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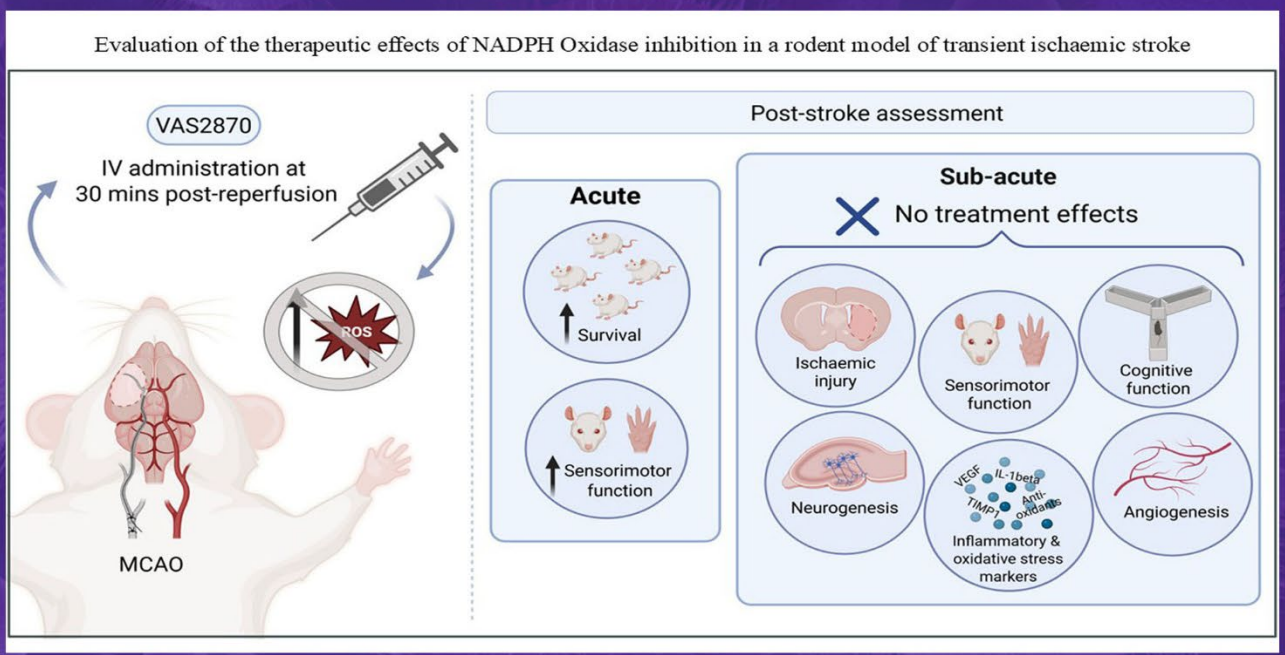

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ABOUT JCTR

Aims and scope

The Journal of Clinical and Translational Research (JCTR) is an open access, peer-reviewed, multidisciplinary scientific journal that publishes studies with at least an ex vivo, in vivo, or clinical component. The published research is centered on any clearly defined clinical problem, which may comprise a disease or the basis of disease, a form of therapy or intervention, and clinical diagnostics or prognostics. Articles (original research, reviews, technical reports, medical hypotheses, commissioned articles, special issue articles, and editorials) are published continuously online and bimonthly in print. Studies performed in cells only will generally not be accepted unless they contain critical data that are in line with the scope of the journal. Some examples of such studies include molecular pathways that lie at the basis of a disease, novel biotechnological approaches for e.g., the production of drugs, or new techniques that improve clinical diagnostics and prognostics. Articles that combine preclinical and clinical data are given priority. Contributions from academic institutions and industry are welcome.

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Journal of Clinical and Translational Research

EDITORIAL

Bridging the artificial intelligence translation gap: From algorithmic promise to clinical reality

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1. Introduction

I was about to write an editorial for the new volume of the *Journal of Clinical and Translational Research*. The role of artificial intelligence (AI) in medical sciences struck me as the most urgent topic at present.[1-3] While considering the best direction for the summer issue, I found it useful to seek AI's assistance. By simply requesting a short editorial, I received a compelling text that addressed the most pressing current issues surrounding the use of AI in clinical and translational medicine.

The fully AI-generated text is presented in the following section, and I am giving credit to the AI for this.

2. AI-generated text

The landscape of clinical and translational research has been dramatically reshaped by AI, yet we stand at a critical juncture where technological capability far outpaces clinical implementation. While machine learning algorithms demonstrate remarkable performance in controlled research environments, the translation to meaningful patient care remains frustratingly elusive.

Recent studies have shown AI systems achieving superhuman accuracy in medical imaging, drug discovery, and diagnostic prediction. However, a sobering reality check reveals that fewer than 5% of Food and Drug Administration-approved AI medical devices are routinely used in clinical practice. This disconnect represents more than a simple adoption lag—it reflects fundamental challenges in how we approach translational research in the digital age.

The primary barrier is not technological sophistication but rather the absence of robust implementation science frameworks specifically designed for AI integration. Traditional clinical translation models, developed for pharmaceutical interventions, prove inadequate for software-based solutions that evolve continuously and operate within complex sociotechnical systems.

We propose three critical areas requiring immediate attention from the translational research community:

First, we must develop new validation frameworks that account for AI's dynamic nature. Unlike static therapeutic interventions, AI systems learn and adapt, raising questions about when and how to assess clinical efficacy. Real-world evidence generation must become integral to AI development, not an afterthought.

