chance of acquiring neurodegenerative illnesses. ACE inhibitors such as captopril, lisinopril, enalapril, ramipril, quinapril, perindopril, and trandolapril have been shown to ameliorate neurodegenerative diseases in animals and humans. Numerous clinical trials have confirmed the therapeutic efficacy of ACE inhibitors in treating these disorders (5). Captopril and perindopril, both ACE inhibitors, lower Ang II levels and can cross the blood-brain barrier (6). Perindopril and captopril were previously shown in studies to reduce or reverse cognitive deterioration in people with late-onset AD. Additionally, captopril therapy was found to be related to a decrease in amyloidogenic processing of the amyloid precursor protein (APP) and a reduction in reactive oxygen species (ROS) levels in the hippocampus of Tg2576 AD mice, as previously described (7). It also acts as a potent anti-inflammatory drug, alleviating cognitive impairment by reducing A β production and increasing microglial activation and phagocytosis (8). In addition, as a sulfhydryl (SH) group containing ACEI, captopril has been shown to have a considerable antioxidant impact (9).

Experimental data suggest that a brain-penetrating ACE inhibitor can prevent cognitive

decline, although its impact on A β deposition and the mechanism of action remain unknown (10). In this study, we evaluated the effects of captopril, a first-generation ACE inhibitor, on ROS products, in vitro acetylcholinesterase (AChE) activity, and amyloid beta peptide formation, using in vitro assays and molecular docking.

METHODS

Chemicals and reagents

2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), xanthine, acetylthiocholine iodide, acetylcholine enzyme (0.3 U/mL) were procured from Sigma-Aldrich, St. Louis, Missouri, USA. Ascorbic acid was purchased from Tokyo Chemical Industry, Chennai, Chennai, India. donepezil hydrochloride was purchased as a tablet from a local pharmacy. All other chemicals, reagents and solvents used were of analytical grade and purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Preparation of captopril, standard ascorbic acid and standard donepezil solutions

Stock solutions of captopril and donepezil hydrochloride were freshly prepared before the assay. Captopril and standard ascorbic acid were dissolved in distilled water to obtain a 10 mM stock solution. In contrast, donepezil hydrochloride was dissolved in 0.1 M phosphate buffer (pH 7.4) to get a 1 mg/mL stock solution. Working solutions were prepared by serial dilution of the respective stock solutions to yield final concentrations of 10, 20, 40, 80, 160, and 320 μ M for captopril and ascorbic acid, and 10, 20, 40, 80, 160, and 320 μ g/mL for donepezil. All solutions were prepared under subdued light conditions to minimise degradation and were stored on ice until use.

In vitro antioxidant activity

DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay

The DPPH assay was performed to evaluate the free radical scavenging activity of the extract following previously described methods (11). A 0.004% DPPH solution was prepared in 100 mL of methanol. 10 μ L of captopril at concentrations of 10 μ M, 20 μ M, 40 μ M, 80 μ M, 160 μ M, 320 μ M was added to 190 μ L of DPPH solution. After vortexing,

the mixture was incubated for 20 mins at 37°C. The control blank contained solvent without the test compound/standard. The decrease in absorbance of the test mixture (due to quenching of DPPH free radicals) was measured at 517 nm. The IC₅₀ value was determined as the concentration of the test mixture that resulted in a 50% reduction in absorbance relative to a control blank. The experiments were repeated in triplicate, and the percentage inhibition was calculated. Ascorbic acid was used as a reference standard. The DPPH radical scavenging activity of the extract was calculated using the equation below.

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

ABTS assay (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging

ABTS radical scavenging activity of captopril was determined according to a method described by Gonzalez-Palma et al., 2016. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was dissolved in water and diluted to 7 mM. ABTS radical cation (ABTS⁺) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in a dark at room temperature for 12–16 h before use. The ABTS⁺ solution was diluted with water to an absorbance of 0.70 (± 0.02) at 734 nm. The reaction mixture consisted of 0.07 mL of the captopril at different concentrations (10–320 µM) and 3 mL of the ABTS radical. After incubation for 6 min, absorbance was measured at 734 nm in a spectrophotometer. Ascorbic acid was used as a reference standard. The antioxidant activity was calculated using the following equation (12).

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of test}}{\text{absorbance of control}} \times 100$$

Xanthine oxidase inhibitory activity

The Xanthine oxidase (XO) inhibitory activity was assayed spectrophotometrically under aerobic conditions, based on the procedure reported earlier (13). The substrate and the enzyme solutions were freshly prepared. The assay mixture, consisting of 50 µL of different concentrations of captopril (10–320 µM), various concentrations of ascorbic acid (10–320 µM), 35 µL of 0.1 mM phosphate buffer (pH = 7.5) and 30 µL of enzyme solu-

tion (0.01 units/mL of XO in 0.1 mM phosphate buffer, pH = 7.5), was prepared immediately before use. After 30 mins of incubation at 25°C, the reaction was initiated by the addition of 60 µL of substrate solution (150 mM Xanthine in 0.1 mM phosphate buffer). The absorption at 295 nm, indicating the formation of uric acid at 25°C, was monitored and the initial rate was calculated. A blank was prepared in the same manner. One unit of XO was defined as the amount of enzyme required to produce 1 mmol of uric acid/minute at 25°C. XO inhibitory activity is expressed as the percentage inhibition of XO in the above system, calculated as (1-B/A) × 100, where A and B are the activities of the enzyme without and with different concentrations of captopril and ascorbic acid. IC₅₀ values were calculated from the mean values of data from three determinations. Ascorbic acid was used as a reference standard.

In vitro AChE inhibition assay

The inhibitory activity of captopril and donepezil hydrochloride against AChE was determined using a modified Ellman's method suitable for microplate analysis (14). The assay was performed in 96-well plates with a final reaction volume of 200 µL. Each well contained 140 µL of 0.1 M phosphate buffer (pH 7.4), 20 µL of 3 mM DTNB, 20 µL of AChE enzyme solution, and 20 µL of test samples or standard at concentrations of 10, 20, 40, 80, 160, and 320 µM for captopril or 10, 20, 40, 80, 160, and 320 µg/mL for donepezil. For the compound interference control, the enzyme was replaced with buffer while maintaining the test compound and substrate. A blank control lacking both enzyme and test compound was included in each run. The plates were pre-incubated for 15 mins at room temperature in the dark to allow enzyme-inhibitor interaction. The reaction was initiated by adding 20 µL of 5 mM acetylthiocholine iodide (ATChI). Immediately after substrate addition, the absorbance was measured at 412 nm for 5 min using a microplate reader. The rate of increase in absorbance (ΔA/min) was obtained from the linear portion of the kinetic curve. Non-enzymatic hydrolysis and compound interference were corrected by subtracting the respective blank or compound control values. The percentage inhibition of AChE activity was calculated using the formula:

$$\% \text{ inhibition} = 1 - \frac{\text{Rate sample} - \text{Rate blank}}{\text{Rate control} - \text{Rate blank}} \times 100$$

Preparation of A β solution

The A β solution was prepared by dissolving the synthetic A β 1–42 peptide in a 0.1% ammonia solution to a final concentration of 250 μ M. To prevent pre-aggregation, the solution was sonicated in an ice bath for 5 mins, divided into 5 cycles of 1 min each. The A β solution was diluted to 25 μ M in a buffer containing 50 mM phosphate (pH 7.5) and 100 mM NaCl.

Thioflavin T fluorescence assay

The Thioflavin (ThT) fluorescence assay was conducted to monitor the rate of fibril formation. A mixture of A β solution (8 μ L) and varying concentrations of captopril (10–320 μ M) or donepezil (10–320 μ g/mL) was added to a ThT solution (1.6 mL) containing 55 μ M ThT and 50 mM NaOH-glycine buffer (pH 8.5). The samples were incubated at 37°C, and the fluorescence levels of the samples were measured using a Biotek Synergy H4 hybrid multimode reader with excitation and emission wavelengths of 446 nm and 490 nm, respectively (15).

Molecular docking analysis

Molecular docking analysis was conducted to explore the binding characteristics of captopril with protein targets linked to AD. The 3D structure of captopril (CID: 44093) was sourced from the PubChem database. Similarly, the structural models of crucial protein targets, including β -amyloid (PDB ID: 8EZD), AChE (PDB ID: 4PQE), and tau protein (p-tau) (PDB ID: 2MZ7), were obtained from the Protein Data Bank in the RCSB. Proteins

were processed by removing water molecules, adding hydrogen bonds, and other charges. After protein and ligand processing, the grid box was placed over the macromolecules at dimensions X, Y, and Z of 203.0, 213.5, and 232.0, respectively. Finally, docking was performed using the Lamarckian genetic algorithm, and outputs were generated (16). Following the docking process, the optimal conformation of captopril for each protein target was identified, and the molecular interactions were visualised using a PYMOL molecular viewer (17).

Statistical analysis

Data analysis was carried out using GraphPad Prism (version 7.0). Based on triplicate experiments, results were reported as Mean $\pm$ SEM, and IC₅₀ values were calculated using linear regression. Differences among means were evaluated using a two-way ANOVA, with a significance threshold of $p < 0.001$. Comparisons with control groups were performed using the Holm-Sidak Test.

RESULTS

DPPH radical scavenging assay

Captopril's free radical scavenging activity was carried out using DPPH radical scavenging assay, ABTS radical scavenging assay, and xanthine oxidase inhibitory assay (Table 1). The results of the free radical scavenging assays exhibited significant ($p < 0.001$) inhibition of the free radical formation at the highest concentration of 320 μ M. A maximum inhibition of $> 75\%$ was observed in all three assays, compared with standard ascor-

Table 1. In vitro antioxidant and neuroprotective activities of captopril compared with standard reference compounds

Parameters	Captopril (IC ₅₀)	Standards (IC ₅₀)
DPPH free radical scavenging activity	71.99 $\pm$ 0.40 μ M	92.32 $\pm$ 0.26 μ M (ascorbic acid)
ABTS radical scavenging activity	106.3 $\pm$ 0.28 μ M	110.1 $\pm$ 0.24 μ M (ascorbic acid)
Xanthine oxidase inhibitory activity	> 320 μ M	> 320 μ M (ascorbic acid)
Acetylcholinesterase inhibitory activity	208.2 $\pm$ 0.36 μ M	79.05 $\pm$ 0.25 μ g/mL (donepezil)
β -Amyloid aggregation inhibition assay	87.69 $\pm$ 0.25 μ M	> 320 μ M (donepezil)

The table presents IC₅₀ values (Mean $\pm$ SEM) for DPPH and ABTS radical scavenging, xanthine oxidase inhibition, acetylcholinesterase inhibition, and β -amyloid aggregation inhibition assays. DPPH, diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

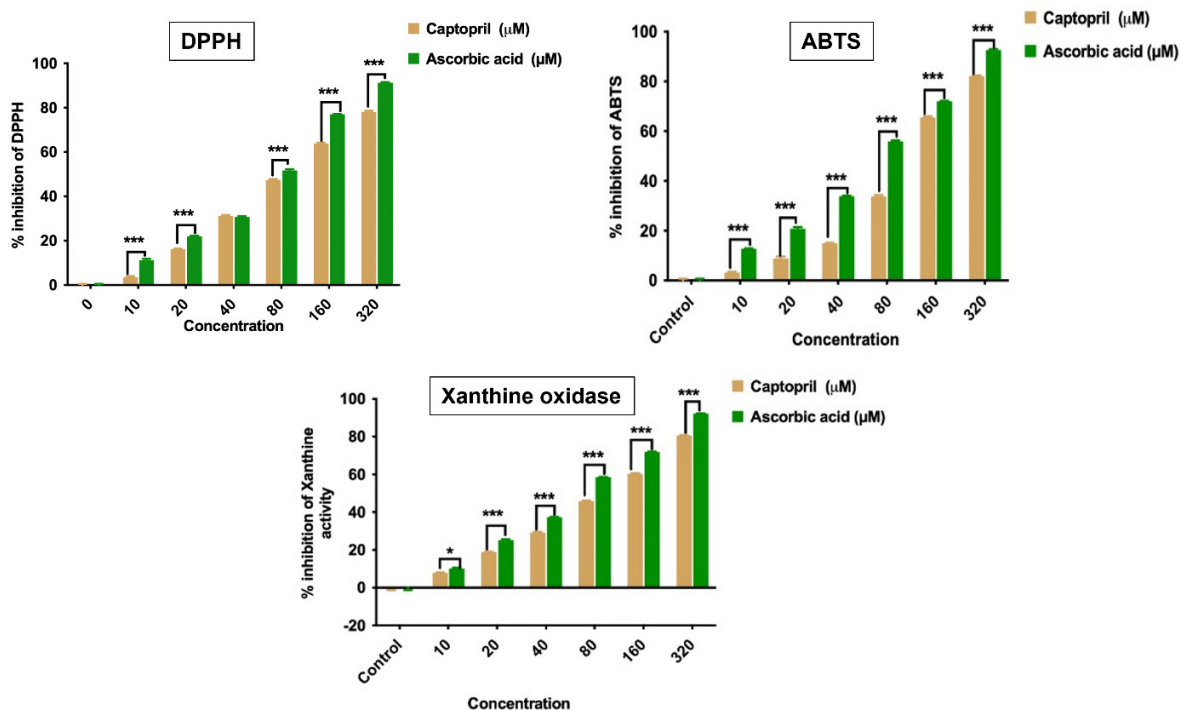


Figure 1. Free radical scavenging effect of captopril at different concentrations compared with standard ascorbic acid. Data are expressed as Mean $\pm$ SEM of triplicate values. Two-way ANOVA was used to assess differences between means at *** $p < 0.001$, and * $p < 0.05$ levels of significance vs. standard ascorbic acid. The means were compared with the standard group using the Holm-Sidak Test.

bic acid, which demonstrated $> 90\%$ inhibition at a similar concentration. The % inhibition of free radical formation exhibited by captopril was concentration-dependent (Figure 1).

In vitro AChE activity

To assess the impact of captopril on AChE activity, various concentrations (10–320 μM) of captopril and the standard donepezil hydrochloride were combined with acetylthiocholine iodide, DTNB, and AChE enzyme. Substantial inhibition of AChE activity was evident across all tested concentrations for captopril and donepezil (Figure 2). Captopril had significantly lower inhibitory activity than donepezil at concentrations ranging from 40 to 320 μM ($p < 0.001$). The inhibitory activity of captopril ranged from 6.98% to 87.03% across the concentration range of 10–320 μM , while donepezil exhibited inhibitory activity in the range of 6.73% to 91.00%.

Effect of captopril on amyloid ($\text{A}\beta$ 1, 42) peptide aggregation

The assessment of captopril's impact on $\text{A}\beta$ (1–42) aggregation revealed a notable inhibition

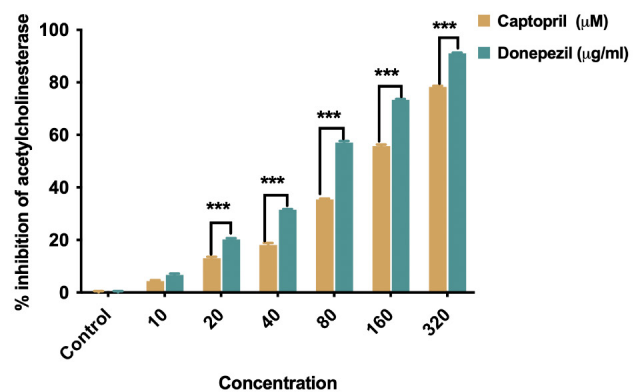


Figure 2. In vitro AChE inhibitory effect of captopril at different concentrations compared with standard donepezil hydrochloride. Data are expressed as mean $\pm$ SEM of triplicate values. Two-way ANOVA was used to assess differences between means at *** $p < 0.001$, levels of significance vs. donepezil. The means were compared with the standard group using the Holm-Sidak Test.

compared with the standard donepezil. At 320 μM concentration, captopril significantly suppressed $\text{A}\beta$ (1, 42) aggregation by 14.45%, while donepezil, at a similar concentration, exhibited 12.22% aggregation. This indicates a substantial reduction in amyloid aggregation by captopril. Concentra-

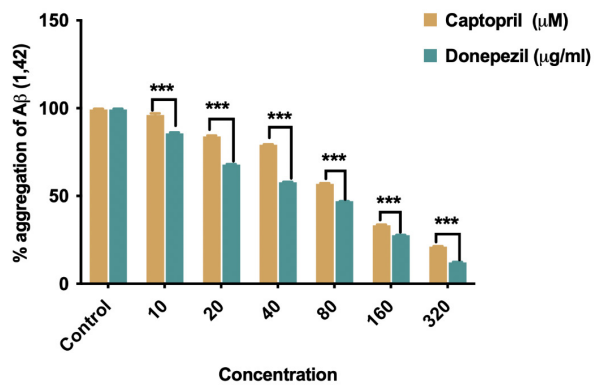


Figure 3. In vitro amyloid A β (1,42) inhibitory effect of captopril at different concentrations compared with standard donepezil hydrochloride. Data are expressed as mean $\pm$ SEM of triplicate values. Two-way ANOVA was used to assess differences between means at *** $p < 0.001$, levels of significance vs. donepezil. The means were compared with the standard group using the Holm-Sidak Test.

tions ranging from 10 to 320 μ M showed a significant ($p < 0.001$) difference in inhibitory activity between captopril and donepezil (Figure 3). Despite this, captopril's inhibitory effect on amyloid aggregate formation was comparable to that of donepezil.

Molecular docking

The docking analysis of captopril with various target proteins revealed differing binding energies and inhibition constants (Figure 4). For beta-amyloid, the captopril formed hydrogen bonds at the binding site at ALA-21 and PHE-20, with a binding energy of -3.73 kcal/mol and an inhibition constant (K_i) of 1.86 mM. In the case of AChE, captopril formed a h-bond at PHE-295 and ARG-296 with a notably lower binding energy of -6.11 kcal/mol and a significantly higher K_i of 33.01 μ M. For phosphorylated p-tau, captopril binds to LYS-267 with a binding energy of -3.83 kcal/mol and a K_i of 1.55 mM. The docking analysis results are shown in Table 2.

DISCUSSION

The present study's results demonstrated captopril's significant antioxidant and neuroprotective properties using an in vitro enzyme inhibition assay, suggesting its potential as a therapeutic agent for neurodegenerative conditions (18). Previous studies have demonstrated that captopril's neuroprotective ability is attributed to its free radical scavenging property (19). An earlier study showed that captopril exhibited a pro-

Table 2. Molecular docking interactions of the ligand with Alzheimer's disease-related target proteins.

Target protein	Binding interactions (h-bond)	Binding energy range	Inhibition constant
Beta-amyloid	ALA-21 (2.115 Å), PHE-20 (2.045 Å) UNL-1 (2.121 Å)	-2.59 to -3.73	1.86 mM
AChE	UNL-1 (2.191 Å) PHE-295 (2.001 Å) ARG-296 (2.199 Å)	-3.2 to -6.11	33.01 μ M
p-tau	LYS-267 (1.954 Å)	-2.68 to -3.83	1.55 mM

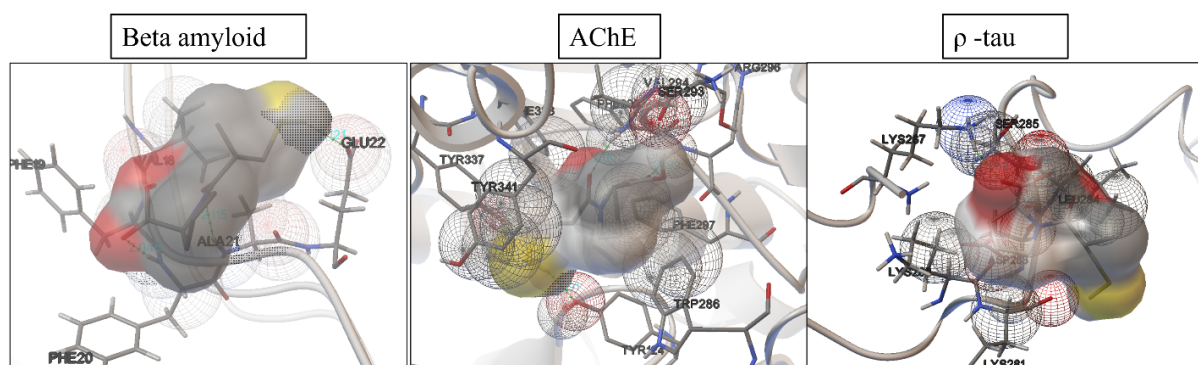


Figure 4. Representative image of molecular docking for captopril against beta-amyloid, AChE, and p- tau receptors

portional response to concentration in the FRAP assay, demonstrating specific antioxidant activity (20). Similarly, another study revealed that captopril attenuated hemolysis induced by free radicals, 2,2'-azobis(2-amidinopropane) (AAPH), and hypochlorite. However, other ACE inhibitors failed to inhibit free radical product formation, suggesting that the unique thiol group present in captopril may account for its antioxidant effect (19). In agreement with the earlier findings, the present findings also substantiate captopril's free radical scavenging capacity at all the tested DPPH, ABTS, and xanthine oxidase assay systems. The antioxidant activity of captopril has been shown to alleviate oxidative stress, thereby reducing the likelihood of the onset of a cascade of events responsible for neurodegeneration.

In AD, a prominent feature is the presence of AChE, often associated with β -amyloid plaques and neurofibrillary tangles (NFT). AChE appears to interact directly with amyloid- β , potentially enhancing its accumulation in insoluble plaques. This newfound function implies that AChE inhibitors could serve as disease-modifying agents (21). Corroborating the given hypothesis, the present study showed that captopril efficiently inhibited AChE activity, suggesting that it might arrest the progression to amyloid accumulation, tau phosphorylation, and the development of AD. Earlier studies have revealed the significant neuroprotective effect of captopril against AD. Captopril has been shown to slow down the accumulation of A β plaques in the hippocampus of mice with AD (7). Captopril treatment not only normalised the elevated levels and activity of the ACE protein associated with AD but also decreased angiotensin II accumulation in the hippocampus and reduced ROS generation. Further, treatment with captopril fully reversed hippocampal neurodegeneration (22). In line with these findings, the present study also substantiates that captopril markedly reduces amyloid peptide aggregation, thereby demonstrating its effectiveness as an Alzheimer's drug.