Second, implementation research must address the human factors that determine AI adoption success. Clinician workflow integration, patient acceptance, and organizational

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readiness are as crucial as algorithmic performance. Without understanding these contextual factors, even the most sophisticated AI tools will gather digital dust.

Third, we need regulatory pathways that balance innovation with safety, while accommodating AI's iterative improvement cycles. Current approval processes, designed for fixed interventions, struggle with software that improves through use.

The *Journal of Clinical and Translational Research* is uniquely positioned to lead this conversation. We encourage submissions that move beyond pure algorithmic development to examine implementation strategies, real-world performance validation, and the complex interplay between AI tools and clinical practice.

The promise of AI in healthcare is undeniable, but promises alone do not heal patients. It is time to apply the same rigor we demand of traditional therapeutics to the translation of AI into clinical reality. Only through this commitment can we bridge the gap between computational capability and patient care.

3. Conclusion

This short text, generated by the AI system Claude, aptly summarizes the urgent need to integrate AI into medical practice. The editorial board welcomes perspectives on AI implementation challenges and invites researchers to submit studies addressing the translational barriers outlined above.

Conflict of interest

Jacek Z. Kubiak is the Editor-in-Chief of this journal. The author declared that he has no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Journal of Clinical and Translational Research

REVIEW ARTICLE

Occult cancer screening in patients with venous thromboembolism: A systematic review and meta-analysis

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Abstract

Background: Unprovoked venous thromboembolism (VTE) can be the first clinical manifestation of an undiagnosed cancer. A cancer diagnosis at an earlier stage could reduce the risk of cancer progression and contribute to improvements in cancer-related mortality. **Aim:** This review analyzes whether extensive screening for undiagnosed cancer in patients with a first episode of unprovoked VTE is effective in reducing cancer-related mortality. **Methods:** Prospective studies in which patients with an unprovoked VTE were allocated to receive specific tests for identifying cancer were eligible for inclusion. To identify studies, PubMed, Web of Science, Cochrane Library, Scopus, EMBASE, Clinical Trials, the International Clinical Trials Registry Platform, and the Cochrane Central Register of Controlled Trials were searched. **Results:** Four randomized clinical trials (RCTs) and six prospective observational studies were included. Rates of cancer diagnosis at initial screening and during the follow-up in RCTs differed statistically between the two groups (odds ratio [OR]: 2.28 [95% confidence interval (CI): 1.37 – 3.82; $p<0.001$] vs. OR: 0.35 [95% CI: 0.16 – 0.77; $p<0.001$], respectively). The analysis of the RTCs indicated early-stage cancer at diagnosis in an extensive screening group, with results statistically significant (OR: 8.5; 95% CI: 2.57 – 28.17; p<0.001). No differences were observed in cancer-related mortality (OR: 1.07; 95% CI: 0.57 – 2.00; $p=0.143$) and overall mortality (OR: 0.80; 95% CI: 0.44 – 1.45; $p=0.567$) at the end of follow-up. **Conclusion:** Extensive testing for undiagnosed cancer in people with a first episode of unprovoked VTE does not reduce cancer-related mortality. **Relevance for Patients:** This study supports tailored cancer screening in VTE patients, potentially reducing harm from overtesting and improving clinical outcomes.

Keywords: Early detection screening; Occult malignancy; Risk; Systematic review; Venous thromboembolism

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1. Introduction

Venous thromboembolism (VTE), including deep vein thrombosis (DVT) of the lower limb and pulmonary embolism (PE), can be the earliest sign of occult cancer.[1-4] Armand Trousseau first reported the relationship between thrombosis and cancer in 1865, describing thrombophlebitis in a woman with a gastric neoplasm. Research from cohort studies and broad-scale registries has reported a prevalence of occult cancer of 5 – 10% within 12 months of VTE diagnosis.[5-8] Hence, clinicians should maintain a high level of suspicion of cancer in patients with acute VTE. Lymphomas and solid tumors of the ovary, pancreas, kidney, stomach, and brain are the most common neoplasms identified.[9] The incidence of occult cancer detection varies according to the presence or absence of risk factors for VTE. A systematic review reported a 12-month prevalence of occult cancer of 2.6% (95% confidence interval [CI]: 1.6 – 3.6) and 10% (95% CI: 8.6 – 11.3) in patients with provoked and unprovoked VTE, respectively.[10] The incidence of occult cancer detection also varies depending on whether the VTE is a first episode or a recurrent event. The incidence of cancer in patients with recurrent idiopathic DVT is significantly higher than that in patients with a first idiopathic DVT (odds ratio [OR]: 4.3; 95% CI: 1.2 – 15.3).[5] Studies have also evaluated whether the site of VTE is a risk factor for occult cancer. In a systematic review, the 12-month prevalence of cancer was similar in patients with PE with or without concomitant DVT (OR: 5.2%; 95% CI: 3.2 – 8.2) than in patients with only DVT (OR: 5.6%; 95% CI: 4.4 – 7.2).[11]

The highest incidence of cancer diagnoses is observed within the 1st months after the thrombotic event. A Danish study reported that the risk was substantially elevated for all cancers during the first 6 months of follow-up and declined rapidly after 1 year from VTE diagnosis.[2] Similarly, in a study conducted by Nordström *et al.*,[12] the frequency of malignancy in patients with unprovoked DVT was significantly higher during the first 6 months after thrombosis diagnosis.

It may seem appealing for clinicians to screen patients with unprovoked VTE for occult cancer to detect underlying neoplasms at an early and more treatable stage. However, the extent to which patients with VTE should be screened for occult cancer is controversial. This systematic review will focus on the evidence regarding occult cancer detection in patients presenting with idiopathic VTE.

2. Methods

According to the recommendations of the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) 2020 Guidelines,[13] a research question was formulated following the PICO method: P (patients): Patients with unprovoked VTE; I (intervention): Extensive tests; C (comparator): Tests at physician's discretion; O (outcomes/results): Occult cancer.

2.1. Literature search

Using PubMed (https://pubmed-ncbi-nlm-nih-gov.bvsspa.idm.oclc.org), Web of Science (https://www.webofscience.com), Cochrane Library (https://www.cochranelibrary.com/), Scopus (https://www.scopus.com), EMBASE (https://www.embase.com), the international clinical trials registration platform Clinical Trials (https://clinicaltrials.gov/), the International Clinical Trials Registry Platform (https://www.who.int/clinical-trials-registry-platform), and the Cochrane Central Register of Controlled Trials (https://www.cochranelibrary.com/central), we searched for literature on occult cancer detection in patients with a first VTE from January 2000 to December 2023. The following retrieval strategy (MeSH word or text word) was employed: DVT, occult malignancy, occult cancer, PE, screening, VTE, and thrombosis. The search strategy applied in each database comprised a combination of these terms in the heading. The search was limited to studies on humans without language restrictions.

2.2. Study selection

To be eligible, studies must investigate the effectiveness of extensive screening for undiagnosed malignancies in reducing cancer-related mortality in adult patients with a first episode of VTE. These studies can compare comprehensive screening strategies with a limited approach. Eligibility requires that the VTE diagnosis be objectively confirmed through ventilation-perfusion scans, computed tomography (CT), pulmonary angiography for PE, and ultrasonography for diagnosing lower extremity DVT. A minimum follow-up period of 12 months is required. Exclusion criteria were as follows: (i) review articles; (ii) duplicate publications; (iii) studies without usable data; (iv) cross-sectional studies; (v) small case series and case reports; and (vi) conference abstracts. Three reviewers (A.F.M., J.M., and C.L.A.A.) screened titles and abstracts of all retrieved records and, subsequently, full-text articles, independently and in duplicate. A fourth reviewer (A.M.C.M.) resolved discrepancies when necessary. To aid the screening process, the reviewers used a standardized screening.

2.3. Data extraction

Using a standardized form, data from the included studies were extracted by A.F.M., J.M., and C.L.A.A. and reviewed by A.M.C.M. The following data were extracted: (i) study setting (country, year of publication, data collection

period); (ii) study population characteristics (sample size, age, gender); (iii) screening strategy; (iv) follow-up period after the episode of VTE; (v) occult-cancer detection; (vi) cancer-related mortality; and (vii) overall mortality.

2.4. Outcomes

The primary outcome was cancer-related mortality. Secondary outcomes were the incidence of previously undiagnosed solid or hematological cancer at initial screening or during follow-up and overall mortality. Cancer was confirmed by histology or cytology or unequivocally diagnosed by imaging.

2.5. Assessment of risk of bias in the studies

We assessed the risk of bias in randomized controlled trials (RCTs) using the Jadad scale.[14] This scale, also known as the Oxford quality scoring system, focuses on evaluating the descriptions of randomization (up to two points), double-blinding (up to two points), and withdrawals and dropouts (up to one point). Studies are scored on a scale from zero to five, with higher scores indicating better quality. A score of three or above is the reference point for adequate trial quality. The Newcastle–Ottawa scale (NOS) was used for prospective studies.[15] The NOS contains eight items, categorized into three dimensions, including selection, comparability, and—depending on the study type—outcome (cohort studies) or exposure (case-control studies). For each item, a series of response options is provided. A star system is used to assess study quality, such that the highest quality studies are awarded a maximum of one star for each item, except for the item related to comparability, which allows the assignment of two stars. Therefore, the NOS ranges from zero to nine stars. The risk of bias is low, with ≥7 stars. Judgments were made with consensus among four reviewers (A.F.M., J.M., C.L.A.A., and A.M.C.M.). Disagreements were resolved by consensus.

2.6. Statistical analysis

This review aimed to compare the performance of the limited and extensive cancer screening strategies for occult cancer detection in patients presenting with idiopathic VTE.

For each study, we retrieved data, and a weighted pooled analysis was performed. The diagnostic OR, an overall performance measure, was calculated and pooled to create a global estimate for each strategy. This estimate displays the probability that VTE patients who underwent the extensive screening had occult cancer, compared with those who underwent a limited screening. We expressed data with their 95% CI intervals. Statistical analyses were performed using SPSS software, version 29.0 (SPSS, IBM Corp, United States of America) and R, version 4.3.2 (R Foundation for Statistical Computing, Austria; www.r-project.org).

3. Results

3.1. Search results

Initially, 889 studies were identified, from which 461 remained after removing duplicates. Following a full-text review to assess eligibility, 10 studies were included in the meta-analysis. These comprised four RCTs.[16-19] and six prospective studies.[20-25] (Figure 1). The definition of unprovoked VTE was consistent across the studies. It primarily included symptomatic PE or DVT that could not be attributed to known risk factors, such as recent surgery, lower extremity trauma, recent immobilization, known thrombophilia, pregnancy, or the puerperium.

A total of 2621 patients were enrolled, with 1644 participating in RCTs and 977 in observational prospective studies. Among these, 1506 patients underwent extensive screening, while 1115 received limited screening. The comprehensive screening protocols comprised CT,[16-18,21-23] abdominal ultrasonography,[20] or whole-body positron emission tomography-CT (PET/CT).[19,24,25] Furthermore, two studies included gastrointestinal endoscopic examinations.[16,21] The limited screening approaches involved the collection of medical histories, physical examination, basic blood tests, chest radiography, and age- and gender-specific cancer screenings (including those for breast, cervical, and prostate cancers).