The binding energies and inhibition constants indicate how effectively captopril binds and potentially inhibits these target proteins implicated in AD. Negative binding energies indicate favourable interactions, with AChE showing the most energetically favourable binding among the three proteins. Additionally, captopril's lowest K_i value

against AChE suggests that comparatively very low concentrations are required for effective inhibition. However, the concentration (33.01 μ M) obtained is higher than the reported concentration of the established AChE inhibitor, donepezil, which showed an IC_{50} value of 0.71 μ M (23). Structurally, a key conserved interaction was identified among captopril, coumarin, and donepezil that forms a hydrogen bond with the backbone of the pivotal PHE-295 residue within the active site. This common binding feature suggests the involvement of a shared molecular recognition motif, likely a C=O $\cdots$ H-N hydrogen bond, which may contribute to anchoring both ligands in a favourable orientation (23). On the other hand, the high K_i values observed with p-tau and β -amyloid suggest that higher captopril concentrations may be required to inhibit these targets. These differences highlight the specificity of drug-protein interactions and indicate that captopril could have varied therapeutic potential against different pathogenic targets in AD.

Comprehending the antioxidant and neuroprotective qualities of captopril in vitro holds significance for possible therapeutic approaches in neurodegenerative conditions like Parkinson's and AD (24). Given its complex pharmacological effects, investigation of captopril as an adjuvant therapy in conjunction with conventional treatments for neurological conditions may be taken into consideration. More studies are required to clarify the precise molecular mechanisms by which captopril provides its neuroprotective and antioxidant benefits.

Although captopril may exhibit neuroprotective effects, further research is needed to determine the specificity and applicability of these effects across a range of neurological disorders and diseases. The mechanisms underlying neuroprotection are diverse and can differ among various pathologies. More preclinical and clinical research is required to determine the precise neuroprotective effects of captopril in humans.

CONCLUSIONS

In conclusion, the present study reinforces captopril's significant antioxidant and neuroprotective properties, particularly in neurodegenerative diseases such as Alzheimer's. The study's findings align with previous research, demonstrating captopril's ability to scavenge free radi-

cals, inhibit AChE activity, and reduce amyloid peptide aggregation, all of which are critical in slowing the progression of Alzheimer's. The binding energy analysis further highlights captopril's potential to target specific proteins associated with the disease, particularly p-tau, suggesting its therapeutic potential at lower concentrations. While these in vitro results are promising, further preclinical and clinical studies are needed to fully elucidate captopril's mechanisms of action and its potential as a neuroprotective agent across various neurological disorders. The potential of captopril as an adjuvant therapy alongside conventional treatments warrants additional investigation to optimise its use in clinical settings.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

V.R.S.H.: investigation, data curation, formal analysis, writing — original draft; R.P.P.: conceptualization, designing, methodology, investigation, data curation, formal analysis; R.M.: conceptualization, designing, methodology, investigation, data curation, formal analysis; A.R.: conceptualization, designing, data curation, formal analysis, resources, supervision, writing — review & editing.

All authors consent to the publication of the identifiable details of the text.

DATA AVAILABILITY STATEMENT

The original findings of this study are provided in the article and the supplementary material. For additional inquiries, please contact the corresponding authors.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable

INFORMED CONSENT STATEMENT

Not applicable

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Influence of the Frequency of Speech Therapy on the Velopharyngeal Gap in Cleft Lip and Palate Patients After Primary Palatoplasty

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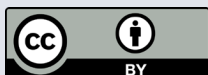
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ABSTRACT

OBJECTIVE The craniofacial conditions of cleft lip and cleft palate (CLP) have been found to be common worldwide, especially in Asia and Thailand. Nevertheless, there are few studies of the association between the frequency of speech therapy and the impact on the velopharyngeal (VP) gap and speech outcome. This study aimed to evaluate the association between the frequency of speech therapy and the VP gap after primary palatoplasty.

METHODS This retrospective cohort study collected data on 95 patients with CLP or isolated cleft palate who underwent primary palatoplasty at Maharaj Nakorn Chiang Mai Hospital between 2006 and 2018. Log risk regression analysis was carried out to identify the effect of frequency of speech therapy in reducing the VP gap including relative risk (RR) and 95% confidence interval (95%CI).

RESULTS From univariable analysis, influence of speech therapy frequency on the presence of a VP gap showed a crude RR = 0.70 (95%CI = 0.49-0.98). Additionally, the frequency of speech therapy in the multivariable analysis showed an adjusted RR = 0.74, 95%CI = 0.46-1.19. The associated anomalies and syndromes showed a statistically significant correlation with a VP gap (adjusted RR = 1.60, 95%CI = 1.12-2.28).

CONCLUSION Patients who receive speech therapy 5-10 times or more showed a decrease in the incidence of a VP gap of at least 26%; however, this did not reach statistical significance. Nevertheless, it is recommended that speech therapy with cleft lip and cleft palate patients should be continued for at least 5 to 10 sessions.

KEYWORDS frequency of speech therapy, velopharyngeal gap, primary palatoplasty, cleft lip and cleft palate, isolated cleft palate

INTRODUCTION

Cleft lip and cleft palate (CLP) are deformities of the lips and palate that develop during pregnancy (1). CLP is recognized as a common craniofacial disease worldwide (1:700), especially in Asia

and Thailand. According to the 2012 Thai population database, the incidence of CLP in Thailand is 1 to 1.5 per 1,000 live births, which equates to about 800 to 1,200 newborns per year. The incidence of CLP in Chiang Mai has been recorded as

having an incidence of 3.9 per 1,000 live births (2).

CLP is related to facial functions including hearing, swallowing and feeding as well as problems of malocclusion. CLP has also been found to have an effect on physical health (e.g. infection, oral and dental health) and the psychological health of both the individual and family. In addition to facial function and overall patient and family health, poor speech outcome and speech development have been found to be the most common problems in CLP patients (3). More than half of CLP patients need speech therapy (Figure 1). Speech therapy is a critical therapeutic modality for reducing velopharyngeal gap and achieving good speech outcomes in cases of cleft palate. It constitutes the first-line intervention in patients presenting with a velopharyngeal gap or unsatisfactory speech outcome.

For the purpose of optimizing speech outcome of patients, both speech therapy and surgery are essential (4). With regard to surgery, palatoplasty, the primary surgical correction for cleft palate, is usually performed at between 9 to 12 months of age to ameliorate cleft palate deformities (5). This early intervention can help normalizing speech and language as well as reducing hearing and psychological problems (6). Outcomes after palatoplasty are commonly assessed by nasal emission, nasal tone of voice, articulatory defects and nasal resonance. However, these measurements of speech outcomes are operator-dependent, indicating a degree of subjectivity in the assessment of speech outcomes.

A numerical measurement, post-operative velopharyngeal gap (VP gap) which is the length

between soft palate and posterior pharynx (Figure 2A and 2B), would be useful in order to evaluate the speech outcome more precisely. Previous studies have found that the speech outcome and VP gap are significantly associated (7-9). In this study, the VP gap was investigated as a reflection of speech outcome and used as an outcome variable in this study.

With regard to factors that are related to VP gap and speech outcomes, studies have shown factors that have an impact on the VP gap and speech outcome include age at primary palatoplasty, type of cleft and associated syndromes (10-12). However, previous studies have been very limited with regard to demonstrating any association between frequency of speech therapy and VP gap. Relationships between Speech outcome and clear-cut points of frequency of speech therapy for CLP patients have not yet been clarified (12). This study was conducted to investigate the association between the frequency of speech therapy and the VP gap and to improve protocol regarding the frequency of speech therapy in order to minimize VP gap in CLP patients after primary palatoplasty (13).

METHODS

Study design

This is a retrospective cohort study. The inclusion criteria included CLP or isolated cleft palate patients who underwent primary palatoplasty from 2006 to 2018 at Princess Sirindhorn IT Foundation Craniofacial Center, Chiang Mai University. Only patients who had a successful initial nasendoscopy were included in the study. Patients

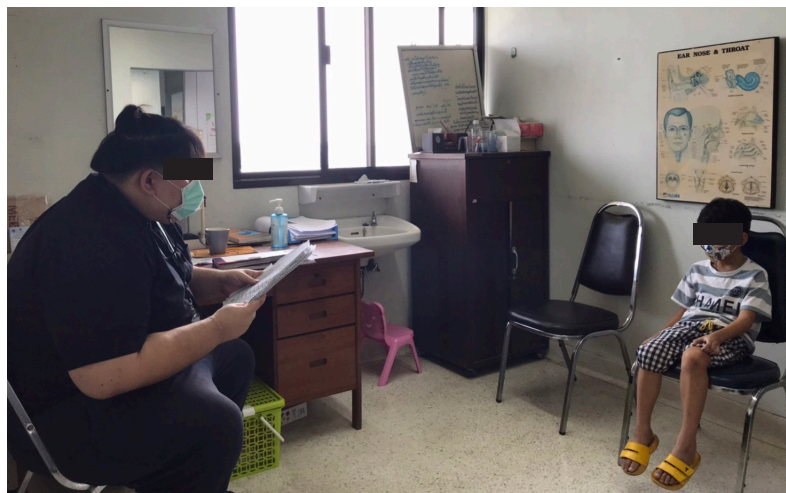


Figure 1. Post operative speech therapy

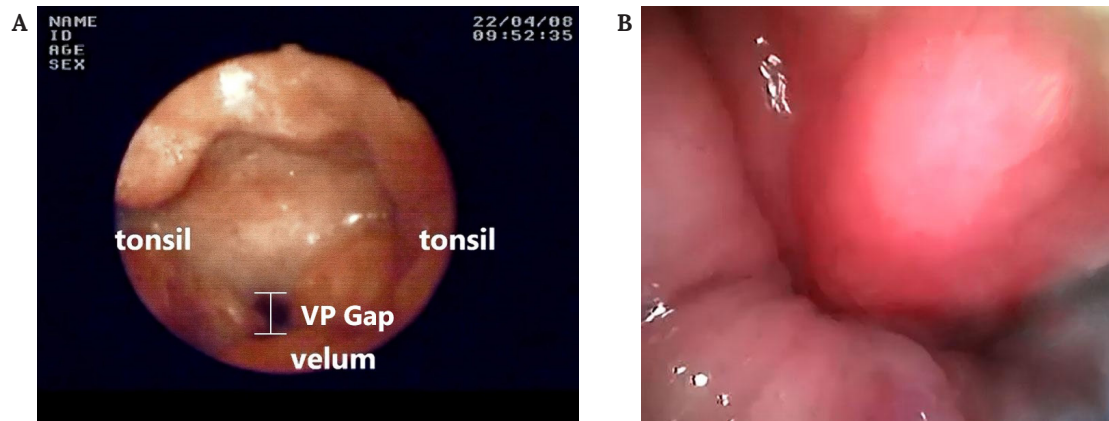


Figure 2. (A) Presence of VP gap in cleft lip and cleft palate patient, (B) not presence of VP gap in cleft lip and cleft palate patient

with incomplete data were excluded. The surgical procedures were performed by a single plastic surgeon with extensive experience in palatoplasty. The surgeon consistently performed the Bardach two-flap palatoplasty technique as the operative method. Following this center's protocol, nasendoscopic evaluations are performed at age 5 years and above.

Ethical considerations

The present study has been approved by the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University, certificate of ethical approval number 486/2021.

Data collection

Data was collected from the medical records of Maharaj Nakorn Chiang Mai Hospital including both the Digicard program and data from the Princess Sirindhorn IT Foundation Craniofacial Center, Chiang Mai University. All data was recorded on Microsoft Excel spreadsheets.

Sample size calculation

Based on a pilot review of 30 cases from the database of Princess Sirindhorn IT Foundation Craniofacial Center, Chiang Mai University, the required sample size needed to address the main research question, i.e., the frequency of speech therapy in both groups (with and without VP gap), was calculated as follows:

- Estimated sample size for a two-sample means test
- Satterthwaite's t test assuming unequal variance

- $H_0: M_2 = M_1$ versus $H_a: M_2 \neq M_1$
- Study parameters
 - o $\alpha = 0.05$, power = 0.80, delta = 8.72
 - o $M_1 = 5.69$ (Mean frequency of speech therapy in the gap group)
 - o $M_2 = 14.41$ (mean frequency of speech therapy in the no gap group)
 - o $SD_1 = 5.73$ (SD of the frequency of speech therapy in the gap group)
 - o $SD_2 = 15.85$ (SD of the frequency of speech therapy in the no gap group)
 - o $N_1/N_2 = 1.00$
- Estimated sample sizes:
 - o $N = 62$ (Total sample size)
 - o $N_1 = 31$ (patients in the no gap group)
 - o $N_2 = 31$ (patients in the gap group)
- The investigator recruited a sample of 62 cases

Statistical analysis

The categorical data is shown as frequency and percentage. Fisher's exact test was used for comparison between groups. Continuous data is shown as mean and standard deviation (normal distributions) or median and interquartile range (non-normal distributions), and were compared using Student's t-test or Wilcoxon's rank-sum test, respectively. Log risk regression (univariable and multivariable analysis) was performed to identify the effect of frequency of speech therapy on reducing the velopharyngeal gap, and also to adjust for potential confounders. A p -value < 0.05 was considered statistically significant. STATA version 16.0 was used for data analysis.

RESULTS

A total of 95 patients who received palatoplasty at Maharaj Nakorn Chiang Mai Hospital between 2006 and 2018 were recruited into this study. Of those patients, 56 patients (58.95%) presented with a VP gap after the first successful nasendoscopy for post primary palatoplasty and 39 patients (41.05%) did not present with a VP gap. The gender balance of patients was 52 males and 43 females. The age at primary palatoplasty, cleft type, gender and distance from speech therapy center to patients' address between patients who presented with VP gap and those who did not present with VP gap were not statistically significantly different. However, there was a statistically significant difference in the presence of associated anomalies between the two groups. The presence of a VP gap in patients with and without associated anomalies was 100% and 58.95%, respectively ($p = 0.039$). The number of patients who first started speech therapy before 2019 (80.00%) and those who started therapy in or after 2019 (20%) was also statistically significant ($p = 0.038$). (Table 1).

Among patients who received their first speech therapy before 2019, 94.03%, 70.59% and 9.09% had a frequency of speech therapy of < 5 times, 5-10 times and > 10 times, respectively. Of patients who had their first therapy in 2019 or later, the frequency of speech therapy was < 5 times (5.97%), 5-10 times (29.41%) and > 10 times

(90.91%), respectively. The difference between the date of the first speech therapy and the frequency of speech therapy between patients who underwent their first speech therapy before 2019 and those who had their first therapy in 2019 or later was significant ($p < 0.001$) (Table 2). Two postoperative factors were evaluated: the type of surgery and the frequency of speech therapy, the independent factors in this study. The patients who underwent palatoplasty and cheilo-palatoplasty developed a VP gap, however the outcome was not statistically significant. The frequency of speech therapy was divided into three groups: < 5 times, 5-10 times and > 10 times. In the group of patients who received speech therapy < 5 times, the frequency of a VP gap was 65.67%. In the group which received speech therapy 5-10 times, 52.94% presented with a VP gap. Among the patients who had more than 10 sessions of speech therapy, only 27.27% had a VP gap ($p = 0.054$) (Table 3).

Univariable analysis found that factors associated with the presence of a VP gap included the frequency of speech therapy, type of cleft palate and the time of first speech therapy. Frequency of speech therapy (crude RR = 0.70, 95%CI = 0.49-0.98, $p = 0.040$) and of associated anomalies (crude RR = 1.80, 95%CI = 1.49-2.16, $p < 0.001$) significantly affected the manifestation of the VP gap.

Table 1. Baseline characteristics of the study population

Characteristics	Patients with VP gap (n = 56)	Patients without VP gap (n = 39)	p-value
Age at primary palatoplasty (year), median (IQR)	1.20 (0.99-1.74)	1.20 (0.98-1.43)	0.586
Gender, n (%)			1.000
Male	31 (59.62)	21 (40.38)	
Female	25 (58.14)	18 (41.86)	
Cleft type, n (%)			0.312
Isolated cleft palate (including soft palate)	14 (73.68)	5 (26.32)	
Unilateral CLP	28 (52.83)	25 (47.17)	
Bilateral CLP	14 (60.87)	9 (39.13)	
Associated anomalies or syndromes, n (%)			0.039
Yes	7 (100.00)	0 (0.00)	
No	49 (58.95)	39 (41.05)	
Distance from speech therapy center (km), median (IQR)	109 (34.4-177)	86 (47.2-136)	0.292
The time of speech therapy, n (%)			0.038
Before 2019	49 (64.47)	27 (35.53)	
Since 2019	7 (36.84)	12 (63.16)	

IQR, interquartile range; VP gap, velopharyngeal gap; CLP, cleft lip and cleft palate

The time of first speech therapy was associated with the gap presentation as a crude RR = 0.57, 95%CI = 0.31-1.06, but the association was not statistically significant ($p = 0.075$). All the prognostic factors were adjusted in the multivariable analysis between the VP gap and non-VP gap groups. Greater frequency of speech therapy was

associated with a slightly reduced the presence of a VP gap with the relative risk being 0.74, 95% CI = 0.46-1.19, but these findings were again not statistically significant ($p = 0.557$) (Table 4).

DISCUSSION

The main focus of this study was independent factors, including the frequency of speech therapy, and how that frequency affected the VP gap among the patient groups. It was found that the greater the frequency of speech therapy, the lower the percentage of patients who presented with a VP gap, as shown in Table 3. Among patients who received speech therapy fewer than five times, 34.33% did not develop a VP gap, whereas 72.73% of those who received more than ten sessions did not present with a VP gap. Univariable analysis

Table 2. Association between period of first speech therapy and frequency of speech therapy

Frequency of speech therapy	The time of first speech therapy, n (%)		p-value
	Before 2019	Since 2019	
< 5 times	63 (94.03)	4 (5.97)	< 0.001
5-10 times	12 (70.59)	5 (29.41)	
> 10 times	1 (9.09)	10 (90.91)	

Table 3. Postoperative factors

Characteristics	Patients with VP gap (n = 56)	Patients without VP gap (n = 39)	p-value
Type of surgery, n (%)			0.137
Palatoplasty	37 (66.07)	19 (33.93)	
Cheilopalatoplasty	19 (48.72)	20 (51.28)	
Frequency of speech therapy, median (IQR)	2 (1-4)	4 (1-9)	0.054
< 5 times, n (%)	44 (65.67)	23 (34.33)	
5-10 times, n (%)	9 (52.94)	8 (47.06)	
> 10 times, n (%)	3 (27.27)	8 (72.73)	

IQR, interquartile range; VP gap, velopharyngeal gap

Table 4. Association between frequency of speech therapy among patients with and without velopharyngeal gap and adjusted prognostic factors

Prognostic factors	Univariable analysis			Multivariable analysis		
	Crude RR	95%CI	p-value	Adjusted RR	95%CI	p-value
Frequency of speech therapy	0.70	0.49-0.98	0.040	0.74	0.46-1.19	0.557
< 5 times						
5-10 times						
> 10 times						
Age at palatoplasty	1.04	0.98-1.10	0.204	1.02	0.96-1.10	0.476
Gender	1.03	0.73-1.44	0.885	1.27	0.67-2.40	0.462
Type of cleft palate						
Isolated cleft palate (including soft palate)	1.00			1.00		
	(reference)			(reference)		
Unilateral CLP	0.72	0.83-1.36	0.309	0.86	0.39-1.89	0.701
Bilateral CLP	0.83	0.39-1.73	0.613	1.09	0.42-2.81	0.858
Type of surgery	1.36	0.93-1.97	0.111	1.22	0.80-1.85	0.355
Distance from speech therapy center	1.00	0.99-1.00	0.297	1.00	1.00-1.01	0.257
Associated anomalies and syndromes	1.80	1.49-2.16	< 0.001	1.60	1.12-2.28	0.009
The time of first speech therapy since 2019	0.57	0.31-1.06	0.075	0.72	0.35-1.50	0.385

CLP, cleft lip and cleft palate

found the frequency of speech therapy was significantly associated with the VP gap ($p = 0.040$). Adding emphasis to this point, the RR values from the univariable and multivariable analyses were nearly identical (crude RR = 0.70; adjusted RR = 0.74).

The factors that significantly impacted the presence of a VP gap were associated anomalies which is consistent with the findings of a study by Tache, et al. and Sie, et al. (10, 11). The associated anomalies and syndrome included the Pierre Robin sequence, VACTERL syndrome, and congenital cardiac valve defects. According to a study by Kocaaslan, et al, the Pierre Robin sequence is associated with more significant velopharyngeal insufficiency compared to patients without this sequence (14).

Furthermore, the timing of first speech therapy showed a statistically significant correlation with the VP gap. This factor was included because of the establishment of the Princess Sirindhorn IT Foundation Craniofacial Center at Chiang Mai University in 2019. That center provided an increased number of speech therapy sessions. The frequency of speech therapy was significantly increased after the center was inaugurated ($p < 0.001$) (Table 2). Although the adjusted RR in the multivariable analysis indicated that a higher frequency of speech therapy decreased the likelihood of a VP gap, this relationship did not reach statistical significance (Table 4).

The frequency of speech therapy in our center was considered to be an important factor as the proportion of patients who had received therapy sessions since 2019 remained low (20.00% of all patients). Thus the number of patients who had speech therapy more than 10 times much lower than the other groups (< 5 times and 5-10 times). Future studies should include more samples with over 10 therapy sessions. A multi-center collaboration could potentially enhance the evaluation of the significance of the association between frequency of speech therapy and the presence of a VP gap. Additionally, if the patients had undergone nasendoscopy before speech therapy, the reduction in the length of the VP gap could have been evaluated more definitively, allowing a clearer determination of the true effect of speech therapy.

CONCLUSIONS

Patients who received speech therapy either 5-10 times or more than 10 times showed at least a 26.00% reduction in the presence of a VP gap, although this did not reach statistical significance. However, it is recommended that speech therapy be provided at current levels in clinical practice for patients with cleft lip and cleft palate.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

S.J.: investigation, data curation, formal analysis, writing — original draft preparation, writing — review & editing; C.K.: investigation, writing — original draft preparation; W.K.: methodology, formal analysis, validation, writing — review & editing; N.S.: conceptualization, methodology, writing — review & editing; K.K.: conceptualization, methodology, resources, writing — review & editing, supervision, project administration

All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The present study has been approved by the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University, bearing the certificate of ethical approval number 486/2021.

INFORMED CONSENT STATEMENT

Informed consent was not obtained from all participants, as the study was conducted retrospectively.

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Comparison of Contrast-Enhanced MRI Using Hepatocyte-Specific Contrast with Contrast-Enhanced CT in Evaluation of Hepatobiliary Space-Occupying Lesions

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ABSTRACT

OBJECTIVE This study aimed to evaluate the diagnostic accuracy of multiphasic contrast-enhanced computed tomography (CECT) and contrast-enhanced magnetic resonance imaging (CEMRI) in assessing hepatic mass lesions.

METHODS A cohort of 120 patients underwent imaging with both CEMRI and CECT to compare their diagnostic capabilities. Findings were analyzed for lesion size, enhancement patterns, sensitivity, specificity, and predictive values. Statistical tests were conducted to assess the significance of differences between the modalities, with *p*-values reported for critical metrics. To ensure consistency and minimize bias, a standardized imaging protocol was followed, and radiology experts independently reviewed all images.

RESULTS CEMRI demonstrated higher sensitivity (90%) and specificity (87%) compared to CECT (85% and 80%, respectively), with statistically significant differences (*p* < 0.05). CEMRI also outperformed CECT in positive predictive value (88% vs. 82%) and negative predictive value (90% vs. 84%). While the mean lesion size detected by CEMRI (4.0 ± 2.0 cm) was slightly smaller than that detected by CECT (4.4 ± 2.2 cm), the difference was not statistically significant (*p* = 0.45). Additionally, CEMRI identified 112 lesions compared to 108 lesions detected by CECT, further highlighting its superior diagnostic accuracy, particularly in detecting heterogeneous lesions.

CONCLUSION CEMRI outperforms CECT in diagnostic sensitivity, specificity, and predictive values, making it the preferred modality for lesion detection and characterization. However, both modalities performed similarly in assessing lesion size and total lesion count. These findings underscore the value of CEMRI in clinical scenarios requiring detailed lesion evaluation. Future studies with larger cohorts and a broader range of pathologies are recommended to confirm and extend these results for greater clinical applicability.

KEYWORDS contrast-enhanced computed tomography, contrast-enhanced magnetic resonance imaging, diagnostic accuracy, lesion characterization, imaging modalities

INTRODUCTION

Hemangiomas and benign adenomas are examples of liver mass lesions, alongside malignant forms such as hepatocellular carcinoma and metastatic disease (1). Accurate diagnosis and categorization of these lesions are essential for guiding clinical management, including treatment planning and prognosis evaluation, forming the basis of effective patient care (2). Advances in modern imaging techniques, particularly contrast-enhanced magnetic resonance imaging (CEMRI) and multiphasic contrast-enhanced computed tomography (CECT), have significantly improved the detection and characterization of liver abnormalities (3).

CECT has long been the preferred imaging method for diagnosing hepatic disorders due to its widespread availability, fast data acquisition, and high spatial resolution (4, 5). This modality captures images during arterial, portal venous, and delayed phases of contrast enhancement, enabling differentiation of lesion types based on enhancement patterns. However, CECT is associated with potential risks, such as ionizing radiation exposure and nephrotoxicity caused by iodinated contrast agents (6, 7).

CEMRI, on the other hand, offers superior contrast resolution for soft tissues while minimizing radiation exposure. Recent technological advancements, such as hepatobiliary phase imaging and diffusion-weighted imaging, have further enhanced the ability to detect and characterize liver lesions (8). These innovations, coupled with liver-specific contrast agents, have made CEMRI particularly effective in identifying and describing lesions. Studies suggest that CEMRI outperforms CECT in diagnostic accuracy, especially for small lesions with complex enhancement patterns (9).

Research indicates that CEMRI is more sensitive and specific than CECT for diagnosing hepatocellular carcinoma, particularly in patients with cirrhosis (10, 11). Additionally, CEMRI demonstrates superior performance in detecting and characterizing hepatic metastases, particularly those associated with colorectal cancer. Its overall effectiveness makes it a valuable alternative to CECT, enhancing diagnostic accuracy and supporting clinical decision-making (12).

The choice between CEMRI and CECT depends on factors such as the patient's health status, available imaging modalities, and clinical context

(13). For example, CECT may be preferred in emergency settings due to its rapid imaging capabilities, whereas CEMRI is advantageous for patients with kidney impairment due to its reduced nephrotoxicity. To provide evidence-based recommendations for evaluating hepatic mass lesions, a comprehensive comparison of these imaging modalities is essential (14, 15).

This study aimed to compare the diagnostic accuracy of multiphasic contrast-enhanced computed tomography and contrast-enhanced magnetic resonance imaging for assessing hepatic mass lesions.

METHODS

Study design

This study was a 12-month retrospective comparative analysis conducted from December 2022 to November 2023. A total of 120 participants with hepatic mass lesions, diagnosed using either multiphasic CECT or CEMRI, were included. Exclusion criteria included pregnancy, a history of liver surgery, medical conditions contraindicating contrast media, or insufficient medical records. To reduce selection bias in imaging interpretation, radiologists were blinded to clinical data, and consecutive sampling was utilized. Key factors analyzed included imaging modality, lesion size and number, enhancement patterns, and final diagnosis based on histological findings or follow-up imaging.

Data collection

Retrospective data were collected from the facility's electronic health records, which included demographic details, clinical histories, imaging findings, and histology results. Patients diagnosed with hepatic mass lesions were primarily evaluated using multiphasic CECT or CEMRI as the diagnostic modalities of choice. Imaging techniques were standardized, with CEMRI incorporating T1-weighted, T2-weighted, and diffusion-weighted imaging, while CECT included arterial, portal venous, and delayed phases. Diffusion-weighted imaging was also incorporated into the imaging protocols.

Statistical analysis

Data analysis was performed using SPSS version 21.0. Means, frequencies, percentages, and standard deviations were calculated for the varia-

bles. The diagnostic performance of CEMRI and CECT was compared by evaluating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Statistical analysis was carried out using Chi-square tests to compare the modalities, with a significance threshold set at $p < 0.05$.

RESULTS

The demographics and clinical characteristics of the study participants ($N = 120$) are summarized in Table 1. The mean age of the participants was 54.0 ± 11.5 years, indicating a wide age range. Slightly more than half of the participants were male ($n = 70$), while 50 participants were female, suggesting a slightly higher prevalence of the condition under investigation among males. The mean body mass index (BMI) was 26.5 ± 3.0 kg/m², classifying most participants as overweight. Additionally, 65% of the participants had a history of chronic liver disease, emphasizing its relevance within the study cohort.

Table 2 presents the imaging findings using CECT and CEMRI. CECT detected 115 lesions, while CEMRI identified 105 lesions, indicating a higher detection rate for CECT. The mean lesion size identified by CECT was 4.4 ± 2.2 cm, compared to 4.0 ± 2.0 cm detected by CEMRI; however, this difference was not statistically significant ($p = 0.45$). Regarding enhancement patterns, 25% of lesions detected by CECT demonstrated heterogeneous enhancement, while 40% exhibited homogeneous enhancement. Conversely, 62% of lesions identified by CEMRI exhibited heterogeneous enhancement, while 38% demonstrated homogeneous enhancement. These findings underscore differences in the capabilities of the two imaging modalities.

Table 1. Participant demographics and clinical characteristics

Characteristic	Value
Mean age (years), mean $\pm$ SD	54 $\pm$ 11.5
Gender, n (%)	
Male	70 (58.3)
Female	50 (41.7)
Mean BMI (kg/m ²), mean $\pm$ SD	26.5 $\pm$ 3.0
Prevalence of liver disease, n (%)	78 (65.0)

BMI, body mass index

The comparative diagnostic performance of CEMRI and CECT is detailed in Table 3. CEMRI demonstrated a higher sensitivity (85%) compared to CECT (80%), with a statistically significant difference ($p = 0.01$). Similarly, CEMRI exhibited greater specificity (87%) than CECT (80%), indicating its superior ability to identify true negatives ($p = 0.04$). The PPV of CEMRI was 88%, compared to 82% for CECT ($p = 0.03$), demonstrating higher accuracy in identifying true positives. The NPV was also higher for CEMRI (90%) than for CECT (84%), with statistical significance ($p = 0.02$). The number of lesions detected by the two modalities was similar, with CEMRI identifying 112 lesions and CECT detecting 108 lesions ($p = 0.30$).

The receiver operating characteristic (ROC) curve analysis revealed that CEMRI exhibited superior diagnostic efficacy, with an area under the curve (AUC) of 0.89 compared to 0.83 for CECT. This result indicates that CEMRI achieved higher true positive rates with lower false positive rates, reflecting enhanced sensitivity and specificity. While CECT demonstrated strong diagnostic efficacy (AUC = 0.83), it was less accurate than CEMRI in distinguishing true positives from false positives (Figure 1).

Table 2. Imaging findings

Mode of imaging	CECT	CEMRI
Number of lesions	115	105
Mean size (cm)	3.5 $\pm$ 2.0	3.5 $\pm$ 2.0
Enhancement pattern		
Homogeneous	40%	38%
Heterogeneous	60%	62%

CECT, contrast-enhanced computed tomography; CEMRI, contrast-enhanced magnetic resonance imaging

Table 3. Sensitivity and specificity analysis of both imaging modalities

Parameters	CECT (%)	CEMRI (%)	p-value
Sensitivity	85	90	0.01
Specificity	80	87	0.04
PPV	82	88	0.03
NPV	84	90	0.02
Mean lesion size (cm)	4.4 $\pm$ 2.2	4.0 $\pm$ 2.0	0.45
Number of lesions identified	108	112	0.30

CECT, contrast-enhanced computed tomography; CEMRI, contrast-enhanced magnetic resonance imaging; PPV, positive predictive value; NPV, negative predictive value

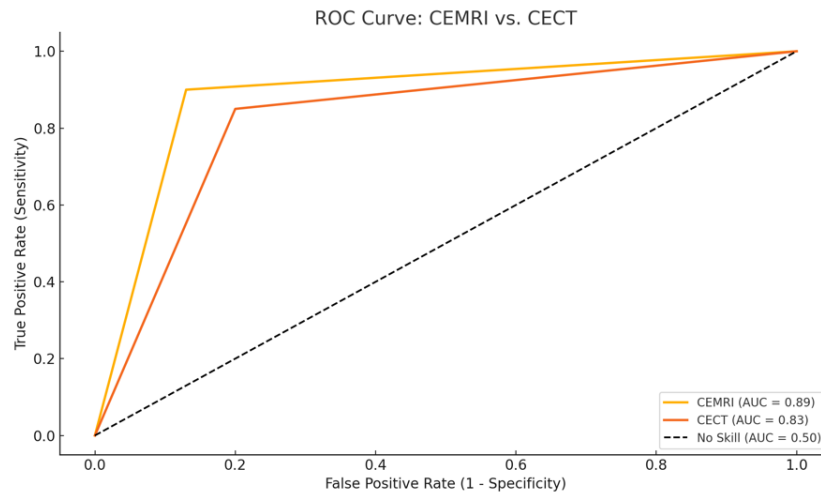


Figure 1. Receiver operating characteristic (ROC) curve

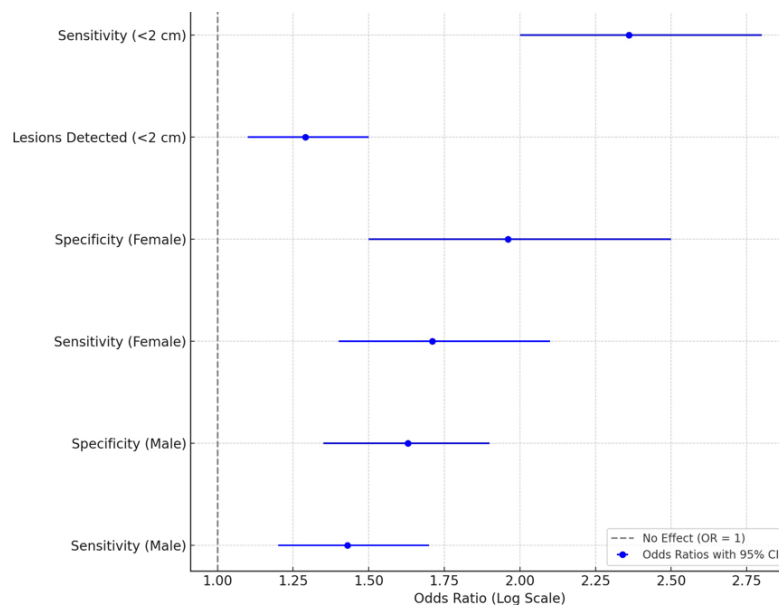


Figure 2. Forest plot

The forest plot analysis further highlighted the diagnostic advantages of CEMRI over CECT across various criteria, including lesion size categories and subgroup analyses. CEMRI demonstrated significantly higher sensitivity for small lesions (< 2 cm), with an odds ratio (OR) greater than 2.0, indicating that it was more than twice as likely as CECT to detect small lesions. This finding was statistically significant, as the 95% confidence interval (CI) did not overlap the no-effect line (OR = 1). Additionally, CEMRI detected more total lesions in this category (OR ~1.25), further confirming its superior performance. Subgroup analyses revealed that CEMRI exhibited significantly higher specificity for female patients (OR ~2.0),

indicating a notable reduction in false positives within this group. Female sensitivity was also considerably higher for CEMRI (OR ~1.7), reflecting its ability to identify true positives. Among male patients, CEMRI demonstrated moderate but consistent superiority in sensitivity (OR ~1.4) and specificity (OR ~1.5). Across all metrics, the confidence intervals for CEMRI remained above the no-effect line, confirming the statistical significance of its superior diagnostic performance. These findings highlighted CEMRI's precision in detecting small lesions and improving specificity in gender-based assessments, establishing it as the preferred imaging modality in clinical practice (Figure 2).

DISCUSSION

This study comprehensively compared CECT and contrast-enhanced magnetic resonance imaging (MRI) to evaluate hepatic space-occupying lesions. The findings highlighted the superior diagnostic capabilities of CEMRI in terms of sensitivity, specificity, and predictive values while acknowledging some comparable aspects between the two modalities.

CEMRI demonstrated higher sensitivity (90%) and specificity (87%) compared to CECT (85% and 80%, respectively), with statistically significant differences ($p < 0.05$). These results align with prior research, emphasizing the enhanced diagnostic accuracy of CEMRI, particularly in detecting hepatic lesions. Studies by Wang et al. and Sica et al. similarly reported higher sensitivity and specificity for CEMRI, attributing this advantage to its superior soft-tissue contrast and hepatobiliary-specific contrast agents (16, 17). Furthermore, the current findings corroborate those by Coenegrachts, who noted that CEMRI excels in identifying smaller or heterogeneous lesions often missed by CECT (18).

The PPV and NPV of CEMRI (88% and 90%, respectively) were superior to those of CECT (82% and 84%, respectively), highlighting CEMRI's reliability in distinguishing true positives and negatives. These findings are consistent with observations by Choi et al., who attributed CEMRI's superior lesion characterization to its ability to depict dynamic enhancement patterns across multiple imaging phases (19).

While the assessment of lesion size revealed no statistically significant differences between the two modalities ($p = 0.45$), with mean lesion sizes of 4.0 ± 2.0 cm for CEMRI and 4.4 ± 2.2 cm for CECT, this finding aligns with research by Kim et al., which reported comparable lesion size measurements between the two techniques (20). However, the slightly higher number of lesions detected by CEMRI (112 vs. 108 by CECT) underscores its ability to identify lesions with subtle enhancement patterns, as supported by prior studies.

A key finding of this study was the results of analysis of lesion enhancement patterns. CEMRI identified a higher proportion of lesions with heterogeneous enhancement (62%) than CECT (60%), highlighting its sensitivity in detecting

complex or mixed lesion textures. This observation is consistent with research by Vogl et al., which emphasized the utility of CEMRI's multi-sequence imaging capabilities and advanced contrast agents in detecting hepatocellular carcinoma (HCC) and metastases (21).

In addition to underscoring its advantages, the study also highlighted some limitations of CEMRI, including longer acquisition times and higher costs compared to CECT. However, the absence of ionizing radiation in CEMRI and its superior soft-tissue resolution offer significant benefits, particularly for patients with chronic liver disease or renal impairment, as noted by Thian et al. (22), although CECT remains a preferred modality in emergency settings due to its rapid image acquisition and greater accessibility.

Limitations

The relatively small sample size of 120 participants limits the generalizability of this study to broader populations or different lesion types. Additionally, the study did not account for potential confounding factors such as advancements in imaging technology, operator expertise, or variations in patient clinical presentations, which may have influenced the findings. The cross-sectional design further restricts the evaluation of the long-term utility of these imaging modalities, including their role in monitoring lesion progression. While specific results achieved statistical significance, their clinical relevance should be interpreted cautiously. Furthermore, as the study focused on a particular type of lesion, the findings may not directly apply to other conditions or imaging techniques. Future research with larger sample sizes, standardized imaging protocols, and longitudinal follow-up is essential to provide more comprehensive insights.

CONCLUSIONS

This study reinforced the superior diagnostic accuracy of CEMRI compared to CECT in detecting hepatic mass lesions. With its higher sensitivity, specificity, and predictive values, CEMRI emerges as the modality of choice for the characterization of liver lesions, particularly in patients with chronic liver disease or cirrhosis. However, CECT remains an invaluable tool for rapid imaging, especially in acute clinical scenarios. Further studies

are recommended to explore the clinical outcomes and cost-effectiveness of these modalities to guide their integration into clinical practice.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

V.P.: conceptualization, formal analysis, writing—review & editing, supervision; S.K.J.: methodology, investigation; S.S.: data curation, writing—original draft.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

This study was conducted in accordance with institutional ethical standards. (Ref. No.: IRB/125/2024).

INFORMED CONSENT STATEMENT

Informed consent was obtained from all individual participants included in the study.

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Performance of Diagnostic Methods for Detection of Drug Resistance Among New Cases of *Mycobacterium tuberculosis*

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ABSTRACT

OBJECTIVE The increasing incidence of new cases of drug resistant-TB (DR-TB) infection is worrisome, with high rates of new pulmonary morbidities occurring mostly among the vulnerable populations. The performance of diagnostic methods of GeneXpert MTB/RIF assay and phenotypic Drug Susceptibility Test (pDST) for detection of drug resistance among new cases of *Mycobacterium tuberculosis* (MTB) infections was evaluated.

METHODS Sputum samples (n = 546) from newly suspected MTB cases were analysed for phenotypic drug resistance using BACTEC MGIT (B-MGIT) and genotyped with xpert MTB/RIF assay. The predictive performance of B-MGIT culture-based methods and xpert MTB/RIF assay for MTB detection were determined.

RESULTS Of the collected sputum samples (n = 546), the highest rates of phenotypic drug resistance (66.7%) were AFB3+ smear positive and MTB with detection rates of 98.2% and 92.7%, respectively, with B-MGIT and Xpert MTB/RIF ($p = 0.63$). B-MGIT detected higher resistance rates to isoniazide (*inh*) and rifampicin (*rif*) of more than 60.0%, and less than 20% resistance to ofloxacin (*ofx*) and protionamide (*proth*) compared to Xpert ($p < 0.05$). B-MGIT had a higher detection rate (98.2%), sensitivity (87.8%), specificity (91.4%), positive predictive value (PPV) (95.8%) (95%CI: 0.778-1.04; $p = 0.001$) and likelihood of detection of rifampicin resistance (OR:5.76;95%CI:1.01-7.56) compared to Xpert MTB/RIF methods. B-MGIT provided higher AUC-rifampicin of 0.9339 (95%CI: 0.8398 to 1.000) than gene xpert (AUC-rifampicin = 0.9019 [95%CI: 0.7899 to 1.000]). B-MGIT is needed for further confirmation of new cases of MTB identified from acid-fast bacilli (AFB) smear positive sputum.

CONCLUSIONS The inclusion of B-MGIT examination of sputum in new TB cases would enhance early detection of drug resistant MTB, mostly from rifampicin negative sputum investigated with GeneXpert and mostly in low resource settings.

KEYWORDS acid-fast bacilli, tuberculosis, MDR-*Mycobacterium tuberculosis*, rifampicin, resistance predictor

INTRODUCTION

Tuberculosis (TB), caused by the *Mycobacterium tuberculosis* (MTB), remains a global health challenge. Continuing health campaigns, detection and treatment have improved in several specific locations in developing countries, particularly in Nigeria (1). In 2020, Nigeria was ranked first in Africa with a rising death rate of up to 1.5 million and 4th globally in reported TB cases (1, 2). The increasing incidence of new cases of drug-resistant TB (DR-TB), resistant to either or both of the 2 first-line drugs, isoniazid and rifampicin, among vulnerable Nigerian populations continues to complicate the TB infection prevalence as well as TB treatment. New *Mycobacterium tuberculosis* complex (MTBC) infections characterized with multidrug-resistance pose challenges to both local and global TB control (3). There are few reports of baseline resistance globally among new TB cases, but resistance is becoming a worrisome situation with a record of one out of five new cases showing resistance (4). DR-TB is defined as a laboratory-confirmed resistance to one or more of the first-line anti-TB drugs, indicating a mono- or poly-resistance pattern (5, 6).

In the recent past, multidrug-resistant tuberculosis (MDR-TB) has become a leading cause of pulmonary morbidity and death, particularly among the populace with declining socio-demographic status (7, 8). Self-medication for respiratory infections without diagnosis among various age groups and both genders further enhances drug resistance patterns, mostly among lower economic status populations, thereby intensifying resistance to MTB and isoniazid (9, 10). Despite the use of acid-fast bacilli (AFB) microscopy in several locations, the reliability of that method is fast declining due to the variability of its sensitivity, inadequate expertise and poor-quality controls, leading to misdiagnosis of TB cases (11). A culture-based method is the gold standard for detection of TB, one which would also provide testing for drug resistance. That would, however, require expensive laboratory infrastructure and expertise (12). The continuing emergence of drug resistance MTB among new cases is a challenge to public health. Application of rapid molecular methods for diagnosis and identification of rifampicin resistance such as the GeneXpert (Xpert) MTB/RIF system is considered an effective di-

agnostic method. This system is currently employed for pulmonary TB detection among the Southwest Nigerian population. The use of MTB resistance as a surrogate marker for GeneXpert MTB/RIF assay and the phenotypic Drug Susceptibility Test (pDST) in Bactec mycobacteria growth indicator tube (B-MGIT) provide precise and reliable detection for new TB cases with resistance (13).