3.2. Randomized controlled trials related to cancer investigations for patients with unprovoked VTE

Four RCTs, enrolling 1644 participants, fulfilled the eligibility criteria for inclusion in this review.[16-19] Three studies assessed the effect of extensive tests, including CT scanning, versus tests at the physician's discretion,[16-18] while one study evaluated PET/CT scanning versus standard testing[19] (Table 1).

The SOMIT study was this field's first major randomized study.[16] In the study, 99 participants were randomized to the extensive screening group, and 102 were randomized to the control group. Of these, 50.2% were female, and the mean age was 66.4 ± 13.1 years. The exhaustive screening strategy evaluated in the SOMIT trial included ultrasound and CT-scan of the abdomen and pelvis, upper and lower gastrointestinal endoscopies, sputum cytology, tumor markers, mammography, Papanicolaou smear in women, and prostate-specific antigen (PSA) and transabdominal prostate ultrasound in men. Patients in the control group were investigated at the physician's discretion. All tests were completed within 4 weeks of the VTE diagnosis. The

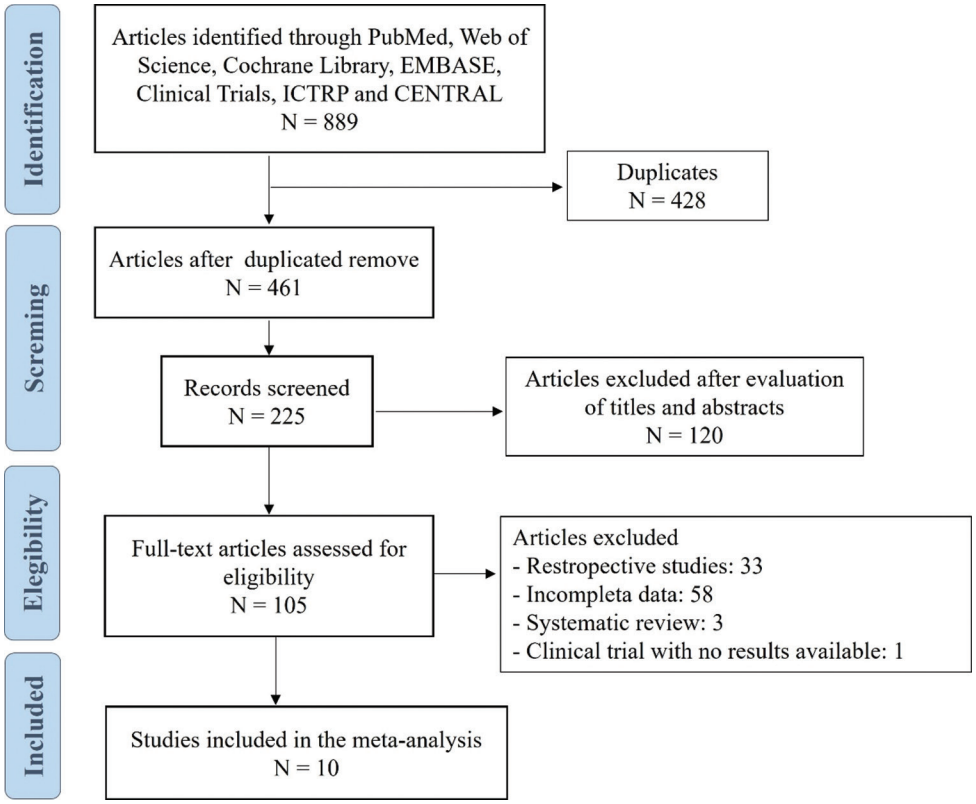

Figure 1. Flow diagram of included studies
Abbreviations: ICTP: International clinical trials registry platform.

exhaustive screening strategy did not provide a clinically significant benefit. In the SOMIT trial, cancer was detected in 13 out of 99 participants (13.1%) who underwent extensive testing. During the follow-up period, a single malignancy (1.0%) emerged in the comprehensive screening group, whereas 10 malignancies (9.8%) became symptomatic in the limited screening group (relative risk: 9.7; 95% CI: 1.3 – 36.8; $p<0.01$). Within this trial, 2 out of 99 participants (2.0%) in the comprehensive testing group and 4 out of 102 (3.9%) in the group tested at the physician's discretion died of cancer (absolute difference: 1.9%; 95% CI: −5.5 - 10.9).

The Screening for Occult Malignancy in Patients with Idiopathic VTE (SOME) trial was a multicenter, open-label, RCT that compared a screening strategy for occult cancer using CT scan of the abdomen and pelvis to a limited screening in patients with a first episode of VTE.[17] The mean age was 53.5 ± 14.0 years, and 32.5% were female. Limited screening included blood testing, chest radiography, and targeted cancer screening based on age and gender: Breast examinations and mammography for women over 50 years old, papanicolaou testing and a pelvic exam for sexually active women aged 18 – 70 years, and prostate examination and PSA testing for men over 40 years old. In the limited-screening-plus-CT group, 14 out of 423 patients (3.3%) received a cancer diagnosis at initial screening, compared to 10 of the 431 patients (2.3%) in the limited-screening group (p=0.28). The extensive screening strategy missed five occult cancers (1.2%), while the limited screening missed four (0.9%) (p=1.0). Cancer-related mortality rates were 0.9% and 1.4%, respectively (p=0.75).