The application of a trained the receiver operating characteristic curve (ROC) model for predictive performance of commonly GeneXpert MTB/RIF and B-MGIT at designated centres, mostly in rural communities in Southwest Nigeria, needs to be evaluated. Evaluation of the comparative performance of these diagnostic tools would help prevent inconsistencies between phenotypic detection (pDST) and genotyping (Gene Xpert), would better inform clinical management of new resistance cases, and would reduce ineffective treatment. To that end, this study aims to review and evaluate the performance of the diagnostic methods GeneXpert MTB/RIF assay and pDST using B-MGIT for detection of drug-resistant *M. tuberculosis* infections among new TB cases.

METHODS

Ethical approval

Approval for the study protocols and collection of the samples was obtained from the Covenant Health Research Committees, Covenant University, Ota, Nigeria (CHREC/055/2022) and the Federal Medical Centre, Abeokuta (FMCA/470/HREC/02/2022). These Institutional Review Boards granted ethical approval with the caveat of the exclusion of the patient's personal data and identifiers. All personal information about the participants was treated confidentially.

Sample collection

Between February 2022 and June 2023, a total of 546 AFB positive sputum samples were collected from both suspected and clinically diagnosed MTB patients at the outpatient clinic of the Federal Medical Centre, Abeokuta, which serves as referral facility for the management and treatment of respiratory infections in Southwest Nigeria. This facility also received samples from two annexed DOT-TB Treatment centers and 23 Primary Health Care facilities providing health services to rural communities in southwest

Nigeria. Patients were initially screened for TB at various adjoining facilities using ZN-AFB sputum smear microscopy. For smear-positive new cases collected using a generalized and randomized method, further confirmation was performed with Xpert MTB/RIF assay and phenotypic drug sensitivity testing (pDST Bactec MGIT) at referral centre. This study has limitations related to insufficient data from adjoining facilities, mainly due to difficulty with diagnostic regents, patients' unwillingness and/or inability to provide sputum and inadequate access to required equipment (GeneXpert) in some communities.

Xpert MTB/RIF assay

AFB smear-positive samples received were further analysed for MTBC and Rifampicin (rif) drug resistance using GeneXpert (Cepheid Inc., Sunnyvale, CA, USA) as previously discussed (14, 15). Briefly, each sample maintained in a leak-proof sputum collection container was analysed with GeneXpert MTB/RIF sample reagent in a 2:1 (v/v) ratio (14). The mixture was transferred to a GeneXpert cartridge containing the required solution for nucleic acid amplification which simultaneously detects MTB complex DNA and the *rpoB* gene associated with MTB resistance (15, 16).

Phenotypic drug sensitivity testing (DST)

Drug susceptibility of the identified MTB/RIF isolates was determined using MTB using the Bactec MGIT 960 system (B-MGIT). Briefly, LJ-culture positive MTB/RIF isolates were sub-cultured and 100 µL aliquot suspension of the isolate was prepared and cultured in an MGIT 960 system. Preparations of reconstituted antibiotics from lyophilized drugs were made into a different final concentration of MTB, 1.0 µg/mL, following previously described protocol (17). To the control tubes, 0.5 mL of Growth Control working solution was added and labeled as drug-free MGIT, and the other drug-containing tubes were labeled S, I, R and E. The results were interpreted in growth units (GU). As the growth control (GC) reached a minimum value of 400 and the GU values of both DST sets were read and recorded. Quality control was observed by testing the batch of MGIT medium, SIRE Kit and PZA kit with *M. tuberculosis* H37Rv strain (18). Safety precautions taken in-

cluded that the liquid culture using the BACTEC MGIT system assay was performed in a biosafety cabinet (BSL-2⁺) with appropriate personal protective equipment (PPE). All procedures were conducted in accordance with specific protocols approved by the Covenant Health Research Ethics Committee, Covenant University, Ota, Nigeria (CHREC/055/2022), and the Federal Medical Centre, Abeokuta (FMCA/470/HREC/02/2022). Strict aseptic techniques were employed to prevent contamination and aerosolization. All biological waste was decontaminated before disposal.

Data analysis

Independent variables from the hospital database, including age and gender (male or female), smear AFB reports and DR-TB patterns, were categorized and analysed using descriptive tools. Comparative evaluation of the detection rate of the Gene Xpert and MGIT960 culture-based method was used to determine the positive predictive values (PPV) at 95% confidence interval ($p < 0.05$). GeneXpert and MGIT960 outcomes for new cases were analysed using multivariate regression for likelihood detection of resistance to MTB and estimation of odds ratios at the 95% confidence interval (CI), establishing a significant association at a $p < 0.05$. Predictive performance was determined using the area under the curve (AUC) of the receiver operating characteristic curve (ROC). The ROC, which operates on the coding of the strain as susceptible and resistant at the susceptibility level used for the phenotypic DST, was derived from the co-ordinates and reliability provided by the AUC. drawn at values closer to 1 and unreliable at 0.50 or less were considered (19).

RESULTS

Detection of MDR-TB among new cases

Of the 546 sputum samples, preliminary AFB staining indicated AFB 3+ (66.7%), AFB +2 (10.1%) and AFB +1 (23.2%) as smear positive. A total of 506 (92.7%) were confirmed *M. tuberculosis* positive with Gene Xpert and 534 (98.2%) with B-MGIT with no significant difference in detection rates (Figure 1A and 1B). Overall drug susceptibility testing performed with B-MGIT indicated higher significant resistance rates of more than 60.0%

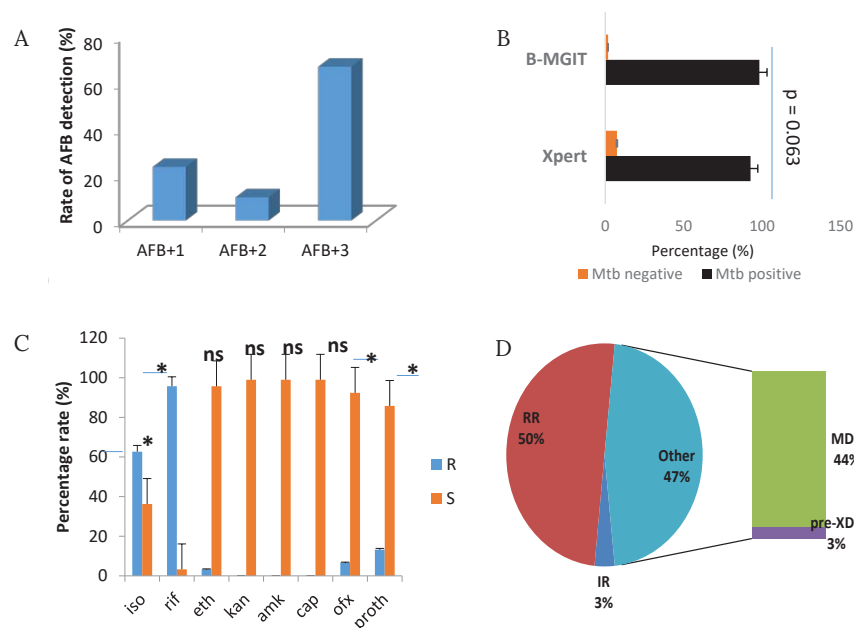


Figure 1. (A) Distribution of AFB smear positive cases recorded in new infections, (B) proportion of confirmed MTB cases, (C) anti-TB resistance rates using the B-MGIT pDST, (D) distribution of the recorded RR, IR, MDR and pre-XDR (R, resistance; S, susceptible; ns, not significant; iso, Isoniazid; RIF, rifampicin; eth, ethambutol; kan, kanamycin; AMK, amikacin; cap, capreomycin; OFX, ofloxacin; proth, protionamide; RR, rifampicin resistance; IR, isoniazid resistance; MDR, multi-drug resistance; XDR, extensive drug resistance. * $p < 0.01$; ** $p < 0.05$).

to isoniazide (inh) and MTB and less than 20% resistance to ofloxacin (ofx) and Protionamide (proth) (Figure 1C) compared to MTB (RR; 50.0%), isoniazid (IR; 3.0%), MDR (44.0%) and pre-XDR (3%) recorded with GeneXpert (Figure 1D).

MTB infection demography by gender and age

Among the new culture-confirmed pulmonary TB cases with resistance to anti-TB drugs, a higher percentage (29.7%) showed significant MTB resistance infection (RR) compared to MDR (23.1%) (OR [95%CI:] = 53.1 [42.5-60.2]) among the male subjects. Of the female subjects, 26.4% and 19.8% were infected with MTB resistant-MTB and MDR-MTB (OR [95%CI:] = 48.4 [31.6-61.5]) strains, respectively, while 3.3% pre-XDR were observed (* $p < 0.01$; Figure 2A). Among the newly detected cases, high rates of MTB resistance (RR) (32.9%) and MDR (26.4%) were recorded for age groups 16-30 years (6.6%) (OR [95%CI:] = 3.63 [0.25-5.05]) and 31-50 years (26.4%) (OR [95%CI:] = 3.81 [1.01-7.62]), respectively. More than 3.0% of MDR, RR and pre-XDR strains were recorded among ages 0-15 years (OR [95%CI:] = 3.27 [0.82-7.39]) (Figure 2B).

Comparative performance of Xpert MTB/RIF assay and B-MGIT960 culture-based methods

Of the new cases of MTB drug resistance infection, B-MGIT had a higher detection (98.2%), sensitivity (87.8%), specificity (91.4%), and PPV (95.8%) (95%CI: 0.778-1.04; $p = 0.001$) compared to Xpert MTB/RIF methods (Table 1). Likelihood of detection of resistance to MTB by B-MGIT assay was higher (OR: 5.76; 95%CI: 1.01-7.56) compared to Xpert MTB/RIF methods (OR : 2.64; 95%CI: 0.39-6.32) based on multivariate regression analysis (Table 1).

Predictive performance of B-MGIT and Gene Xpert for detection of resistance

Predictive performance of the assay methods for the detection of resistance was determined based on the calculated ROC, AUC. The predictive performance of B-MGIT using the ROC model was higher for AUC-MTB at 0.9339 (95%CI: 0.8398-1.000) compared to Gene Xpert at 0.9019 (95%CI: 0.7899-1.000) as shown in Figure 3.

DISCUSSION

The present study considered the comparative performance of Xpert MTB/RIF and B-MGIT for the detection of drug resistant MTB among

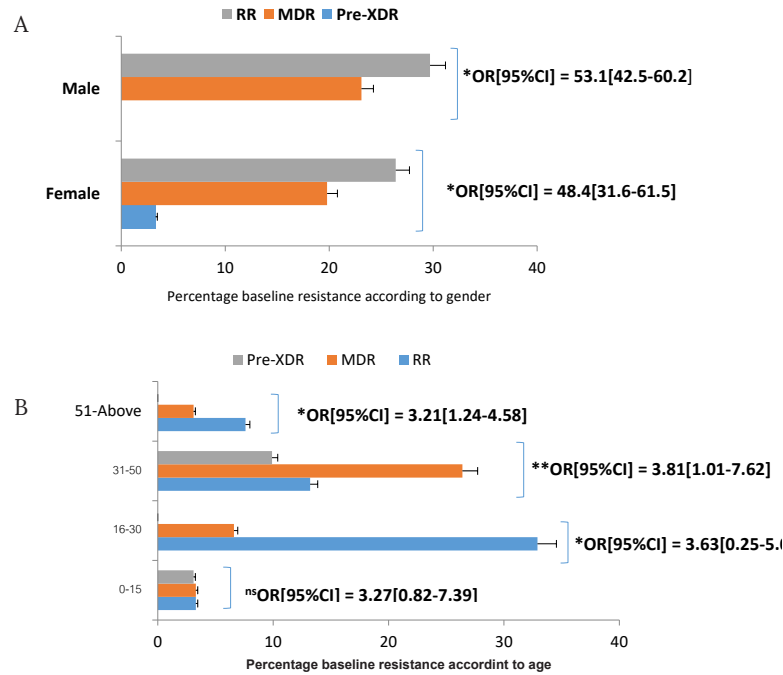


Figure 2. (A) Resistant MTB infection rates by gender distribution, (B) proportion of MTB resistance pattern based on age distribution. (ns, not significant; * $p < 0.01$; ** $p < 0.05$, RR, rifampicin resistance; XDR, extensive drug resistance; MDR, multi-drug resistance; mono-DR, mono-drug resistance)

Table 1. Comparative performance of Xpert MTB/RIF assay and MGIT960 culture-based methods for detection of drug resistance strains among new cases

Method	Detection performance					
	Detection rate (%)	Sensitivity	Specificity	PPV	95%CI	p-value
Xpert MTB/RIF	92.6	81.6	80.1	81.3	0.812-0.984	0.001
B-MGIT	98.2	87.8	91.4	95.8	0.778-1.040	
	Multivariate regression analysis					
	B	SE	t-value	p-value	Odds ratio	95%CI
Xpert MTB/RIF	1.43	0.49	0.001	0.023	2.64	0.39-6.32
B-MGIT	1.57	0.54	0.001	0.001	5.76	1.01-7.56

PPV, positive predictive value

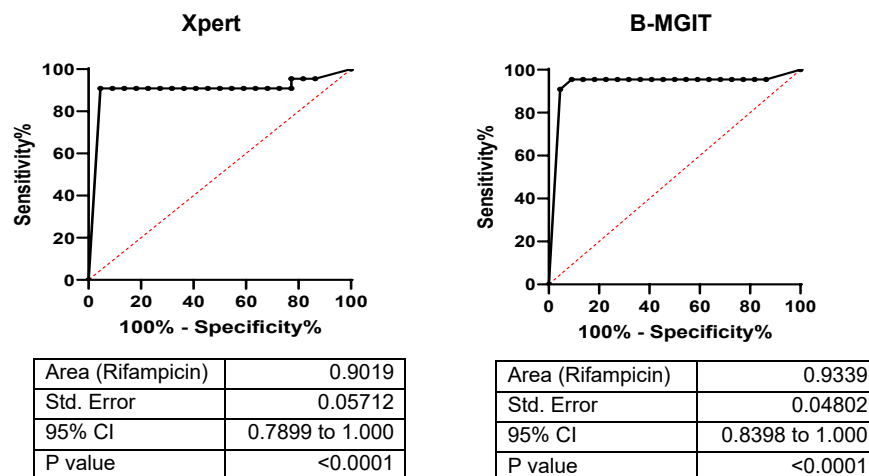


Figure 3. Predictive performance of rifampicin detection by diagnostic method

new TB cases from designated locations in south-west Nigeria. The smear AFB is considered a routine diagnostic method for the early phenotypic detection of MTB in many facilities, most located in rural settings. Due to low detection rates and other limitations of smear microscopy for MTB diagnosis, false TB detection continues to enhance the spread of the infection and misdiagnosis has grave implications for mortality rate, transmission and delay of appropriate therapy (20). As clinical pulmonary morbidity persists among the patients, there is an increase in the use of drugs (antibiotics) to treat any perceived respiratory infections, thereby causing development of more resistance strains (21). This is further substantiated by the high resistance observed among new cases of MTB infections to the first-line anti-TB drugs (MTB and isoniazid), suggesting a new mono-resistance pattern. The recorded MDR and pre-XDR among the newly detected strains is a new report which is similar to that reported in Mumbai, India (22). The observed MDR resistance among the new cases of MTB call for urgent and strategic population-based surveillance, including appropriate control of antibiotic use for pulmonary infections.

The gender and age demography further reveals the infection pattern among the population. Clinically diagnosed new MTB/RIF (Rifampicin resistance *M. tuberculosis*) among male and female subjects could be attributed to the use of antibiotics for the treatment of bacterial infections other than MTB (23, 24). Mono-DR MTB infections among male and female patients in the age groups 16–30 and 31–50 years has similarly been reported (25, 26), suggesting possible spread and transmission of MTB due to the mobility of these age groups, motivated by a desire for socio-economy improvement (27). As the MTB infection rate increases among both genders and various age ranges, there is a need for more precise diagnostic methods.

GeneXpert MTB/RIF assay is as the most rapid test for diagnosis of MTB approved by the WHO (28). It has priority as a diagnostic method because of its speed, reliability, and economic advantages (16). GeneXpert employs a DNA-PCR technology for simultaneous detection of MTB and MTB resistance (*rpoB* gene) (5). Gene Xpert is commonly used in several facilities due to the advantages of being rapid with lower cost, B-MGIT

showing higher detection, sensitivity, specificity and likelihood of the detection of resistance to MTB than the Xpert MTB/RIF methods. Several reports have stated that the GeneXpert MTB/RIF assay has proved to be both sensitive and specific, with considerable PPV for the detection of MTB resistant MTB (29–31). However, the B-MGIT culture-based method with phenotypic DST (pDST) offers accurate and timely pulmonary MTB detection, drug sensitivity testing and effective treatment (32). The assessment of Gene Xpert performance observed in this study indicates it has a lower sensitivity and specificity compare to B-GMIT for detection of new MTB cases. This outcome could be attributed to low abundance of MTB in the submitted samples for Gene Xpert detection. Adequate training and periodic re-training of technical staff handling the genotyping protocol could effectively enhance detection performance. B-MGIT (pDST) is more sensitive and specific, and is available in referral centres for drug resistance detection and estimation. It is not usually distributed to resource-limited communities because it requires advanced laboratory infrastructure, trained staff and adequate quality assurance for detection of low level (smear or Gene Xpert negative) resistant isolates and standardized minimum inhibitory concentration (MIC) evaluation (33).

The ability of B-MGIT to predict resistance in MTB is higher (AUC-MTB = 0.9339) compared to Gene Xpert (AUC-MTB = 0.9019). The high predictive power of B-MGIT to infer MTB resistance in new cases of MTB infection could enhance disease prognosis, therapeutic management of pulmonary MTB, reduction in pulmonary morbidity, and identification of resistant mutations at a very early stage and reduce ineffective treatment (34, 35). Because of the discriminatory potential of the ROC model for mono-drug resistance of MTB by pDST and Gene Xpert described in this study, it would be possible to predict the phenotypic and genotypic occurrence of drug-resistant MTB. This improved predictive performance would aid identification of essential diagnostic methods for the prevention of unfavorable resistance outcomes, development of patient-centered prognostic assessment and would also increase the possible chances for MTB cure (36). The application of ROC also provides prediction of potential MTB

resistance in new cases of MTB, and strengthens the prevention of MDR-TB transmission in many communities (37). It would also afford an advantage in the selection of single and combinations of appropriate anti-TB drugs for clinical management and treatment. However, this study has some limitations. Sample size calculation was not employed in the study due to the limited number of patients already presumed to have TB with clinical presentations and smear AFB positivity but with negative Gene Xpert outcomes. This population was over-estimated during clinical assessment, and sputum culture, the gold standard, was not performed at an early stage of the infection, leading to the possible exclusion of some new TB cases.

CONCLUSIONS

The use of the AFB smear report for assessment of new cases of MTB infections in studied locations is not adequate for MTB diagnosis. Analysis of gender and age demography further support the increasing incidence of resistant MTB among the populace, thereby suggesting a need for more precise diagnostic methods rather than AFB microscopy alone. Further assessment with B-MGIT would enhance detection and provide precise evaluation of drug resistant MTB in new cases of TB infections and supposed negative cases. Sputum outcomes of the GeneXpert MTB/RIF assay deployed to some health facilities may not be sufficient for diagnosis of MTB among AFB smear positive cases which could miss atypical types. Investigation of GeneXpert with B-MGIT examination could further confirm both MTB strains and resistance profiles for more adequate clinical management.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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AUTHOR CONTRIBUTION

P.A.A.: conceptualization, methodology, data curation, project administration, supervision, investigation, resources, visualization, writing – original draft preparation, software, writing – review & editing; O.E.: methodology, data curation, project administration, supervision, investigation, formal analysis, resources, visualization, writing – original draft preparation, software, writing – review & editing; O.C.A.: methodology, data curation, project administration, visualization, writing – original draft preparation, software, writing – review & editing; K.S.A.: methodology, data curation, investigation, formal analysis, writing – original draft preparation, software, writing – review & editing; J.O.A.: conceptualization, methodology, data curation, resources, visualization, software, writing – review & editing; O.E.O.: data curation, project administration, investigation, formal analysis, visualization, writing – review & editing; C.P.N.: investigation, formal analysis, resources, visualization, software, writing – review & editing; Z.I.A.: data curation, investigation, formal analysis, resources, visualization; A.A.A.: methodology, data curation, investigation, visualization, writing – original draft preparation, software, writing – review & editing; T.T.B.: conceptualization, methodology, project administration, supervision, investigation, formal analysis, resources, software, writing – review & editing

All authors consent to the publication of the identifiable details of the text.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

Approval for the study protocols and collection of the samples were obtained from the Covenant Health Research Committees, Covenant Univer-

sity, Ota, Nigeria (CHREC/055/2022) and Federal Medical Centre, Abeokuta (FMCA/470/HREC/02/2022). These Institutional Review Boards granted ethical approval with evidence of the exclusion of the patient's personal data and identifiers.

INFORMED CONSENT STATEMENT

Informed consent of all the recruited participants was at the commencement of the study. All personal information about the participants was treated confidentially.

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Etoricoxib in the 21st Century: An Old Molecule With New Possibilities

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ABSTRACT

Chronic pain conditions, including osteoarthritis (OA), rheumatoid arthritis (RA), ankylosing spondylitis (AS), and acute gouty arthritis (AGA) are responsible for a substantial proportion of global disabilities and health-care burdens. Etoricoxib, a selective cyclooxygenase-2 (COX) inhibitor, has shown better efficacy in these conditions due to its anti-inflammatory and analgesic properties. Its pharmacokinetic properties allow for rapid absorption and a long half-life, the latter enabling once-daily dosing, which has the impact of enhancing both adherence and symptom relief. Recent pharmacological investigations have evidenced the efficacy of etoricoxib intervention in other indications such as dental pain, low back pain (LBP), and postoperative analgesia. Clinical trials and meta-analyses have revealed etoricoxib's superiority over traditional non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen. Novel delivery systems such as nanostructured lipid carriers have been shown to have improved anti-inflammatory effects and decreased cardiotoxicity in preclinical studies. Etoricoxib has shown synergistic outcomes when etoricoxib is combined with tramadol, pregabalin, or thiocolchicoside in the treatment of both acute as well as chronic musculoskeletal pain. This article assembles the latest therapeutic advances and clinical and safety information on etoricoxib, highlighting its emerging role as an important drug in personalized management of pain and inflammation.

KEYWORDS etoricoxib, ankylosing spondylitis, acute gouty arthritis, back pain, osteoarthritis, rheumatoid arthritis, third molar extraction

INTRODUCTION

Pain is a major and growing cause of global morbidity and disability (1). Among the available pharmacological strategies, non-steroidal anti-inflammatory drugs (NSAIDs) remain a backbone for the management of both acute and chronic pain in conditions such as osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute gouty arthritis as well as a variety of perioperative and non-arthritic pain syndromes (2). Their clinical usefulness emerges from the inhibition of cyclooxygenase (COX) enzymes, resulting in reduced prostaglandin synthesis and attenuation of in-

flammatory and nociceptive pathways. Yet the widespread use of conventional, non-selective NSAIDs has been tempered by well-documented gastrointestinal (GI) toxicity, principally ulceration and bleeding due to COX-1 inhibition, and, more recently, by recognition of cardiovascular (CV) risks, particularly in elderly or comorbid populations (3, 4).

To address these safety issues, selective COX-2 inhibitors were designed to retain anti-inflammatory activity without damaging the gastric mucosa via COX-1 inhibition (5); however, this inhibition introduced new concerns over cardiovascular

safety, rooted in the prostacyclin-thromboxane imbalance hypothesis. The withdrawal of rofecoxib and valdecoxib underscores the importance of balancing efficacy against the long-term CV risk with this drug class (6). Etoricoxib, marketed since the beginning of the 2000s, is a selective COX-2 inhibitor. With one of the highest COX-2 to COX-1 selectivity ratios among approved agents, it offers potent analgesic and anti-inflammatory effects while maintaining a more favorable GI safety profile compared to non-selective NSAIDs (7, 8). Its pharmacokinetic properties allow for once a day administration because of its rapid absorption and long elimination half-life (Table 1) (5). The landmark dose finding study determined that 60 mg/day was the dose that provided the optimal balance of efficacy and tolerability in knee osteoarthritis (OA), a key finding that has since been confirmed in numerous randomized controlled trials (9). The strong analgesic and anti-inflammatory properties of etoricoxib in OA, rheumatoid arthritis (RA), ankylosing spondylitis (AS), and acute gouty arthritis (AGA), as well as in some non arthritic pain conditions including

dental postoperative pain, low back pain (LBP), dysmenorrhea, and preoperative models, have been documented in many investigations, often describing them as effective as or better than standard NSAIDs (10-15).

Within the coxib class, etoricoxib's pharmacodynamic and pharmacokinetic characteristics distinguish it from agents like celecoxib, parecoxib, and rofecoxib. While its higher COX-2 selectivity may underpin its stronger anti-inflammatory potency, it also raises cardiovascular safety concerns, particularly in high-risk populations (16, 17). Celecoxib, by contrast, exhibits lower COX-2 selectivity and a different metabolic profile, potentially influencing both its safety and its efficacy balance (18). These distinctions have direct implications for clinical decision-making, especially in complex therapeutic scenarios. This review focuses on etoricoxib analgesic efficacy in various pain conditions and the role of etoricoxib in contemporary clinical practice, whilst also suggesting opportunities for the potential use of etoricoxib in other unique patient groups Figure 1.

Table 1. Pharmacological profile of etoricoxib

Parameter	Details
Mechanism of action	Suppresses prostaglandin synthesis involved in pain and inflammation, with minimal COX-1 effect (19).
Primary indications	Osteoarthritis, rheumatoid arthritis, gouty arthritis, ankylosing spondylitis, acute dental/postoperative pain (20).
Pharmacokinetics	Oral bioavailability ~100%; Tmax: 1-2 hrs; t _{1/2} : ~22 hrs; hepatic metabolism via CYP3A4; renal excretion as metabolites (21).
Pharmacodynamics	Dose-dependent inhibition of COX-2; spares platelet aggregation and gastric mucosa damage at therapeutic doses (22).
Adverse effects	Cardiovascular risks, edema, rare but serious dermatological reactions, e.g., SJS, TEN (23-25).
Contraindications	Established cardiovascular disease, active peptic ulcer, severe hepatic impairment (26).
Regulatory/clinical status	Approved in multiple countries, restricted/withdrawn in some due to CV safety concerns. Recommended for patients at low CV risk (27).
Formulation and Dosing	Available as oral tablets in dosages of 30, 60, 90, and 120 mg (28)
Fixed-dose combinations approved by DCG	Etoricoxib 10 mg + methyl salicylate 100 mg + menthol 50 mg + linseed oil 30 mg per gm. of gel (for acute musculoskeletal pain), etoricoxib 10 mg + methyl salicylate 20 mg per gm. of cream (acute musculoskeletal pain), etoricoxib + paracetamol (60 mg + 500 mg) tablets (for short term use in acute painful and inflammatory conditions), thiocolchicoside + etoricoxib (4 mg + 60 mg) fc tablets (for the acute treatment of inflammatory musculoskeletal disorders associated with painful muscle spasm in adults), etoricoxib 1%+ menthol 5% spray (for the topical treatment of acute musculoskeletal pain) (29)

COX, cyclooxygenase; Tmax, time to reach maximum plasma concentration; t_{1/2}, elimination half-life; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; CV, cardiovascular; DCG, drugs controller general; FC, film-coated

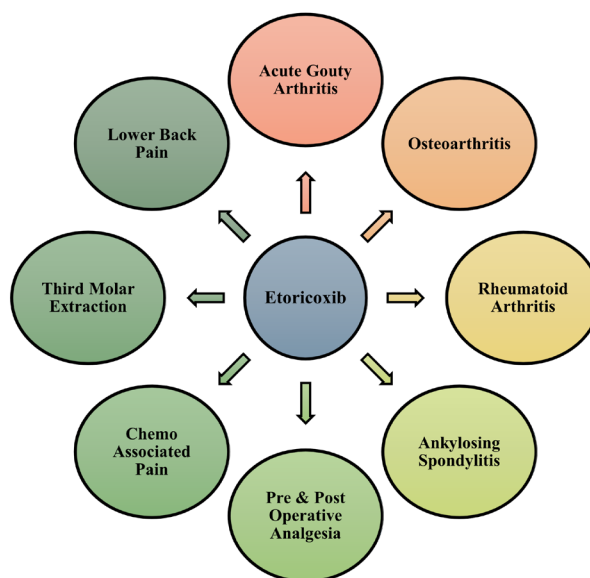


Figure 1. Etoricoxib uses in different pain conditions

ETORICOXIB AND OSTEOARTHRITIS

Etoricoxib is helpful in the symptomatic treatment of OA, a disease in which articular cartilage degenerates and subchondral bone remodels, leading to inflammation in the synovium (30). OA pathophysiology is characterized by disequilibrium between catabolic and anabolic pathways that result in structural changes (cartilage fibrillation, growth and subchondral sclerosis). With an increasing prevalence due to aging populations and obesity, pain relief and functional improvement are the most urgent goals of OA management, since no disease-modifying treatments have been available until now (31, 32). Guidelines from the American College of Rheumatology (ACR) recommend NSAIDs as the first-line treatment; however, their use is challenged by the GI and CV risk they pose (33–35).

The ability of etoricoxib to selectively target COX-2 results in a more favourable GI safety profile compared with non-selective NSAIDs, which makes etoricoxib a particularly attractive NSAID for use in high-risk patients such as the elderly or patients with a history of GI events (36–39).

In clinical trials, etoricoxib has been shown to be effective at doses of 30–90 mg/day, with 60 mg/day identified as optimal for the relief of pain and improvement of function in knee and hip OA (15, 40–42). Comparative trials, such as

the etoricoxib versus diclofenac sodium gastrointestinal tolerability and effectiveness study, have indicated its non-inferiority compared with diclofenac and its superiority compared to ibuprofen in analgesia, with lower GI adverse event (AE) rates (43, 44). Although the rate of discontinuation due to hypertension is higher with etoricoxib, its CV risk profile is similar to that of other NSAIDs, requiring cautious monitoring in high-risk patients (45, 46).

Among the large meta-analyses evaluating the analgesic effect of NSAIDs for OA pain, the 2017 Lancet review ranked etoricoxib amongst the most effective analgesics for OA pain, alongside diclofenac, which has marginal superiority in functional outcomes (47). Rapid onset of action, as early as 4 hours post-dosing, also increases its clinical usefulness. Etoricoxib is recommended as an alternative option to NSAIDs for moderate to severe OA, where topical treatments are unsuitable or GI concerns prohibit the use of non-selective NSAIDs. Although discontinuation is advantageous, this preparation is not without risk in cases of marked renal/hepatic failure (48, 49). Etoricoxib has a good balance of efficacy and tolerability, and provides a strategic therapeutic option for the treatment of OA, although ongoing CV risk assessment is crucial for achieving optimal patient outcomes Table 2.

Table 2. Efficacy and safety of etoricoxib vs other NSAIDs

Comparison area	Etoricoxib efficacy vs traditional NSAIDs
Acute GA	As effective as indomethacin in improving pain and other symptoms (12). Provides an analgesic and anti-inflammatory effect similar to indomethacin, diclofenac, and naproxen (50)
OA	Similar efficacy to naproxen and diclofenac in improving pain and physical function (51) Attenuates OA development and reduces nociception in experimental rat models (52)
RA	At least as effective as naproxen (26). One study found etoricoxib to be significantly superior to naproxen for all primary efficacy end-points and clinically relevant response (53)
AS	Effective in patients refractory to other traditional NSAIDs (54)
Acute (postoperative dental)	Superior to acetaminophen/codeine and oxycodone/acetaminophen (55)
GI safety	Better GI safety profile (51), less likely to result in treatment cessation due to NSAID-type GI symptoms (54)
CV safety	Increased risk of CV disorders (hypertension, heart failure, myocardial infarction, stroke) (56, 57). Traditional NSAIDs (e.g., ibuprofen, diclofenac) also carry CV risks (58)
Renal safety	Increased risk of renal adverse effects (48). Traditional NSAIDs also have renovascular effects (e.g., edema, blood pressure elevation, acute renal failure) (59)

GA, gouty arthritis; OA, osteoarthritis; RA, rheumatoid arthritis; AS, ankylosing spondylitis; GI, gastrointestinal; NSAIDs, nonsteroidal anti-inflammatory drugs; CV, cardiovascular

ETORICOXIB AND RHEUMATOID ARTHRITIS

RA, an autoimmune disease with chronic synovial inflammation, joint destruction, and systemic manifestations, can present in either seropositive or seronegative clinical forms (60–63). Recent European Alliance of Associations for Rheumatology (EULAR) recommendations endorse a treat-to-target approach with prompt use of disease-modifying anti-rheumatic drugs (DMARDs), including methotrexate, leflunomide, and sulfasalazine, and short-term glucocorticoids. Additional therapy is required as an adjunct to DMARDs when pain is uncontrolled, with paracetamol followed by NSAIDs.

Etoricoxib stands as a possible alternative in cases of refractory forms of pain (64, 65). Its effectiveness in the treatment of RA has been confirmed in clinical trials (Table 3). In two 12-week randomized trials, etoricoxib at 90 mg/day was superior to placebo and showed similar or greater efficacy than naproxen 500 mg twice daily. All three primary endpoints for tender and swollen joints, Patient Global Assessment of Disease Activity, and Investigator Global Assessment showed statistically significant improvements, with higher ACR20 responses for etoricoxib-treated patients compared to naproxen and placebo in the US population. In that study, fast onset and efficacy were observed (21). An 8-week trial determined

that 90 mg/day was the optimal dose, with efficacy equivalent to diclofenac (12). Etoricoxib 90 mg/day not only relieves pain but also improves functional outcomes, as measured by improved Health Assessment Questionnaire scores (66). In the light of conflicting transatlantic efficacy findings, the place of etoricoxib in the management of RA is currently that of a useful therapeutic option, especially when traditional NSAIDs are contraindicated or ineffective, in agreement with recommendations aimed at improving quality of life (QoL) in patients with a chronic or incapacitating disorder.

ETORICOXIB AND ANKYLOSING SPONDYLITIS

AS, a chronic inflammatory axial spondyloarthritis, involves sacroiliac joints, facet joints, and spinal ligaments with gradual loss of flexibility and destruction (67, 68). Its pathogenesis includes genetic predisposition (particularly to HLA-B27), immune dysregulation, and environmental factors, is more prevalent in Central Europe and in the male population (69, 70). Clinical findings consist of inflammatory back pain, stiffness, enthesitis, and extra-articular features such as uveitis and idiopathic bowel disorders (71). Current assessments of the Spondylo Arthritis International Society (ASAS) and EULAR guide-

lines emphasize NSAIDs as first-line therapy, advocating chronic use at maximum tolerated doses for symptomatic relief, including inhibition of radiographic progression and reduced CV risk in spondyloarthropathies (72).

Etoricoxib is more effective and better tolerated in the control of AS. In a pivotal 52-week trial, the change in pain with etoricoxib 90 mg/day compared to naproxen 1,000 mg/day was assessed. The design consisted of a 6-week placebo-controlled phase followed by a 46-week active comparator phase. At the end of 6 weeks, etoricoxib exerted a significantly better influence when compared with placebo on the primary end-point parameters such as reduction in spinal pain, improvement in the disease activity, as well as the final Bath Ankylosing Spondylitis Functional Index (BASFI) scores. Etoricoxib was also more effective than naproxen in back pain and disease activity and that superiority was maintained for all 3 domains at 52 weeks. Improvements in ASAS20 were significantly greater, and fewer patients withdrew due to lack of efficacy (73).

A meta-analysis of 26 studies found that more participants achieved pain relief with etoricoxib than with any other NSAID, and that relief was rapid and sustained. Etoricoxib's COX-2 selectivity minimizes side effects, critical for the 5-10% of AS patients with comorbid inflammatory bowel disease (IBD). Data confirmed no increased risk of IBD exacerbation with etoricoxib, bringing the safety profile in line with placebo Table 4 (74).

Cost-effectiveness analyses have shown etoricoxib to be more cost-effective than celecoxib and diclofenac or naproxen, primarily due to lower costs of management of adverse events and improved quality-adjusted life years associated with better Bath Ankylosing Spondylitis Disease Activity Index (BASFI) outcomes (75).

ETORICOXIB AND ACUTE GOUTY ARTHRITIS

Acute gouty arthritis (AGA) is caused by hyperuricemia, which is a serum urate level above saturation of monosodium urate crystal deposits in synovial and periarticular tissues (76-78). In contemporary populations, hyperuricemia is much more common in men than in women (79), leads to gout and also rises dramatically with age (80). Gout clinical presentations include acute and often severe joint pain (especially nocturnal) ac-

companied by erythema, swelling, and systemic inflammation, and can last for days, resulting in significant functional loss, and frequent emergency hospital visits (81, 82).

Recent ACR and EULAR guidelines recommend a treat-to-target approach centered on achieving pain relief as soon as possible and preventing tissue damage mediated by urate crystals. NSAIDs play a key role in treatment during acute flares and in prophylaxis because of their strong anti-inflammatory as well as their analgesic action (83, 84). A systematic review of 22 studies comparing COX 2 selective with non-selective NSAIDs showed that there is no significant difference in effectiveness across drugs, although COX 2 inhibitors have a significantly lower risk of adverse effects. Etoricoxib is as effective as indomethacin, diclofenac, and naproxen in terms of pain relief, inflammation, and safety as it has an improved safety profile, which is crucial since gout patients are often burdened with many comorbid conditions (85).

The efficacy of etoricoxib 120 mg once daily (OD) in the treatment of acute gouty arthritis flares has been demonstrated in two randomized, double blind, comparator-controlled clinical trials. Eligible patients had a recent onset of flare, satisfied the ACR diagnostic criteria, and were experiencing moderate to severe pain. The treatment was for 8 days, with a comparison of etoricoxib 120 mg OD and indomethacin 50 mg three times a day. A greater proportion of etoricoxib-treated patients reported no or mild pain, reflecting the more rapid onset of its analgesic effect relative to indomethacin (86, 87).

On the basis of these findings, the European Medicines Agency has authorized etoricoxib 120 mg OD for AGA (88). Exploratory findings further found significantly more rapid improvement of erythema by 8 days, and post hoc analyses suggest that treatment benefits were due to anti-inflammatory and analgesic effects rather than spontaneous improvement. Hence, etoricoxib 120 mg OD is a well tolerated, effective alternative for rapid symptom relief in patients with acute gout which combines the efficacy of conventional NSAIDs with a favourable adverse event profile in patients who are often elderly and frequently comorbid with gout (86, 87).

ETORICOXIB AND RECENT PHARMACOLOGICAL STUDIES

Recent pharmacological studies have demonstrated the pharmacological potential of etoricoxib in the treatment of several acute and chronic painful conditions. Apart from its uses in traditional conditions, etoricoxib seems to be a consistent therapeutic option in specific situations such as colonoscopy pain management, taxane-related acute pain syndrome in breast cancer patients, lumbar laminectomy recovery, dental surgeries, LBP, postoperative recovery, and post-bariatric surgery pain. Its selective inhibition of COX-2 produces potent analgesic effects while minimizing the risk of gastrointestinal toxicity typically associated with non-selective NSAIDs (Figure 2).

ETORICOXIB AND DENTAL PAIN MANAGEMENT

Etoricoxib has shown a wide range of analgesic effectiveness in both acute and chronic pain states, in addition to those in arthritis for which it is approved. In pain management in dentistry, it has been postoperatively demonstrated that

one dose of oral etoricoxib produces a faster and more pronounced relief than traditional non-selective NSAIDs alone, with better pain scores and patient satisfaction compared with ibuprofen or diclofenac (89, 90). In a study of 56 third molar extraction patients, preoperative administration (120 mg) significantly reduced pain on days 3 and 7, but exerted no difference on inflammation, trismus, or oral health QoL compared with when the drug was given after the surgery (91). In dental implant surgery, etoricoxib was superior to ibuprofen, nimesulide, and acetaminophen in terms of postoperative pain and rescue analgesic consumption (2), and in a different third molar investigation, preemptive administration of etoricoxib resulted in better analgesic and anti-inflammatory effects than naproxen (92). Eight high-quality clinical trials are included in a meta-analysis that compared etoricoxib with traditional NSAIDs following third molar surgery. That study reported a strong association between etoricoxib and decreased use of rescue analgesics, and that etoricoxib at 120 mg was the most effective dose, more effective than ibuprofen at 400 mg and sim-

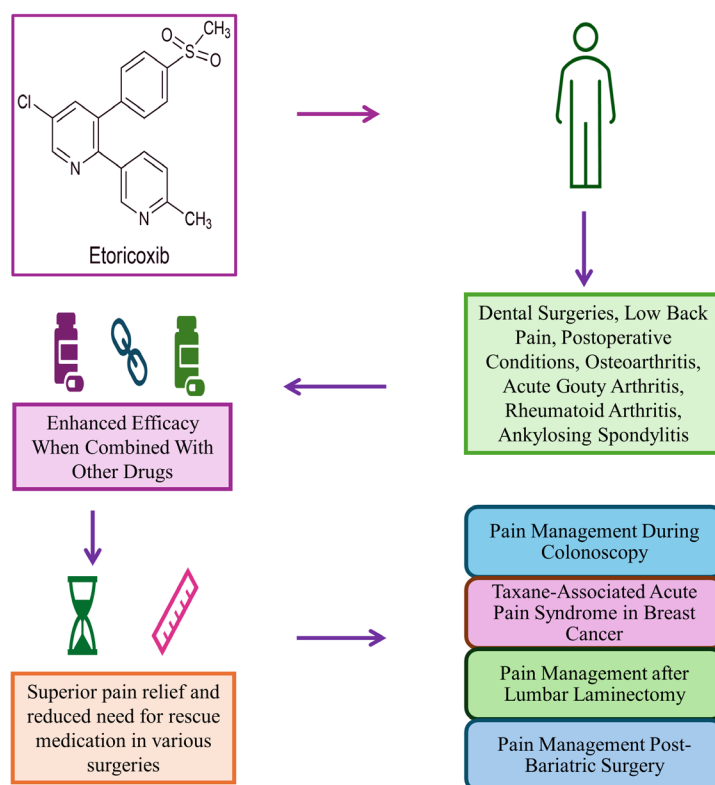


Figure 2. Clinical usefulness and benefits of etoricoxib in pain management and its enhanced efficacy when combined with other drugs

ilar to ibuprofen at 600 mg and naproxen sodium at 550 mg (93).

ETORICOXIB AND LBP

In LBP, 60–90 mg OD of etoricoxib has a stable analgesic effect on pain reduction in both acute and chronic conditions, including spondyloarthritis-associated and axial osteoarthritic back pain (94). In a multimodal Phase III study, fixed-dose etoricoxib/tramadol (90 mg/50 mg) OD was compared with paracetamol/tramadol (975 mg/112.5 mg) thrice daily for 7 days and showed better pain relief on days 3 and 5, a higher proportion of responders by day 3, and similar disability reduction (Oswestry and Roland-Morris scores) and tolerability (95). In chronic LBP, over 21 days etoricoxib 90 mg daily was superior to lornoxicam 8 mg twice daily for pain dimension, allodynia, and central sensitization reduction (96). Real-world data from 383 Indian patients receiving intramuscular etoricoxib (90 mg/mL) confirmed its effectiveness and safety across diverse acute pain scenarios, with clinicians rating it on par with or superior to existing NSAID injections (97).