In a small-scale RCT, two diagnostic strategies were compared: A CT-based strategy involving thoracic, abdominal, and pelvic scans combined with a fecal occult blood test, and a strategy based on physicians' clinical judgment.[18] The study involved 195 participants with a mean age of 69.3 ± 14 years, of whom 94 (48.2%) were females. Occult cancer was detected in 10.2% (10/98) of patients assigned to the CT-based strategy, compared to 8.2% (8/97) in the control group (absolute difference: 2.0%; 95% CI: −7.2 - 11.1; p=0.81). Over a follow-up period of up to 24 months, two patients in each group were diagnosed with cancer. The overall mortality rates were 7.1% (7/98) in the CT-based group and 11.3% (11/97) in the control group (absolute difference: −4.2%; 95% CI: −12.3 – 3.9; p=0.67), with two and four cancer-related deaths in each group, respectively.

Table 1. Randomized controlled trials related to cancer investigations for patients with an unprovoked venous thromboembolism

Author, year	Country	Study period	Sample size		Mean age/female, *n*		Intervention		Follow-up	Cancer detection at initial screening, *n* (%)		Cancer detection during follow-up, *n* (%)		Cancer-related mortality, *n* (%)		Overall mortality, *n* (%)	
			Extensive tests	Limited tests	Extensive tests	Limited tests	Extensive tests	Limited tests		Extensive tests	Limited tests	Extensive tests	Limited tests	Extensive tests	Limited tests	Extensive tests	Limited tests
Piccioli *et al.*, 2004 (SOMIT study[16]	Italy	January 1993 to December 1997	99	102	66.2/45	66.6/56	Ultrasound and CT-scan of the abdomen and pelvis, gastroscopy, colonoscopy or sigmoidoscopy, barium enema, sputum cytology, and tumor markers (CEA, α-FP, and CA125); mammography and Papanicolaou smear for women, and transabdominal ultrasound of the prostate and PSA test for men	Tests at the physician's discretion	24 months	13 (13.1)	0	1 (1.0)	10 (9.8)	2 (2.0)	4 (3.9)	NA	
Carrier *et al.*, 2015 (SOME study)[17]	Canada	October 2008 to April 2014	423	431	53.4/124	53.7/154	Limited tests in combination with a CT scan of the abdomen and pelvis	Medical history, physical examination, blood testing, chest radiography, and screening for breast, cervical, and prostate cancer	12 months	14 (3.3)	10 (2.3.)	5 (1.2)	4 (0.9)	4 (0.9)	6 (1.4)	5 (1.2)	6 (1.4)

(Cont'd...)

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Table 1. ***(Continued)***

Author, year	Country	Study period	Sample size		Mean age/female, *n*		Intervention		Follow-up	Cancer detection at initial screening, *n* (%)		Cancer detection during follow-up, *n* (%)		Cancer-related mortality, *n* (%)		Overall mortality, *n* (%)	
			Extensive tests	Limited tests	Extensive tests	Limited tests	Extensive tests	Limited tests		Extensive tests	Limited tests	Extensive tests	Limited tests	Extensive tests	Limited tests	Extensive tests	Limited tests
Prandoni *et al.*, 2016[18]	Italy	January 2006 to May 2008	98	97	69.3/44	69.0/50	Thoracic, abdominal, and pelvic CT-scan in combination with fecal occult blood test	Tests at the physician's discretion	24 months	10 (10.2)	8 (8.2)	2 (2.0)	2 (2.0)	2 (2.0)	4 (4.1)	7 (7.1)	11 (11.3)
Robin *et al.*, 2016 (MVTEP study)[19]	France	March 2009 to August 2012	197	197	64/92	62/95	Limited tests plus PET/CT	Medical history, physical examination, usual laboratory tests, and basic radiographs	24 months	11 (5.6)	4 (2.0)	1 (0.5)	9 (4.7)	2 (1.0)	5 (2.5)	8 (4.1)	8 (4.1)

Abbreviations: α-FP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; CT: Computed tomography; NA: Not available; PET/CT: Positron emission tomography/computed tomography; PSA: Prostate-specific antigen.

Finally, in the MVTEP study, 394 patients with unprovoked VTE were randomized into two groups: one combining a limited screening strategy with PET/CT imaging (197 participants) and the other following a limited screening strategy alone (197 participants).[19] The trial included 47.5% females, with participants' ages ranging from 49 to 76 years, with a mean age of 63 years. The limited screening involved medical history assessment, physical examination, routine laboratory tests, and a chest X-ray, with additional age- and sex-specific cancer screenings (PSA testing for men over 50 years old, mammography for women over 50 years old, and Papanicolaou smear for all women). After the initial screening assessment, cancer was diagnosed in 11 (5.6%) patients in the PET/TC group and four (2.0%) patients in the limited screening group (absolute risk difference: 3.6%; 95% CI: −0.4 - 7.9; p=0.07). Furthermore, one (0.5%) occult malignancy was detected in 186 patients who had a negative initial screening in the PET/CT group, compared with nine out of 193 patients (4.7%) in the limited screening group (absolute risk difference: 4.1%; 95% CI: 0.8 - 8.4; p=0.01). During follow-up, 16 patients died, eight in each group. Two (1.0%) patients in the PET/CT group and five (2.5%) in the limited screening group had cancer-related deaths. In the PET/CT group, non-cancer-related deaths included myocardial infarction (one patient), pneumonia (three patients), congestive heart failure with hemorrhagic shock (one patient), and unknown causes (one patient). The limited screening group reported deaths due to myocardial infarction (one patient), ischemic stroke (one patient), and sudden death (one patient).