Preoperative 60 mg dosing also enhanced postoperative QoL in a 60-patient trial (98), while a 39-volunteer trial found that 120 mg etoricoxib significantly reduced both pain and trismus within 48 hours (unlike a placebo), with a short-term benefit on facial swelling comparable to prednisolone (99).

ETORICOXIB AND OPERATIVE ANALGESIA

The preoperative benefit of etoricoxib may also apply to colonoscopy, where 120 mg administered 30 minutes before the procedure significantly reduced pain during cervical biopsy and curettage at both 10 minutes and 24 hours post-procedure without increasing adverse events (100), and in spinal surgery it provided better pain control at 12, 24, and 48 hr after lumbar laminectomy and reduced postoperative morphine consumption (101). But its absorption can be impaired following bariatric surgery due to the change in gastric pH; in this scenario, celecoxib or etodolac can be a better choice (102). In a total knee arthroplasty study, etoricoxib 120 mg/day provided long-acting analgesia, reduced morphine consumption, and had fewer adverse events than both ketoprofen and a placebo (103). A Phase II trial with 144 breast can-

cer patients revealed that etoricoxib significantly decreased both the incidence and severity of taxane-associated acute pain (104).

ETORICOXIB AND COMBINATION THERAPIES

Combination strategies further heighten etoricoxib's clinical usefulness. In healthy volunteers, a combination of etoricoxib 90 mg and tramadol 50 mg in FDC formulation was the bioequivalent of the combined administration of etoricoxib 90 mg and tramadol 50 mg with a comparable pharmacokinetics and safety profile (105). In vitro, etoricoxib combined with cannabidiol delivered via PLGA nanoparticles on two glioblastoma cell lines, U-138 MG and T98G, the combination significantly reduced cell survival and promoted apoptosis, showing a strong antitumor effect (106).

Etoricoxib-loaded nanostructured lipid carriers (ET-NLCs) significantly reduced radiation-induced cardiac inflammation in rats by enhancing antioxidant activity. The ET-NLCs provided better anti-inflammatory and cardioprotective effects than the plain etoricoxib, indicating that ET-NLCs can be a safer and more potent therapeutic approach (107). Presurgical FDC with etoricoxib 90 mg and dexamethasone 4 mg in third molar extractions enhanced patients' satisfaction, postponed rescue analgesia and decreased swelling (108). In head to head FDC trials, low dose etoricoxib 30 mg plus paracetamol 325 mg showed similar analgesia to ibuprofen/paracetamol, but had a better GI safety profile (109). Etoricoxib 60 mg plus thiocolchicoside 4 mg was better than chlorzoxazone/diclofenac/paracetamol triple FDC in severe LBP (110), and pregabalin/etoricoxib FDC gave greater pain relief, functional improvement, and rescue use reduction than etoricoxib alone in chronic LBP (111). Preoperative etoricoxib in arthroplasty patients showed lower Visual Analogue Scale scores at rest and with activity, and reduced blood loss compared to ibuprofen without an increase in adverse effects (112).

Preclinical studies support that calcium gluconate (50 mg/kg) enhances anti-inflammatory activity of low dose etoricoxib (5 mg/kg) in animal models, showing efficacy similar to higher doses of etoricoxib (113); however, comparison of etoricoxib and curcumin against cisplatin induced nephrotoxicity showed that curcumin was

able to provide better protection against oxidative stress and renal dysfunction than etoricoxib,

which could only provide marginal benefit (114).

Table 3. Clinical trials findings of etoricoxib

Study	Design	Participants	Intervention	Key findings
IUD insertion pain	RCT, double-blind, placebo-controlled	130 women	Etoricoxib 120 mg vs. placebo	No significant difference in pain scores during/after insertion (115)
MEDAL program (OA/RA CV safety)	Multinational RCTs	34,701 patients	Etoricoxib (60/90 mg) vs. diclofenac 150 mg	CV risk non-inferior (HR 0.96) Fewer GI AEs More hypertension with etoricoxib (116)
Postoperative dental pain	RCT, double-blind	225 patients	Etoricoxib 120 mg vs. oxycodone/acetaminophen	Superior pain relief Longer duration (> 24 h vs. 7.4 h) Less nausea/vomiting (117)
Periodontal surgery (cross-over)	RCT, double-blind	20 patients	Etoricoxib 120 mg vs. dexamethasone 8 mg vs. placebo	Reduced pain vs. placebo 4-8 h post-op (118)
Rheumatoid arthritis (multinational)	RCT, double-blind	891 patients	Etoricoxib 90 mg vs. naproxen 500 mg BID	Similar efficacy to naproxen, fewer GI AEs (66)
Third molar surgery (QoL)	RCT, double-blind	60 patients	Etoricoxib 60 mg pre-op vs. placebo	Lower pain scores Better QoL Less rescue meds (98)
Mandibular surgery	RCT, triple-blind	97 patients	Etoricoxib vs. diclofenac vs. placebo	Superior pain reduction at 2 h/12 h/48 h postoperative (119)
Periodontal surgery (Parallel)	RCT, double-blind	60 taken, 56 completed	Etoricoxib 120 mg vs. celecoxib 200 mg vs. placebo	Lower pain vs. placebo (2-7 h) Fewer rescue meds No pain reduction difference between celecoxib and etoricoxib (120)
Single-implant surgery	RCT, triple-blind	135 patients	Etoricoxib, ibuprofen, nimesulide, and acetaminophen against placebo	Best pain relief from 12 h onward (2)
RA	RCT, double blind	816 patients	Etoricoxib 90 mg, naproxen 500 mg and placebo	Etoricoxib was superior to both placebo and naproxen (121)
Mucogingival surgery	Randomized parallel double-blind	58 patients (out of 60 initially enrolled)	Placebo, 8 mg dexamethasone, and 90 mg etoricoxib	Etoricoxib showed lower pain scores than dexamethasone (122)
Mandibular surgery	RCT	39 volunteers	Etoricoxib 120 mg, prednisolone 10mg, and control group	Etoricoxib showed significant pain reduction and reduction of trismus (99)
Minor oral surgery	RCT, double-blind	40 subjects	Etoricoxib 120 mg and naproxen 500 mg	No difference between etoricoxib and naproxen in decreasing post-extraction pain (92)

Table 3. Clinical trials findings of etoricoxib (continued)

Study	Design	Participants	Intervention	Key findings
RA	RCT, double-blind	761 randomized patients (out of 1014 screened)	Etoricoxib 90 mg, Etoricoxib 10/30/60 mg, placebo	Demonstrated dose-dependent pain improvement with etoricoxib (123)
AS	RCT, double-blind	1015 subjects	Naproxen 1000 mg vs. etoricoxib 60 and 90 mg	Non-inferior pain relief; similar safety (124)
Third molar surgery	RCT, double-blind	56 patients	Etoricoxib 60 mg pre-op vs. placebo	Lower pain (0-12 h) Less rescue use (125)

IUD, intrauterine device; RCT, randomized controlled trial; OA, osteoarthritis; RA, rheumatoid arthritis; AS, ankylosing spondylitis; CV, cardiovascular; HR, hazard ratio; GI, gastrointestinal; AE, adverse event; BID, twice daily; QoL, quality of life; h, hour; mg, milligram

PRECAUTIONS AND ADVERSE EFFECTS

Administration of etoricoxib in specific medical conditions is not recommended due to potential risks. These conditions include active gastrointestinal bleeding or ulceration (126), ischemic heart disease, varying degrees of heart failure (26), peripheral arterial disease, and uncontrolled hypertension consistently exceeding

140/90 mmHg (50). Precautions are suggested for breastfeeding women (127), individuals experiencing central nervous system effects (50), hyperkalemia (128), hypersensitivity reactions (127), NYHA class II-IV congestive heart failure (50), pregnancy (128), severe hepatic dysfunction (50), or severe renal impairment (129).

Table 4. Outcome and findings of meta-analysis studies of etoricoxib

Indication	Key efficacy outcomes	Comparative findings
OA- RCT responder analysis	Higher % achieving > 15-70% pain relief vs. placebo; NNT 3.2-8.9 depending on pain threshold; mean WOMAC pain reduction	No statistically significant differences found in direct comparisons of NNTs among etoricoxib 60 mg and naproxen 1000 mg, etoricoxib 30 mg and celecoxib 200 mg, or etoricoxib 30 mg and ibuprofen 2400 mg (130)
OA - network meta-analysis	Relative efficacy and tolerability based on patient with-drawals due to lack of efficacy or AEs	No significant efficacy/tolerability difference vs. celecoxib or naproxen (131)
Acute gouty arthritis	No significant differences were found in pain relief, tenderness, swelling, or global assessments between etoricoxib and NSAIDs	Etoricoxib 120 mg daily had the same efficacy on acute gout as indometacin and diclofenac. Etoricoxib had a lower incidence of overall adverse events (132)
Laparoscopic cholecystectomy	↓ Pain at 12-24 hrs; reduced analgesic consumption	More effective analgesia without added AEs risk vs. placebo (133)
Third molar surgery	Better analgesia vs. ibuprofen/diclofenac in some trials; fewer rescue analgesics needed	Etoricoxib reduced the number of patients needing rescue analgesics compared to NSAIDs. Adverse effect evaluation showed no statistical difference between etoricoxib and nonselective COX-2 NSAIDs (93)
Postoperative pain after third molar extraction	Lower efficacy treatments (paracetamol 500 and 1,000 mg) showed NNTs that rapidly increased at higher MEC, while high efficacy treatments (ibuprofen plus paracetamol combinations) showed greater separation at higher ME	NNTs for etoricoxib 120 mg were stable. Etoricoxib 120 mg and ibuprofen 200/400 mg plus paracetamol 500/1,000 mg produced the lowest NNTs in the dental pain mode (134)

Table 4. Outcome and findings of meta-analysis studies of etoricoxib (continued)

Indication	Key efficacy outcomes	Comparative findings
Gouty arthritis (acute gout)	No significant differences were detected in pain score change, tenderness, swelling, or patient's/investigator's global assessments between etoricoxib and indometacin	Etoricoxib and indometacin had comparable effects on pain relief. Etoricoxib had a significantly lower risk of overall AEs (135)
Gastrointestinal safety of etoricoxib in OA and RA	Gastrointestinal adverse events (GAEs), defined as events within 28 days after the last dose of study medication	Etoricoxib did not increase the risk of GAEs compared with placebo. Etoricoxib reduced the GAE risk effectively compared with diclofenac and naproxen (136)
CV and GE effects of etoricoxib in OA	Etoricoxib had a significantly lower risk of GI events compared to naproxen	Etoricoxib did not increase the risk of CV events compared to placebo, celecoxib, ibuprofen, or naproxen. The risk of GI events with etoricoxib was comparable to placebo, celecoxib, and ibuprofen (137)

OA, osteoarthritis; RCT, randomized controlled trial; NNT, numbers needed to treat; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index; AE, adverse event; NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; MEC, minimum effective concentration; RA, rheumatoid arthritis; GAE, gastrointestinal adverse event; CV, cardiovascular; GI, gastrointestinal

DISCUSSION

Etoricoxib is a highly selective COX-2 inhibitor designed to provide potent analgesic and anti-inflammatory effects. It has a COX-1/COX-2 IC₅₀ ratio of 344, which is higher than other coxibs, e.g., celecoxib and valdecoxib (138). This high selectivity means it primarily inhibits COX-2 activity without significantly affecting COX-1 activity. The primary advantage of selective COX-2 inhibitors, including etoricoxib, is their improved GI safety profile compared to non-selective NSAIDs. Pharmacokinetically, etoricoxib is rapidly absorbed, has a long half-life of approximately 20–24 hours, enabling convenient once-daily dosing, and is primarily metabolized by CYP3A4 (139).

Clinically, etoricoxib is approved and effective for a wide range of acute and chronic pain conditions, including osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, acute gouty arthritis, chronic LBP, postoperative dental pain, and primary dysmenorrhea (140). It demonstrates efficacy non-inferior or superior to traditional NSAIDs like diclofenac, naproxen, and ibuprofen across these indications, often with a faster onset in acute settings like dental pain (43, 44). Combination therapies, such as fixed-dose combinations with tramadol, pregabalin, or paracetamol, enhance analgesia and reduce rescue medication needs in musculoskeletal and postoperative

pain (105, 111, 134). Novel delivery systems like etoricoxib-loaded nanostructured lipid carriers (NLCs) show promise for enhanced anti-inflammatory and potential cardioprotective effects in preclinical models (107).

Clinical trials (Table 3) and meta-analysis (Table 4) indicate that etoricoxib provides effective analgesia for osteoarthritis, acute gout, and postoperative pain, with efficacy generally comparable to other NSAIDs and COX-2 inhibitors. It often shows a gastrointestinal safety advantage over traditional NSAIDs and does not appear to elevate cardiovascular risk relative to placebo or common comparators. In acute gout, its tolerability profile is more favorable, with fewer adverse events than indometacin or diclofenac (135).

CONCLUSIONS

Etoricoxib continues to hold significant clinical value as a selective COX-2 inhibitor, offering potent and sustained analgesic effects across diverse pain conditions. Its demonstrated efficacy in chronic inflammatory diseases such as osteoarthritis, rheumatoid arthritis, and ankylosing spondylitis, as well as acute conditions like gout and dental pain, reinforces its utility in both primary care and specialized settings. Importantly, its favorable gastrointestinal safety profile compared to traditional NSAIDs makes it a preferred

option in patients at higher risk for GI complications. Emerging formulations and combination therapies, such as those with tramadol or pregabalin, have further enhanced its therapeutic range, allowing for more personalized and tolerable pain management strategies.

Despite its established role in pain control, several avenues remain open for expanding the pharmacological and clinical applications of etoricoxib. Future investigations should explore its potential as part of disease-modifying protocols, particularly in chronic inflammatory states where structural progression is a concern. The development of advanced drug delivery systems, including nanocarriers and targeted-release formulations, may improve site-specific action and reduce systemic exposure. Moreover, the safety and efficacy of etoricoxib in underexplored contexts, such as cancer-related or neuropathic pain, warrant rigorous clinical trials. Integrating pharmacogenomic data could also refine patient selection and optimize risk-benefit outcomes in long-term use.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M.A.A.: conceptualization, methodology, software, writing & editing, data curation, writing original draft preparation, visualization, investigation; A.K.: data curation, writing original draft preparation, supervision, project administration, review, validation, formal analysis, resources.

All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

All data used in this review were obtained from prior publications and are openly accessible through the referenced literature, ensuring the

transparency of the discussion.

INSTITUTIONAL REVIEW BOARD STATEMENT

This review article does not present any new experimental research involving humans or animals, and therefore ethics approval was not necessary.

INFORMED CONSENT STATEMENT

This review did not include studies involving human subjects, so informed consent was not required.

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Is Tui Na a Promising Approach for Autoimmune Diseases? A Review of Clinical Studies and Mechanisms

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ABSTRACT

Autoimmune diseases, characterized by immune system dysfunction targeting healthy tissues, affect millions worldwide. While conventional treatments have advanced, unmet needs persist. Tui Na, a traditional Chinese massage therapy, has gained attention as a potential complementary treatment. This review explores Tui Na's role in managing autoimmune conditions. A comprehensive literature search was conducted to identify studies investigating the use of Tui Na for autoimmune diseases in several databases. The available evidence suggests that Tui Na may offer potential benefits for individuals with autoimmune diseases. Studies have reported improvements in pain, fatigue, and overall well-being. Combining Tui Na with conventional treatments has often yielded enhanced outcomes. However, the evidence base is limited by a paucity of high-quality randomized controlled trials. Tui Na's potential as a complementary therapy for autoimmune diseases is promising. Its ease of application and reported benefits warrant further exploration. However, the underlying mechanisms remain unclear, and the evidence base is insufficient to establish definitive recommendations. Future research should focus on rigorous study designs, larger sample sizes, and mechanistic investigations. Integrating Tui Na into comprehensive care plans requires careful consideration of patient-specific needs and potential interactions with conventional treatments. In conclusion, Tui Na shows promise as a complementary therapy for autoimmune diseases, offering potential benefits in symptom management and quality of life. While preliminary findings are encouraging, robust research is needed to establish its efficacy and safety.

KEYWORDS Tui Na, traditional Chinese massage, autoimmune diseases, rheumatoid arthritis, multiple sclerosis, amyotrophic lateral sclerosis, ankylosing spondylitis

INTRODUCTION

Autoimmune diseases arise from a dysregulation of the adaptive immune system, leading to a pathological response where autoantibodies erroneously target and bind to the body's own healthy tissues, triggering an immune response against these self-antigens (1).

Over 80 autoimmune diseases have been identified, with recent scientific evidence suggesting a number potentially exceeding 100 distinct autoimmune conditions (2-5). It is estimated that it affects around 10% of the population, ranking as the third most common disease category after cancer and cardiovascular disease (2, 6). Recent

research (7) suggests a marked increase in the incidence of autoimmune diseases, resulting in a growing burden on individuals, society, and healthcare systems.

Pathogenesis seems to be related to an interplay between genetic predisposition (such as gender, ethnicity, and family history) and environmental factors (such as pollution, stress, and diet quality) (3, 6, 8, 9).

Furthermore, the incidence of specific autoimmune diseases varies according to geodemographics (10). For example, the incidence of type 1 diabetes is 10 to 20 times higher in Europe and the United States of America compared to the Middle East and Asia. Grave's disease incidence is almost four times higher in the Middle East/Asia compared to Europe/United States of America. Additionally, the female/male incidence ratio also varies with the specific autoimmune disease. For instance, primary biliary cirrhosis has a ratio of 10/1, Grave's disease has a ratio of 5/1, Coeliac disease has a ratio of 1/1, and Crohn's disease has a ratio of 1/1.2 (10, 11).

Despite recent advances in the treatment of autoimmune diseases, some barriers persist (12–15). Continued exploration of complementary therapeutic strategies is warranted to ensure equitable access to affordable and effective care for patients worldwide.

Tui Na, or Chinese massage, is a technique of Traditional Chinese Medicine. It is mentioned in several medical classics such as the Huangdi Neijing (Inner Canon of the Yellow Emperor) (16).

This form of therapeutic massage involves several soft-tissue and bone adjustment manipulations (17) and is often used in conjunction with other techniques such as acupuncture, moxibustion, cupping, Guasha, and herbal therapy.

Traditionally, Tui Na harmonizes the Yin and Yang, promotes, invigorates and regulates the flow of Qi and blood, expels, clears, dissipates and dredges pathogenic factors, releases and relaxes the sinew channels, and lubricates and improves the movement of joints (18).

These therapeutic indications based on traditional Chinese medicine theories are better understood when we treat them as a logical model of a system's biology with a structural mathematical language (19, 20). In summary, by equating 'yin' to 0 and 'yang' to 1, we can map these binary concepts onto human physiological regulation, pro-

viding a framework for understanding vegetative functional tendencies (19).

This review aims to elucidate the role of Tui Na in managing autoimmune diseases by exploring its potential therapeutic applications, its underlying mechanisms, and the methodological approaches of current research.

METHODS

This narrative review synthesizes the current literature on Tui Na for the management of autoimmune diseases. The information presented was primarily sourced from a search of major biomedical databases, including PubMed, Scopus, Web of Science, and CNKI. The search strategy employed a combination of keywords and Medical Subject Headings (MeSH) terms related to the key concepts, e.g., "Tui Na", "Chinese Massage", "autoimmune diseases", "rheumatoid arthritis", "ankylosing spondylitis", "multiple sclerosis", "systemic lupus erythematosus", and "amyotrophic lateral sclerosis".

Studies were included if they were peer-reviewed clinical articles published in English, Portuguese, Spanish, French, or Chinese. Articles were excluded if they were not relevant to the specified topic or were not available in a peer-reviewed format. Identified articles were screened based on title and abstract, with relevant full-text articles being retrieved for a comprehensive narrative synthesis.

TUI NA FOR AUTOIMMUNE DISORDERS

The increasing prevalence and complex nature of autoimmune diseases necessitate a comprehensive exploration of complementary therapeutic approaches. The present study focuses on discussion of the scientific evidence assessing Tui Na as a potential treatment for various autoimmune conditions such as rheumatoid arthritis, multiple sclerosis and amyotrophic lateral sclerosis, and ankylosing spondylitis. The included studies are summarized in Table 1.

Rheumatoid arthritis

Rheumatoid arthritis is a chronic, systemic disease primarily affecting the synovial joints, particularly those in the hands and feet. Pathologically, it is characterized by synovial hyperplasia, pannus formation, and subsequent cartilage and

Table 1. Characteristics of included studies

References	Wang, Yang (21)	Hong and Xiao-xiang (22)	Chen (23)	Cai (24)
Condition	Rheumatoid arthritis	Rheumatoid arthritis	Rheumatoid arthritis	Rheumatoid arthritis
Type of study	Randomized controlled trial	Clinical observational study	Randomized controlled trial	Randomized controlled trial
Number of participants	n = 60 (30 in the treatment group/30 in the control group)	n = 48	n = 80 (40 in the study group/40 in the control group)	n = 60 (20 in the experimental group/20 each in control groups 1 and 2)
Intervention	Massage “leading Yang into Yin” combined with a breath guided method	Warm Needle acupuncture and Chinese massage	Drug bamboo pot therapy combined with acupoint massage	Long’s bone setting massage combined with acupuncture and traditional Chinese medicine
Control	Placebo (vitamin C)	No control group	Drug bamboo pot therapy	Control 1: conventional Western medicine; Control 2: acupuncture and traditional Chinese medicine.
Main results	The group that received combined massage and breath-guided therapy experienced a significantly greater reduction in anxiety scores (HAMA scores) compared to the control group ($p < 0.05$). The total effectiveness rate of the treatment group was 86.67%, which was significantly higher than the 23.33% effectiveness rate of the control group ($p < 0.05$).	Clinical Recovery: 6 cases (symptoms and signs eliminated, normal work resumed). Remarkable effectiveness: 14 cases (most symptoms and signs abated, light work possible). Improvement: 24 cases (symptoms and signs improved slightly). Ineffectiveness: 4 cases (no change in symptoms and signs).	The study group had lower pain scores (VAS) compared to the control group ($p < 0.05$). The study group had better disease activity scores (SDAI) compared to the control group ($p < 0.05$). The total effectiveness rate of the study group was higher than that of the control group ($p < 0.05$).	The experimental group total clinical effective rate was higher than that of both control group 1 and control group 2 ($p < 0.05$) and its incidence and recurrence rate of adverse reactions were lower than that of both control group 1 and control group 2 ($p < 0.05$).
Main limitations	The study did not explore long-term effects or follow-up outcomes. High risk of performance bias due to the difficulty in blinding manual therapy interventions. The sample size was relatively small.	No control group. Subjective criteria for outcomes. The sample size was relatively small. Combined-modality treatment.	The sample size and study duration was relatively small.	The sample size and study duration was relatively small. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Combined-modality treatment.

Table 1. Characteristics of included studies (continued)

References	Qin, Li (25)	Wang, Rong (26)	Wang (27)	Nie, Han (28)
Condition	Rheumatoid arthritis	Rheumatoid arthritis	Rheumatoid arthritis	Rheumatoid arthritis
Type of study	Randomized controlled trial	Randomized controlled trial	Randomized controlled trial	Randomized controlled trial
Number of participants	n = 82 (41 in the study group/41 in the control group)	n = 80 (40 in the study group/40 in the control group)	n = 60 (30 in the treatment group/30 in the control group)	n = 84 (42 in the study group/42 in the control group)
Intervention	External application of Traditional Chinese Medicine powder combined with massage	Tiebaojin massage cream in addition to conventional Western medicine treatment	Herbal fumigation therapy combined with massage	Traditional Chinese medicine, herbal fumigation and massage
Control	Massage only	Conventional Western medicine treatment	Conventional Western medicine treatment	Routine treatment and a local hot compress
Main results	The study found that the combined therapy was significantly more effective than massage alone. The scores for TCM syndromes (joint swelling, adverse joint activity, and muscle soreness) were lower in the treatment group compared to the control group ($p < 0.05$). Knee joint function (Lysholm score) was better in the treatment group ($p < 0.05$). Pain scores (NRS) were lower in the treatment group ($p < 0.05$).	The effectiveness rate was 90.0% in the treatment group, significantly higher than the 72.5% in the control group. The treatment group showed greater improvements than the control group in the number of joint pains, morning stiffness, grip strength, C-reactive protein, erythrocyte sedimentation rate, and rheumatoid factor ($p < 0.05$).	The total efficacy in the treatment group was 93.33%, significantly higher than the 73.33% of the control group ($p < 0.05$). Symptom scores improved in both groups, but the improvement was significantly greater in the treatment group ($p < 0.05$).	The experimental group total effectiveness rate was 90.5%, significantly higher than the 71.4% in the control group ($p < 0.05$). The erythrocyte sedimentation rate and C-reactive protein, and pain scores were significantly lower in the observation group at 3 weeks, 3 months, and 6 months post-treatment ($p < 0.05$). Knee function scores were significantly higher in the observation group at 3 weeks, 3 months, and 6 months ($p < 0.05$). Synovial thickness was significantly less in the observation group at all follow-up times ($p < 0.01$).
Main limitations	The sample size and study duration were relatively small. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Lack of placebo control.	The sample size and study duration were relatively small. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Lack of placebo control.	The sample size was relatively small. The study duration was very short. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Lack of placebo control. Combined-modality treatment.	The sample size was relatively small. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Lack of placebo control. Combined-modality treatment.

Table 1. Characteristics of included studies (continued)

References	Li (29)	An and Liu (30)	Salarvand, Heidari (31)	Ozdelikara and Alkan (32)
Condition	Rheumatoid arthritis	Rheumatoid arthritis	Multiple sclerosis	Multiple sclerosis
Type of study	Randomized controlled trial	Randomized controlled trial	Systematic review and meta-analysis	Quasi-experimental, pretest-posttest design
Number of participants	n = 78 (39 in the study group/39 in the control group)	n = 60 (30 in the treatment group/30 in the control group)	n = 10 RCTs with a total of 421 participants	n = 15
Intervention	Oral Chinese herbal medicine combined with fumigation and therapeutic massage	Modified Taohecheng-qi decoction combined with acupuncture and massage	Slow stroke back massage, Swedish massage, reflexology effleurage massage	Reflexology
Control	Conventional Western medicine treatment	Conventional Western medicine treatment	Usual care, relaxation therapy, sham reflexology, other massages	No control group
Main results	The total effective rate for the treatment group was 94.87%. Post-treatment pain scores were significantly lower in the treatment group (3.05 ± 0.45) compared to the control group (5.82 ± 1.24), with a statistically significant difference ($p < 0.05$). The duration of morning stiffness was statistically significantly shorter in the treatment group (30.12 ± 10.42 min) compared to the control group (43.52 ± 16.59 min) ($p < 0.05$).	The incidence of DVT and skin infection was not significantly different between the two groups ($p < 0.05$). The incidence of subcutaneous ecchymosis was significantly lower in the observation group ($p < 0.05$). ROM, HSS, and VAS scores were all significantly better in the observation group ($p < 0.05$). TCM syndrome scores were significantly lower in the observation group ($p < 0.05$). Hemoglobin was higher and D-dimer was lower in the observation group ($p < 0.05$).	Fatigue: Measured using the Fatigue Severity Scale (FSS) in 6 studies. The meta-analysis showed a statistically significant reduction in fatigue (SMD = -1.62, 95% CI -2.40 to -0.83, $p < 0.0001$). Pain: Measured using Visual Analogue Scale (VAS), Pain Effects Scale (PES), and Numeric Rating Scale (NRS) in 4 studies. Significant reduction in pain severity was observed in 3 studies ($p < 0.05$).	The average FSS score decreased significantly from 40.06 ± 19.27 at baseline to 27.66 ± 21.23 post-intervention ($p = 0.045$). State Anxiety (STAI-S): Scores decreased from 50.33 ± 9.48 to 37.53 ± 10.11 ($p = 0.008$). Trait Anxiety (STAI-T): Scores decreased from 43.33 ± 9.48 to 31.86 ± 10.27 ($p = 0.003$).
Main limitations	The sample size was relatively small. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Lack of placebo control. Combined-modality treatment.	The sample size was relatively small. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Lack of placebo control. Combined-modality treatment.	Significant heterogeneity in study designs, including variations in massage duration, frequency, and outcome measures. Small number of studies included in the analysis, limiting generalizability. Lack of long-term follow-up data. Publication bias was detected.	The study had a small sample size. The absence of a control group limits the ability to compare results with untreated participants.

Table 1. Characteristics of included studies (continued)

References	Zhang, Yan (33)	Marinova, Angelcheva (34)	Siev-Ner, Gamus (35)
Condition	Multiple sclerosis	Multiple sclerosis	Multiple sclerosis
Type of study	Systematic review and meta-analysis of randomized controlled trials	Controlled trial	Randomized controlled trial
Number of participants	n = 10 RCTs with a total of 631 participants	n = 50 (25 experimental group/25 control group)	n = 71 (53 finished the intervention)
Intervention	Manual intervention such as traditional Chinese massage, Swedish massage, and Thai massage	Tui Na therapy	Reflexology
Control	Sham interventions (e.g., sham massage, sham reflexology), usual care, relaxation techniques	No intervention (usual care)	Sham reflexology
Main results	Massage therapy significantly reduced fatigue (SMD: -0.78; $p = 0.001$). Reflexology showed no significant effect on fatigue. Manual therapy (both massage and reflexology) significantly reduced pain (SMD: -0.59; $p = 0.0252$). Reflexology alone was effective in relieving pain (SMD: -0.40; $p = 0.02$). Manual therapy did not significantly alleviate spasticity (SMD: -0.71; $p = 0.0567$). Reflexology did not improve physical function (SMD: -0.08; $p = 0.68$). Reflexology did not improve psychological disorders (SMD: -0.21; $p = 0.94$).	Pain Reduction: The experimental group showed significant improvement in pain intensity compared to the control group. At the end of the study, 15 patients in the experimental group reported no pain, compared to only 2 in the control group. Impact on Daily Work: Pain interference with normal work was significantly reduced in the EG compared to the control group. Quality of life: The experimental group reported better functional abilities, reduced fatigue, and improved psychological and social well-being.	Significant improvement was noted for paresthesia in the reflexology group, and this benefit was sustained at the three-month follow-up. Urinary Symptoms & Spasticity showed significant improvement in the reflexology group. An improvement was observed in muscle strength, though it was of borderline significance.
Main limitations	Studies varied in disease duration, treatment regimens, and evaluation scales, leading to high heterogeneity. High risk of performance bias due to the difficulty in blinding manual therapy interventions. Potential reporting bias as studies with negative results may not have been published. Some studies had small sample sizes, limiting generalizability.	The study excluded patients with contraindications for physical therapy, heart diseases, or advanced multiple sclerosis, which may limit the generalizability of the findings. The study relied on self-reported measures which are subjective and may introduce bias. The sample size was relatively small, and the study was conducted in a single geographic location, which may affect the broader applicability of the results. The long-term effects of Tiuna therapy were not assessed.	The sample size was relatively small. High risk of performance bias due to the difficulty in blinding manual therapy interventions.

Table 1. Characteristics of included studies (continued)

References	Li, Liu (36)	Li, Liu (36)	Gui-yi, Jian-hui (38)	Zou, Wang (39)
Condition	Amyotrophic lateral sclerosis	Ankylosing spondylitis	Ankylosing spondylitis	Chronic neck pain and stiffness during an acute flare-up of ankylosing spondylitis
Type of study	Case report	Clinical observation study	Clinical study	Case report
Number of participants	n = 1	n = 49 (42 finished the intervention)	n = 128 (64 observation group/64 control group (finished the intervention))	n = 1
Intervention	Western medicine (oral riluzole tablets) and Tuina therapy	Fire-needle therapy combined with tuina manipulations	Conventional medication (sulfasalazine and meloxicam) combined with acupuncture and spinal manipulations	Tui Na therapy
Control	No control group	No control group	Conventional medication (sulfasalazine and meloxicam)	No control group
Main results	The patient showed initial improvement in pulmonary function (e.g., forced vital capacity, vital capacity, and maximal voluntary ventilation) after treatment, though some measures decreased slightly by the third test but remained higher than baseline. Limb circumferences increased, except for the left forearm. The patient reported slight improvements in slurred speech, choking while drinking water, and left upper limb weakness. Tongue stirring speed and strength also improved. Skeletal muscle mass decreased slightly, while body fat and body mass percentages increased.	Therapeutic Results Clinical recovery: 16 cases, remarkable effect; 13 cases, some effect; 7 cases, and failure: 6 cases. Total effectiveness rate: 85.7%. Improvement in Symptoms and Signs: Spine mobility: Remarkable improvement in 30 cases, some effect in 7 cases, failure in 4 cases. Morning stiffness: Clinical recovery in 25 cases, remarkable effect in 10 cases, some effect in 3 cases, failure in 3 cases. Other symptoms like anemia, low-grade fever, neck and shoulder pain, fatigue, lumbosacral pain, and joint tumefaction showed varying degrees of improvement.	Symptom Improvement: Both groups showed decreased TCM symptom scores, but the observation group had significantly lower scores. Pain and Physical Functioning: The observation group showed greater reductions in VAS (pain severity), BASFI (functional index), and BASDAI (disease activity index) scores compared to the control group. Biochemical Indicators: Levels of ALP, ESR, CRP, and OPG decreased in both groups, with greater reductions in the observation group. Efficacy Rate: Total efficacy rate was 92.2% in the observation group versus 78.1% in the control group.	The patient suffered catastrophic results, including quadriplegia due to cervical fracture dislocation (C4–C5) and died 7 days after admission due to respiratory failure and cardiovascular dysfunction.
Main limitations	As a case report, the findings are based on a single patient and cannot be generalized. There is no comparison group. The study relied on self-reported measures, which are subjective and may introduce bias.	Lack of a control group or randomized design. Small sample size and single-center study. Results are based on subjective assessments and self-stipulated criteria. Combined-modality treatment.	The study did not explore long-term effects or follow-up outcomes. The sample size was relatively small. The study was conducted in a single hospital, which may have introduced location bias. No placebo group. Combined-modality treatment.	As a case report, the findings are based on a single patient and cannot be generalized. There is no comparison group.

bone destruction (40, 41).

As an autoimmune disease, it is driven by a complex interplay of genetic and environmental factors that activate immune cells, such as T cells and macrophages, to release pro-inflammatory cytokines (e.g., TNF- α and IL-6), leading to chronic synovial inflammation and the enzymatic destruction of cartilage and bone through the activity of enzymes like matrix metalloproteinases and peptidylarginine deiminase (42, 43).

This debilitating disease imposes a substantial burden on both individuals and society (44, 45). At the individual level, the condition leads to significant musculoskeletal impairments, resulting in diminished physical function, reduced quality of life, and increased risk of comorbidities (46, 47). On a societal level, rheumatoid arthritis contributes to a substantial economic burden due to direct medical costs, lost productivity resulting from impaired work capacity, and decreased social participation (48, 49).

Conventional treatment has been growing in efficacy in the last decades (50). However, it still has its limitations and adverse side effects (50, 51).

Regarding Tui Na for rheumatoid arthritis, research is scarce. However, some studies suggest potential benefits.

A study by Wang, Yang (21) investigated the impact of a specific massage technique combined with breathing exercises on anxiety in rheumatoid arthritis patients. The results showed that patients who received this combined treatment experienced a significantly greater reduction in anxiety symptoms compared to those who received a placebo. This suggests that the massage and breathing technique could be a beneficial addition to the treatment plan for rheumatoid arthritis patients.

A preliminary research study (22) investigated the use of Tui Na combined with warm needling acupuncture technique for rheumatoid arthritis. 91.66% of patients showed clinically relevant improvements. Specifically, 12.50% of patients experienced a near-complete elimination of both local and general symptoms, allowing them to resume a normal life routine, while 29.17% of patients experienced a significant reduction in symptoms and signs after treatment ended, allowing them to undertake light work activities and 50.00% of patients experienced a slight improvement in

symptoms and signs following treatment. The remaining 8.33% of patients experienced no change in symptoms or signs after the treatment ended. However, to determine the specific added effect of Tui na, it is necessary to compare the combined therapy with warm needling acupuncture monotherapy.

Chen (23) conducted a clinical trial combining the use of acupuncture point hand stimulation (acupressure or acupoint massage) with herbal cupping for rheumatoid arthritis. The experimental group (combined treatment) showed significantly superior improvements in pain levels (Visual Analogue Scale) and disease activity (Simplified Disease Activity Index) compared to the control group (herbal cupping only).

The 3-arm clinical trial of Cai (24) showed that a specific Tui Na model combined with acupuncture and Chinese herbal medicine was statistically superior in clinical efficacy, adverse reactions and recurrence of symptoms compared to both conventional medicine and acupuncture plus Chinese herbal medicine.

Another study (25) explored the influence of an external application of a traditional Chinese medicine powder combined with Tui Na on the knee joint function of rheumatoid arthritis patients with knee joint damage. Results showed that the experimental group was statistically superior in reducing joint swelling, adverse joint activity, muscle soreness and joint pain compared to the control group (massage only). In addition, the Lysholm Knee Scoring Scale and Numerical Rating Scale for pain showed significant improvement and superior benefits in the experimental group compared to the control group.

Not specifically investigating the effect of Tui Na, Wang, Rong (26) investigated the efficacy of Tiebaojin massage cream in treating rheumatoid arthritis. The roots or stems of the plant Tiebaojin or *Berchemia lineata* are used in traditional Chinese medicine (52), providing anti-inflammatory action (53) and are also indicated for rheumatoid arthritis (54). In that study, the experimental group (conventional medicine combined with Tiebaojin cream) showed a significantly higher efficacy rate compared to the control group (conventional medicine). Benefits were observed in improved symptoms, grip strength, and reduced levels of inflammation markers (Erythrocyte

Sedimentation Rate, C-reactive protein, and rheumatoid factor). This suggests that Tiebaojin cream may be a good addition to Tui Na massage to improve clinical symptoms and reduce inflammation in rheumatoid arthritis patients.

A study by Wang (27) compared the effectiveness of combined moxibustion and Tui Na versus traditional medication (diclofenac and methotrexate) in treating rheumatoid arthritis. The study found that the combination therapy was more effective in reducing symptoms and improving overall condition compared to the traditional medication group. The therapy was also reported to have no adverse side effects.

Additionally, Nie, Han (28) assessed the efficacy of moxibustion combined with Tui Na in treating knee rheumatoid arthritis. Results showed that moxibustion plus Tui Na was significantly superior to the active control (standard care plus hot compress). Benefits were observed in improved levels of inflammation markers (erythrocyte sedimentation rate and C-reactive protein), pain, knee function, and joint swelling (measured by MRI and ultrasound). Neither intervention showed significant adverse effects.

Li (29) went further and compared a combined treatment of oral Chinese herbal medicine, moxibustion, and Tui Na to conventional Western medicine in rheumatoid arthritis patients. The results showed that the combined Chinese medicine approach was more effective in reducing pain, improving overall condition, and shortening the duration of morning stiffness compared to the Western medicine treatment.

An and Liu (30) conducted a study exploring the combination of Taohe Chengqi decoction and acupoint massage in preventing deep vein thrombosis after knee arthroplasty in rheumatoid arthritis. In that study, *Taohe Chengqi* decoction, a traditional Chinese herbal medicine, showed potential cardiovascular benefits (55). Overall, the combination of techniques is suggested to effectively reduce the incidence of deep vein thrombosis after knee arthroplasty in patients with rheumatoid arthritis, promoting postoperative recovery.

To the best of our knowledge, there is a lack specific research on the mechanisms of Tui Na in rheumatoid arthritis. However, the study by Wang, Jun (56) suggests some mechanisms that

may explain the apparent benefits of Tui Na for rheumatoid arthritis. In their study performed on rabbits, the results showed that Tui Na could significantly reduce the pain threshold and improve β -endorphin and cholecystokinin-8 levels. β -endorphins are endogenous opioids that play an important role in pain management (57, 58), while cholecystokinin-8 may elicit nociceptive activation (59). Moreover, the anti-inflammatory actions of β -endorphins are well-established (60), while the anti-inflammatory effect of cholecystokinin-8 has been suggested (61, 62), but requires further investigation.

Multiple sclerosis and amyotrophic lateral sclerosis

Multiple sclerosis is a chronic inflammatory and neurodegenerative disease affecting both white and grey matter within the central nervous system (63). At the cellular level, multiple sclerosis is a cell-mediated autoimmune disease directed against central nervous system myelin antigens, driven by pathogenic Th17- and Th1-type T cells and CD8+ myelin autoreactive T cells, with auto-antibodies potentially playing a secondary or enhancing role (64).

Independent of the form of the disease (relapsing or progressive), multiple sclerosis significantly compromises patients' quality of life (65) with common neurological manifestations including optic neuritis, diplopia, sensory loss, limb weakness, gait ataxia, loss of bladder control, and cognitive dysfunction (66).

Despite advancements in treatment, early and accurate diagnosis remains a significant challenge in managing multiple sclerosis (67). Early diagnosis and treatment are essential for optimizing outcomes, as they can significantly delay disease progression and reduce disability (68).

The study by Marinova and Angelcheva (34) assessed the influence of Tui Na therapy on pain regarding the quality of life of patients suffering from multiple sclerosis. In this study, the authors observed that Tui Na was significantly beneficial for reducing the intensity of pain and improving the functional state and quality of life compared to patients not receiving Tui Na intervention. Furthermore, the authors state that Tui Na therapy also demonstrated positive effects on patients' psychological, social, and physical well-being.

Participants not only reported significant reductions in pain but also increased energy levels and improved self-confidence and self-esteem. These findings suggest that Tui Na not only alleviates subjective pain but also empowers patients to adapt to chronic neurological deficits by influencing their behaviour and overall quality of life.

The systematic review and meta-analysis conducted by Zhang and Yan (33) studied the efficacy and safety of several manual therapies for multiple sclerosis symptoms. Results regarding Tui Na related techniques such as acupressure and reflexology showed significant benefits in reducing pain. These techniques were considered safe and worth combining with conventional treatment of patients with multiple sclerosis.

A previous study, the systematic review and meta-analysis by Salarvand, Heidari (31), reported that reflexology can reduce pain levels, while a study by Ozdelikara and Alkan (32) suggested that reflexology is an effective method for reducing fatigue and anxiety in patients with multiple sclerosis. Finally, a randomized controlled study (35) showed that reflexology can benefit multiple sclerosis patients by alleviating motor, sensory and urinary symptoms.

Amyotrophic lateral sclerosis, also known as Lou Gehrig's disease, however, is a progressive neurodegenerative condition affecting motor neurons. These nerve cells control voluntary muscle movement, and their degeneration leads to muscle weakness, atrophy, and eventually paralysis. Amyotrophic lateral sclerosis ultimately impacts the ability to perform essential functions such as walking, talking, and breathing (69).

The key molecular mechanisms involve the misfolding, aggregation, and cytoplasmic sequestration of RNA-binding proteins such as TDP-43 and mutated SOD1, which leads to cellular toxicity, impaired axonal transport, mitochondrial dysfunction, and ultimately, programmed apoptosis of motor neurons (70, 71). Similar to multiple sclerosis, available treatments may prolong survival, reduce the rate of decline, or help manage symptoms. However, there is currently no known treatment that halts or reverses its progression (69). Literature on the therapeutic management of amyotrophic lateral sclerosis across various modalities remains limited.

Concerning Tui Na, only a single case report

combining this Chinese massage technique with conventional medicine was identified. The results of the study performed by Li and Liu (36) suggest that the combination of Tui Na and conventional care may offer potential benefits in improving speech, swallowing, and upper limb function in amyotrophic lateral sclerosis patients. However, given the limited number of case reports, further rigorous research, including randomized controlled trials, is imperative to establish the efficacy and safety of Tui Na as a complementary therapy for these patients.

The mechanisms of Tui Na and overall massage therapy are still being explored.