Pooling data from all trials revealed an overall cancer incidence of 6.3% (104 out of 1644 patients). The most common cancer sites were colorectal (13.8%), lung (8.5%), breast (5.3%), and prostate (5.3%). At initial screening, cancer was detected in 5.9% (48 out of 817) of patients in the exhaustive screening group, compared to 2.7% (22 out of 827) in those who received standard testing (OR: 2.28; 95% CI: 1.37 - 3.82; p<0.001) (Figure 2). During the follow-up period, cancer was identified in 1.1% (9 out of 817) of the exhaustive screening group, compared to 3.0% (25 out of 827) in the standard testing group (OR: 0.35; 95% CI: 0.16 - 0.77; p < 0.001) (Figure 2). In the extensive screening group, 27 cancers were detected at an early stage, while nine were identified at advanced stages. In contrast, six cancers were detected at early stages in the limited screening group, and 17 were diagnosed at advanced stages (OR: 8.5; 95% CI: 2.57 - 28.17; p<0.001) (Table 2). The all-cause mortality rate during the follow-up period was 2.8% (20 out of 718) in the exhaustive screening group, compared to 3.4% (25 out of 725) in the standard testing group (OR: 0.80; 95% CI: 0.44 - 1.45; p=0.567) (Figure 3). The cancer-related mortality rate was 1.2% (10 out of 817) in the exhaustive screening group, compared to 2.3% (19 out of 827) in the standard testing group (OR: 1.07; 95% CI: 0.57 - 2.00; p=0.143) (Figure 3).

All four studies were randomized. Methods for randomization and double-blinding were described and deemed appropriate. Although none of the studies offered information on withdrawals and dropouts during the follow-up period, they were considered to have a low risk of bias (Table 3).

3.3. Prospective studies related to cancer investigations in patients with unprovoked VTE

Across the six observational prospective studies investigating screening for occult cancer in patients with unprovoked VTE, a total of 977 patients were included.[20-25]

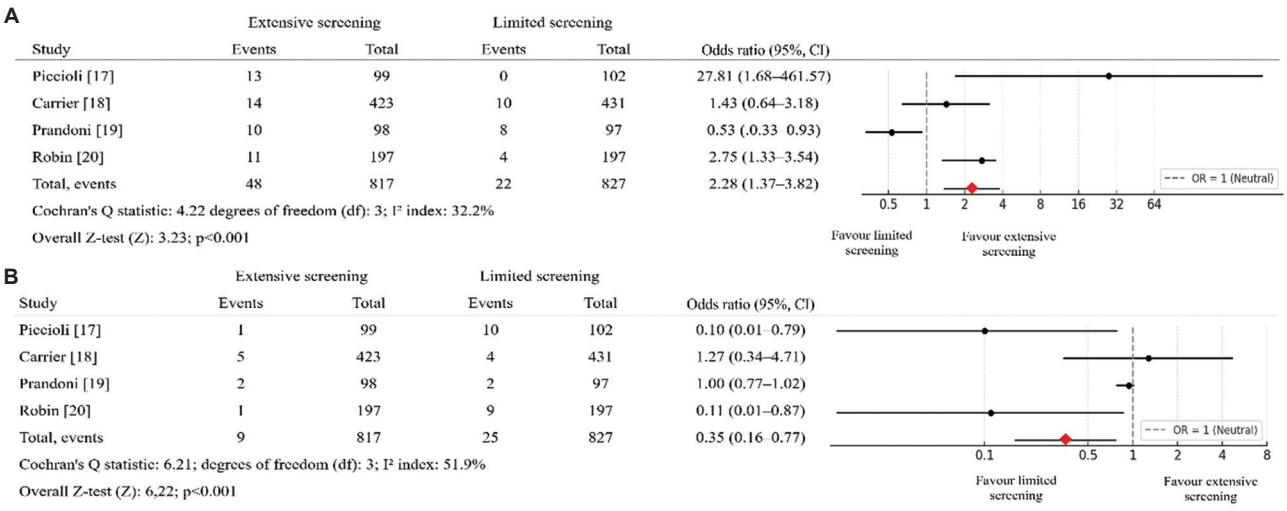

Figure 2. Forest plot comparing extensive versus limited screening for cancer detection at initial screening (A) and during follow-up (B)

Table 2. Comparison of cancer types and stages detected in randomized controlled trials for occult cancer in patients with unprovoked venous thromboembolism

Study	Intervention	Period	Cancers, *n*	Types of cancer (*n*)	Early stages*	Advanced stages**
Piccioli *et al.*[16]	Extensive screening	Initial	13	Bladder (2), lung (2), kidney (1), adrenal gland (1), stomach (1), liver (1), uterus (1), breast (1), ovary (1), colon (1), prostate (1)	12	1
		Follow-up	1	Lung (1)	1	—
	Limited screening	Initial	0	—	—	—
		Follow-up	10	Lung (1), colon (2), prostate (2), pancreas (2), breast (1), stomach (1), bladder (1)	2	8
Carrier *et al.*[17]	Extensive screening	Initial	14	Colorectal (3), cholangiocarcinoma (2), lymphoma (3), breast (2), urologic (3), cancer of unknown primary (1)	NA	NA
		Follow-up	5	Acute leukemia (1), gynecologic (1), colorectal (1), prostate (1), melanoma (1)	NA	NA
	Limited screening	Initial	10	Gynecologic (3), melanoma (1), prostate (2), pancreatic (2), cholangiocarcinoma (1), lymphoma (1)	NA	NA
		Follow-up	4	Acute leukemia (1), gynecologic (1), colorectal (1), pancreatic (1)	NA	NA
Prandoni *et al.*[18]	Extensive screening	Initial	10	Kidney (3), ovary (1), adrenal gland (1), breast and lung (1), lung (2), stomach (1), pancreas (1)	7	3
		Follow-up	2	Stomach (1), bladder (1)	—	2
	Limited screening	Initial	8	Colon (1), liver (1), breast (1), lung (non-small cell) (1), kidney (1), prostate (1), stomach (1), esophagus (1)	NA	NA
		Follow-up	2	Breast (1), colon (1)	NA	NA
Robin *et al.*[19]	PET/CT	Initial	11	Polycythemia (1), testicle (1), pancreas (1), uvula (1), prostate (3), lymphoma (1), lung (1), colon (1), ovary and uterus (1)	7	4
		Follow-up	1	Colon (1)	—	1
	Limited screening	Initial	4	Prostate (1), liver (1), colon (1), endometrium (1)	2	2
		Follow-up	9	Bladder (1), kidney (1), lymphoma (1), polycythemia (1), lung (2), uterus and breast (1), pancreas (1), prostate (1)	2	7

Notes: *Cancers in stages I and II; **Cancers in stages III and IV.
Abbreviations: NA: Not available; PET/CT: Positron emission tomography/computed tomography.

The mean age of participants was 63 years, and 60% were female. Four studies used CT for screening,[20-23] while two used PET/CT.[24,25] Among these, the study conducted by Van Doormaal *et al.*[23] compared extensive screening against limited tests. The other studies focused on evaluating the efficacy of a comprehensive screening.[20-22,24,25] (Table 4).

The study conducted by Jara-Palomares *et al.*[20] enrolled 107 patients with PE, who underwent an occult cancer screening program followed by a 2-year follow-up. The mean age of the participants was 63 years, comprising 56 females. The cancer screening program included a comprehensive medical history review, physical examination, routine blood chemistry, complete blood count, determination of tumor markers (including carcinoembryonic antigen, alpha-fetoprotein, CA19.9, CA125, and PSA for males), chest radiography, and abdominal/pelvic ultrasound examinations. The initial screening detected occult cancer in five patients (4.7%), and four additional cases were identified during the follow-up period. The overall sensitivity of the screening was 55.5%. Unprovoked PE and a shock index of ≥1 variable were independent risk factors for occult cancer. Among patients diagnosed with cancer, 44.4% (4 out of 9 patients) died due to cancer.

Another study enrolled 51 patients with a mean age of 55 years (range: 27 – 84 years), 31% of whom were female[21] The screening protocol comprised a thorough medical

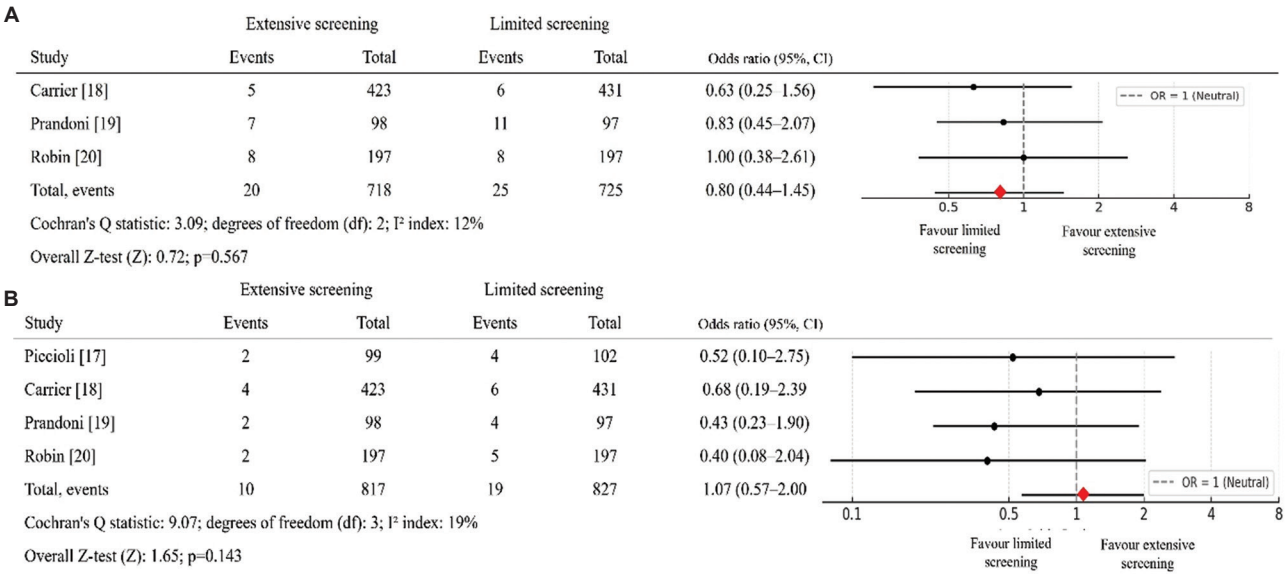

Figure 3. Forest plot comparing extensive versus limited screening for overall mortality (A) and cancer-related mortality (B)

Table 3. Quality assessment of included randomized controlled trials using the Jadad scale

Study	Described as randomized*	Described as double-blind*	Description of withdrawals*	Randomization method described and appropriate**	Double-blinding method described and appropriate**	Total score	Risk of bias
Piccioli *et al.*[16]	1	1	0	1	1	4	Low
Carrier *et al.*[17]	1	1	0	1	1	4	Low
Prandoni *et al.*[18]	1	1	0	1	1	4	Low
Robin *et al.*[19]	1	1	0	1	0	3	Low