The benefits in terms of improving these conditions may be related to the capacity to increase blood flow, loosen stiff and tense muscles, and soothe and calm the mind, thus promoting relaxation, treating painful muscular conditions, and reducing anxiety (72). The improvements in mood and overall well-being may be due to changes in parasympathetic activity (measured by heart rate, blood pressure and heart rate variability) and hormonal levels (measured by cortisol levels) resulting from massage therapy (73). On the other hand, massage techniques can stimulate lymphatic drainage, potentially modulating serum enzyme levels associated with acute skeletal muscle cell damage and inflammation (74, 75).

Finally, and although more research is needed, massage therapy may also have a positive impact on nerve conduction and plasticity (76-78).

Ankylosing spondylitis

Ankylosing spondylitis is a chronic inflammatory disease primarily affecting the axial skeleton, including the spine and sacroiliac joints. The inflammatory process leads to progressive fibrosis, calcification, and eventual fusion of the affected joints (79). Extra-articular manifestations such as uveitis and inflammatory bowel disease may also occur (80, 81).

Ankylosing spondylitis is driven by genetic factors, particularly the HLA-B27 allele, which, in conjunction with environmental triggers like the gut microbiome, activates the IL-17/IL-23 axis and releases pro-inflammatory cytokines such as IL-17 and TNF- α , leading to chronic inflammation, bone destruction, and new bone formation (79, 82, 83).

The challenges in diagnosing and treating an-

kylosing spondylitis are significantly impacted by the incomplete understanding of its pathogenesis. While advancements in therapeutics have been made, the complex interplay of genetic, immunological, and environmental factors underlying the disease has hindered the development of more targeted and effective treatments (79).

Zhao (37) observed the clinical effect of fire-needle therapy plus Tui Na manipulations for the treatment of ankylosing spondylitis in a clinical case series. The combination of both therapies produced benefits in more than 80% of patients for spine mobility, number of painful joints, thoracic mobility, the occiput-wall distance test, the Gaenslen test (a provocation test that can be used to detect musculoskeletal abnormalities and primary-chronic inflammation of the lumbar vertebrae and sacroiliac joint), and the Schober test (a test used to determine if there is a decrease in lumbar spine range of motion). In that study, more than 60.00% of patients showed benefits in morning stiffness, anaemia, low-grade fever, neck & shoulder pain, fatigue and lethargy, lumbosacral pain, and joint inflammation and swelling.

A study by Guiyi and Jianhui (38) aimed to observe the effects of acupuncture plus spinal manipulations on the physical functioning and biochemical indicators in patients with ankylosing spondylitis. The randomized controlled trial compared the use of conventional medication (sulfasalazine and meloxicam) with conventional medication plus acupuncture and spinal manipulations. The study found that combining conventional medication with acupuncture and spinal manipulations significantly improved symptoms and overall health in patients. This treatment approach led to reduced pain, fatigue, and inflammation, as indicated by lower levels of alkaline phosphatase, erythrocyte sedimentation rate, C-reactive protein, and osteoprotegerin. These biomarkers are often used as indicators of inflammation, bone metabolism, and overall health status (84–87). Patients who received this combined treatment had a higher overall success rate compared to those receiving only conventional medication.

However, to ascertain the specific added benefit of Tui Na in these two studies, it is necessary to compare the combined therapy with either fire-needle or classic acupuncture monotherapy.

In addition, a case study underscores the importance of specialized training and knowledge in applying Tui Na to patients with specific conditions like ankylosing spondylitis (39). The report highlights that compression above the thoracic spine should be avoided in these patients, particularly in cases involving the cervical spine.

The study highlights that avoiding compression above the thoracic spine is essential to prevent potential exacerbation of symptoms. By carefully considering the patient's condition and avoiding potentially harmful techniques, practitioners can maximize the benefits of Tui Na while minimizing risks.

The mechanisms explaining the benefits of Tui Na for ankylosing spondylitis are still unknown. However, Tui Na and acupuncture pain reduction may be explained by the ability to stimulate the release of endorphins (88–90), providing muscle relaxation and alleviating pain by modulating immune response and reducing inflammation (60, 91, 92). This may also improve joint mobility by loosening tight muscles and connective tissue.

Final remarks

Tui Na therapy offers several advantages in managing autoimmune conditions. Its ease of application and affordability contribute to its feasibility in various healthcare settings.

Its application often involves full-body treatment on an unclothed patient in a supine position (72). However, Tui Na and massage therapies can be adapted to suit specific settings. For instance, treatments can be focused on specific areas like the head, hands, feet, or back, and performed on a clothed patient in a seated position, maintaining patient comfort and privacy (72).

Tui Na seems to be a cost-effective complementary approach (93) as suggested by evidence that it provides relief in patients suffering from autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, amyotrophic lateral sclerosis, and ankylosing spondylitis.

According to the results of this review, the effective management of autoimmune diseases necessitates a multidisciplinary approach involving healthcare professionals with specialized knowledge and skills. Therapists and practitioners trained in complementary therapies like Tui Na play a crucial role in tailoring treatment plans by

understanding the specific needs of each patient and developing individualized complementary treatment regimens.

These therapists may play a crucial role in improving functional abilities by helping patients maintain independence and quality of life. However, it is important to note that adjunct therapies such as Tui Na should be used in an integrative medicine setting along with other healthcare professionals and never as a replacement for conventional medical care.

This review identified several methodological limitations. Firstly, the quality of included studies was variable, suggesting the need for improvements in research design and reporting.

Secondly, a significant methodological limitation across the reviewed studies is the multicomponent nature of the interventions. Because Tui Na is nearly always combined with other therapies, such as acupuncture, herbal medicine, or moxibustion, it is difficult to isolate the true therapeutic effects of Tui Na alone, limiting the interpretation of many studies' results. Furthermore, it limits the identification and study of the precise mechanisms underlying the effects of Tui Na.

Another challenge in Tui Na research is the lack of detailed reporting on specific techniques and treatment protocols. Unlike pharmacological trials where the dosage and administration of a drug can be precisely controlled and documented, Tui Na is a complex, practitioner-dependent intervention. Without a clear and detailed description of the manipulations used (e.g., frequency, duration, pressure, specific points or areas targeted), it is difficult for other researchers to replicate a study's methodology or for clinicians to reproduce the treatment in practice. This methodological ambiguity leads to inconsistent and often inconclusive findings, which hinders the accumulation of evidence. Therefore, future research must prioritize the rigorous, step-by-step documentation of treatment conduct, including the operator's skill level, to improve the quality, transparency, and clinical applicability of Tui Na studies, ultimately strengthening its position within evidence-based medicine.

A key limitation of this review is that many of the included studies were conducted exclusively in Chinese populations, which may affect the generalizability of the findings to other ethnic

and demographic groups.

CONCLUSIONS

Tui Na therapy emerges as a potential complementary treatment for autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and ankylosing spondylitis. While research is limited, available studies suggest benefits in reducing pain, improving function, and enhancing overall well-being. The combination of Tui Na with conventional medical treatments appears to yield synergistic effects.

However, the evidence base for Tui Na in these conditions remains relatively small, with most studies involving Tui Na as a standalone intervention and being observational or involving small sample sizes. Furthermore, the precise mechanisms underlying its therapeutic effects are not fully understood. Future research should focus on high-quality randomized controlled trials to establish definitive evidence and elucidate the underlying mechanisms.

Despite these limitations, Tui Na's ease of application, potential cost-effectiveness, and minimal adverse effects make it an attractive complementary therapy. Its integration into comprehensive care plans for autoimmune diseases warrants further exploration.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

H.S.: conceptualization, formal analysis, project administration, resources, visualization, writing—original draft; A.S.: conceptualization, formal analysis, project administration, resources, writing—review and editing; A.M.: conceptualization, formal analysis, resources, writing—review and editing; I.R.S.: conceptualization, formal analysis, resources, writing—review and editing; J.M.R.: methodology, supervision, validation, visu-

alization, formal analysis, project administration, resources, writing—original draft, writing—review and editing.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

INSTITUTIONAL REVIEW BOARD STATEMENT

This review does not require ethical approval.

INFORMED CONSENT STATEMENT

Not applicable

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Visual-Vestibular Rehabilitation Using Orthoptic and Vestibulo-Ocular Reflex Training in Parinaud Syndrome: A Case Report

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ABSTRACT

Parinaud syndrome, or dorsal midbrain syndrome, is a rare neuro-ophthalmological disorder characterized by vertical gaze palsy, convergence-retraction nystagmus, light-near dissociation, and impaired convergence. Rehabilitation literature has primarily focused on motor outcomes in stroke, with limited evidence on visual rehabilitation in this Syndrome. A 39-year-old female presented with acute thalamic-midbrain hemorrhage and developed Parinaud Syndrome with vertical gaze palsy, convergence-retraction nystagmus, diplopia, and impaired near point of convergence. A structured visual-vestibular rehabilitation protocol was implemented, consisting of orthoptic training (pencil push-ups, stereogram fusion, saccadic tracking, smooth pursuits) and vestibulo-ocular reflex (VOR) training (gaze stabilization drills). The program was delivered for 4 weeks, 5 sessions per week. The patient showed partial restoration of upward gaze, improved convergence, reduced diplopia, and enhanced gaze stability. Near point of convergence improved, facilitating better reading ability and daily visual tasks with less fatigue. Functionally, she reported greater independence and confidence in activities requiring visual control. This case demonstrates the effectiveness of targeted visual-vestibular rehabilitation in Parinaud Syndrome, highlighting the role of orthoptic and vestibulo-ocular reflex exercises in improving ocular motility and functional vision. Future studies exploring digital eye-tracking and virtual reality may further enhance rehabilitation outcomes in this rare condition.

KEYWORDS midbrain syndrome, orthoptic exercises, stroke, health and wellbeing, quality of life

INTRODUCTION

Parinaud syndrome, also known as dorsal midbrain Syndrome, is a rare neuro-ophthalmologic disorder that results from lesions affecting the dorsal aspect of the midbrain, particularly structures involved in controlling vertical eye movement. Clinically, it is characterized by vertical gaze palsy, light-near dissociation of the pupils, and convergence-retraction nystagmus, with occasional signs like eyelid retraction and ptosis also reported

(1, 2). Common etiologies include pineal gland tumors, stroke, multiple sclerosis, and traumatic injuries involving the rostral midbrain (3).

Rehabilitation in such patients must therefore address both motor and visual components to achieve functional independence. Effective visual-vestibular rehabilitation promotes good health and well-being by reducing visual disability and improving functional independence. Emerging techniques in visual and vestibular rehabilitation,

such as orthoptic exercises and vestibulo-ocular reflex (VOR) training, have shown promising results in improving visual tracking, eye alignment, and gaze stability, especially in conditions involving the brainstem and cerebellum (4-6). The VOR functions to stabilize visual images on the retina during rapid head movements by generating equal and opposite eye movements. This reflex arc involves the semicircular canals, vestibular nuclei, and ocular motor nuclei, maintaining gaze stability and postural control (7).

Orthoptic training targets ocular coordination and convergence, while VOR exercises improve gaze control during head movements, both of which are essential for enhancing spatial orientation and postural stability (8). Integrating these approaches in stroke rehabilitation may not only support neuroplastic recovery of visual pathways but also improve engagement in activities of daily living (ADLs) (9).

Previous studies have shown that VOR -based rehabilitation improves gaze stability and balance (4, 8), while others report variable outcomes depending on lesion site and training intensity (5). This case report aims to describe the effectiveness of an integrated visual-vestibular rehabilitation program combining orthoptic and VOR training in a patient with Parinaud Syndrome following thalamic-midbrain hemorrhage.

CASE REPORT

A 39-year-old female with no known comorbidities presented with complaints of right-sided upper and lower limb weakness, slurred speech, and deviation of the angle of the mouth, symptoms which began around 11:30 PM, as reported by her husband. On initial examination, the patient appeared drowsy. She was diagnosed with an acute haemorrhagic stroke and was admitted to the intensive care unit for one week for acute medical management until she became medically stable.

Following stabilization, the patient was transferred to the neurology ward for further evaluation and interdisciplinary rehabilitation. A computed tomography (CT) scan revealed an acute intraparenchymal haemorrhage with active bleeding and mass effect involving the left thalamus, Thalamo-capsular region, and extending inferiorly to the left cerebral peduncle and superiorly to the left corona radiata. Perifocal edema was present. The

clinical and radiological findings were consistent with Parinaud's syndrome. CT and magnetic resonance imaging (MRI) scans were repeated every four weeks over a two-month period to monitor progression.

The patient reported blurred vision and difficulty in focusing on near objects since the onset of stroke, with gradual worsening over two weeks. She had no prior visual disorders or comorbidities and was not on any neuroactive medication. Psychologically, she expressed frustration and fear of dependency due to visual disorientation and imbalance.

Neurologically, the patient exhibited impaired upward gaze, resulting in vertical gaze palsy. This limitation significantly impacted her ability to engage in ADLs independently, due to restricted visual tracking.

The multidisciplinary rehabilitation plan focused on patient and caregiver education, outlining the rehabilitation goals, expected outcomes, and duration of inpatient stay. The core goals were to improve ADL independence such as eating, grooming, bathing, dressing, and toileting as well as to promote functional mobility and community reintegration. Emphasis was placed on safety during ambulation and return to independent living.

To address visual deficits related to Parinaud's syndrome, a specialized visual-vestibular rehabilitation protocol was implemented. This included orthoptic exercises to improve convergence and ocular alignment, and VOR training to enhance gaze stability. Light-near dissociation: pupils constrict normally to a near object but have a delayed or abnormal response to light, assessed under both dim and bright conditions. Near point of convergence: it helps assess the overall efficiency of the eye's vergence system, which is crucial for tasks requiring near vision, Diplopia-Double vision when seeing near objects. Written informed consent was obtained from the patient for participation and publication of this report.

METHODS

This case report describes the rehabilitation of a 39-year-old female diagnosed with Parinaud Syndrome secondary to a left thalamic-midbrain haemorrhagic stroke. The patient presented with vertical gaze palsy, right hemiparesis, impaired proprioception, and loss of balance. After initial

medical stabilization in the ICU, she was referred for interdisciplinary inpatient neurorehabilitation. Figure 1 shows the ocular motility examination demonstrating impaired upward gaze in Parinaud Syndrome

Clinical findings

- Vertical gaze palsy (upward gaze restriction)
- Convergence-retraction nystagmus
- Light-near dissociation of pupils
- Diplopia (double vision)
- Near point of convergence deficit.

A comprehensive visual-vestibular rehabilitation protocol was developed, incorporating:

Orthoptic exercises

- Targeted interventions included convergence training, pencil push-ups, saccadic tracking, and smooth pursuit tasks. Sessions were delivered for 30 minutes per day, 5 days a week, focusing on improving upward gaze and binocular coordination.

Rehabilitation

Protocol for stereogram exercise:

1) Initial positioning: The patient positioned a pen in the middle of the images on the stereogram card while holding it at arm's length.

2) Focus on the pen: The patient was told to keep an eye on the pictures on the card without really looking at them, while gently bringing the pen closer to their nose.

3) Image fusion: The images on the card started to double as the pen got closer (for example, two kittens showing up as four). When the two core pictures reach an ideal point of convergence, they combine into a single image.

4) Refusing to look straight at the card: The patient was instructed to stabilise the fused image while keeping their attention on the pen.

5) Progress: The patient worked to make the central image sharper after it was obtained. The pen was gradually taken out while staying focused on the convergence point.

6) Advanced step: To create a third image without the pen, the patient visualised a point halfway between the card and their eyes as a way to practise voluntary convergence.

Pen convergence exercise protocol:

1) Holding a pen: The patient held a pen at arm's length, focusing on its tip.



Figure 1. Ocular motility examination demonstrating impaired upward gaze in Parinaud syndrome

2) Bringing the pen closer: The pen was slowly moved toward the nose while maintaining focus, stopping when double vision occurred.

3) Sustaining convergence: The patient attempted to keep the image single for as long as possible before taking a short break and repeating the exercise.

4) Increasing difficulty: Over time, the patient aimed to bring the pen closer before experiencing diplopia, progressively improving convergence ability (6).

VOR training

- Exercises such as gaze stabilization with head movement (VOR x 1 and VOR x 2 drills), using fixed visual targets while performing horizontal and vertical head movements. Initiated in sitting and later progressed to standing and dynamic postures to integrate balance and eye movement control.

- Duration: 20–25 minutes per session, 5 sessions/week for 4 weeks.

The total rehabilitation duration was 4 weeks, with periodic team reviews and goal setting to adapt treatment based on progress.

Outcomes were assessed pre-and post-intervention using

- Near point of convergence (NPC) -measured using RAF rule (cm).
- Diplopia Rating - self-reported using a 0-10 Visual Analog Scale (VAS).
- Dizziness handicap inventory (DHI) - measures functional, physical, and emotional impact of dizziness.

Baseline and post-intervention results are presented in Table 2

RESULTS

The patient showed partial restoration of upward gaze and improved convergence ability, reducing episodes of diplopia, as summarized in Table 1. Gaze stability improved with VOR training, enabling clearer vision during head movements and enhancing spatial orientation. Near point of

convergence improved, allowing better focus for reading and daily tasks with less visual strain. Table 2 presents quantitative outcomes. Overall, the patient reported greater functional independence and confidence in performing vision-related ADLs. Table 3 summarizes rehabilitation protocol. The patient reported improved reading ability, better environmental navigation, and reduced visual fatigue during daily tasks.

DISCUSSION

Parinaud syndrome presents unique rehabilitation challenges due to the combined visual and motor impairments stemming from midbrain pathology. To strengthen understanding of the observed recovery pattern, the following discussion integrates comparative literature, proposed mechanisms, and clinical implications. In this case, the patient exhibited vertical gaze palsy;

Table 1. Pre-intervention and post-intervention outcome scores

Outcome measure	Pre-intervention	Post-intervention
Vertical gaze (upward movement)	Severely restricted, unable to elevate eyes	Partial restoration, able to sustain brief upward gaze
Convergence (near point of convergence)	Poor convergence, early diplopia at ~20 cm distance	Improved NPC, diplopia only after prolonged near work
Diplopia (double vision)	Frequent during near and distance tasks, affecting ADLs	Markedly reduced, occasional only with fatigue
Light-near dissociation	Pupils sluggish response to light, accommodation impaired	Near accommodation improved, clearer near focus

ADLs, activities of daily living; NPC, near point of convergence

Table 2. Quantitative changes in visual-vestibular function following 4 weeks of orthoptic and vestibulo-ocular reflex training

Outcome measure	Assessment tool	Pre-intervention	Post-intervention	% improvement
NPC	RAF rule (cm)	20 cm	8 cm	60.0%
Diplopia severity	Visual Analog Scale (0-10)	8	3	62.5%
DHI	Questionnaire	64	28	56.0%

NPC, near point of convergence; RAF, Royal Air Force rule; DHI, Dizziness Handicap Inventory

Table 3. Summary of rehabilitation protocol

Phase	Exercise type	Frequency	Duration	Progression criteria
Week 1-2	Orthoptic: pencil push-ups, stereogram fusion	5 days/week	30 min	Maintain single vision for 5 sec
Week 2-3	VOR x1 (horizontal/vertical gaze stabilization)	5 days/week	20 min	Stable gaze without dizziness
Week 3-4	Combined orthoptic + VOR x 2 tasks	5 days/week	45 min	Functional improvement and ADL tolerance

VOR, vestibulo-ocular reflex; ADL, activities of daily living

therefore a targeted visual-vestibular rehabilitation strategy was essential (10).

Comparison with Literature: Similar improvements have been reported in earlier research. Sheikh et al. demonstrated that VOR training enhances gaze stability and balance control following brain injury (4). Porciatti et al. emphasized the role of orthoptic therapy in improving convergence and binocular coordination in neuro-ophthalmologic disorders (5). Conversely, Herdman and Clendaniel noted that retention of VOR adaptation varies depending on lesion location and exercise intensity. These findings suggest that individualized progression and repetition are essential for sustained improvement (8).

Recent studies have illustrated that orthoptic exercises are widely used in patients with neuro-ophthalmologic deficits to improve convergence, accommodation, and saccadic function. Elambirainathan et al. and Jones et al. reported that orthoptic exercises improve visual function. Their inclusion in this case contributed to partial restoration of upward gaze and improved functional visual behavior during ADL performance (6, 11). Additionally, a study discovered that orthoptic exercises help manage convergence and fusion impairments, highlighting the necessity of a planned rehabilitation approach (9).

Mechanisms of Improvement: The patient's progress may be explained by neuroplastic reorganization within the brainstem and cerebellar circuits. Repetitive oculomotor and vestibular stimuli likely enhanced sensory recalibration, strengthening eye-head coordination and gaze stabilization. The integration of orthoptic and VOR tasks promoted both cortical and subcortical adaptations, facilitating smoother visual tracking and reduced diplopia during functional tasks.

Limitations and clinical relevance

Being a single-case report, the findings cannot be generalized to all Parinaud Syndrome patients. The absence of long-term follow-up and lack of advanced eye-tracking assessments are notable limitations. However, this case provides valuable clinical insights for physiotherapists on incorporating visual-vestibular exercises into rehabilitation programs for brainstem stroke. The approach highlights the importance of early, task-specific oculomotor retraining in improving

daily functional vision.

Future investigations should include larger cohorts and objective tracking tools, such as infrared oculography or virtual reality-based gaze assessment, to further validate these outcomes.

CONCLUSIONS

The integrated orthoptic and VOR -based visual-vestibular rehabilitation resulted in measurable improvement in convergence (55.0%), reduced diplopia (62.5%), and better gaze stability, enabling functional visual recovery in Parinaud Syndrome. These results highlight the clinical relevance of physiotherapeutic visual training in neuro-ophthalmologic conditions.

Ethical Approval: The case protocol was reviewed and approved by the Institutional Review Board of Saveetha Institute of Medical and Technical Sciences.

Patient Consent: Written informed consent was obtained from the patient for participation and publication

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

P.J.: conceptualization, methodology, investigation, data curation, writing — original draft; V.J.: supervision, validation, writing — review & editing, project administration; P.S.: formal analysis, resources, visualization, writing — review & editing.

The authors have no conflicts of interest to report.

DATA AVAILABILITY STATEMENT:

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

The case protocol was reviewed and approved by the Institutional Review Board of Saveetha Institute of Medical and Technical Sciences.

INFORMED CONSENT STATEMENT

Written informed consent was obtained from the patient for participation and publication.

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