Notes: *A study receives a score of 1 for "yes" and 0 for "no"; **A study receives a score of 0 if no description is given, 1 if the method is described and appropriate, and -1 if the method is described but inappropriate.

history and physical examination, blood tests, a chest X-ray, sex-specific cancer screenings (including pelvic examination and Papanicolaou smear for women, and PSA testing or digital rectal examinations for men over 40 years old), and a CT scan of the abdomen and pelvis. The CT scan included a virtual colonoscopy and gastroscopy. It identified abnormalities in eight patients (OR: 16%; 95% CI: 7.0 – 28.5), leading to further investigations. These investigations resulted in the diagnosis of pancreatic adenocarcinomas in two patients (OR: 4.0%; 95% CI: 0.5 – 13.5), one of which was detected early (T1-2N0M0). Additionally, three patients were diagnosed with precancerous lesions.

Rieu *et al.*[22] investigated the efficacy of a screening program for occult cancer in patients over 70 years old. The cohort comprised 50 patients undergoing a 12-month prospective follow-up. The screening included a medical history, clinical laboratory tests including tumor markers, abdominal ultrasound, chest X-ray, and thoracic-abdominopelvic CT scan. At initial screening, one patient was identified with chronic lymphocytic leukemia. During follow-up, three additional cancers were detected (rectosigmoid adenocarcinoma with hepatic metastases, hepatocellular carcinoma, and gastric adenocarcinoma, respectively). The mortality rate was 28%, with two deaths attributed to cancer.

Limited and extensive cancer screening strategies were compared in another prospective cohort study.[23] All 630 patients underwent baseline screening, which consisted of a history, physical examination, basic laboratory tests, and chest X-ray. The extensive screening group added abdominal and chest CT scans and mammography. In 12 of the 342 (3.5%) patients in the extensive screening group, cancer was diagnosed at baseline, compared with seven of 288 patients (2.4%) in the limited screening group. Extensive screening detected six additional cancers (OR: 2.0%; 95% CI: 0.7 – 4.3), of which three were potentially curable. During a median of 2.5 years of

Table 4. Prospective studies related to cancer investigations for patients with an unprovoked venous thromboembolism

Author, year	Country	Study period	Sample size	Mean age/ female, *n*	Intervention	Follow-up	Cancer detection at initial screening, *n* (%)	Cancer detection during follow-up, *n* (%)	Cancer-related mortality, *n* (%)	Overall mortality, *n* (%)
Jara-Palomares *et al.*, 2010[20]	Spain	February 2003 to August 2004	107	63/56	Medical history, physical examination, routine blood chemistry, complete blood count, tumor markers (CEA, α-FP, CA19.9, CA125, and PSA), chest radiography, and abdominal/pelvic ultrasound	24 months	5 (4.7)	4 (3.7)	4 (3.7)	NA
Carrier *et al.*, 2010[21]	Canada	May to September 2006 August to December 2007	51	55/35	Medical history, physical examination, laboratory analyses, chest radiograph, and CT scan of the abdomen/pelvis, including a virtual colonoscopy and gastroscopy; in females, pelvic examination and Papanicolaou smear, as well as mammogram (for those over 50 years of age); PSA test for males over 40 years of age	24 months	2 (4.0)	0	NA	NA
Rieu *et al.*, 2011[22]	France	18 months	50	80/31	Medical history, clinical examination, laboratory analyses, tumor markers (CEA, PSA, CA125, and CA15.3), chest radiograph, abdominal pelvic ultrasonography, and a thoraco-abdomino-pelvic CT-scan	12 months	1 (2.0)	3 (6.0)	2 (4.0)	14 (28.0)
Van Doormaal *et al.*, 2011[23]	Netherlands	December 2002 to December 2007	Extensive screening: 342 Limited tests: 288	62/246	Extensive screening: limited screening plus abdominal and chest CT-scan; includes mammography for females Limited tests: history, physical examination, routine blood chemistry, and chest radiograph	30 months	Extensive screening: 12 (3.5) Limited tests: 7 (2.4)	Extensive screening: 13 (3.8) Limited tests: 14 (4.9)	Extensive screening: 17 (5.0) Limited tests: 8 (2.8)	Extensive screening: 26 (7.6) Limited tests: 24 (8.3)
Rondina, 2011[24]	USA	November 2008 to January 2010	40	55/19	PET/CT scan, medical history, physical examination, and laboratory analyses	12 months	1 (2.5)	0	1 (2.5)	2 (5.0)
Alfonso *et al.*, 2013[25]	Spain	January 2007 to June 2010	99	68.4/38	PET/CT scan	24 months	7 (7.0)	2 (2.0)	1 (1.0)	3 (3.0)

Abbreviations: α-FP: Alpha-fetoprotein; CEA: Carcinoembryonic antigen; CT: Computed tomography; NA: Not available; PET/CT: Positron emission tomography/computed tomography; PSA: Prostate-specific antigen; USA: United States of America.

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follow-up, malignancy was diagnosed in 3.7% and 5.0% of the extensive and limited screening groups, respectively. In the extensive screening group, 26 patients (7.6%) died compared with 24 (8.3%) in the limited screening group (hazard ratio [HR]: 1.22; 95% CI: 0.6 – 2.2). Among these deaths, 17 (5.0%) in the extensive screening group and 8 (2.8%) in the limited screening group were cancer-related (HR: 1.79; 95% CI: 0.7 – 4.3).

In a pilot study, the use of PET/CT to screen for occult malignancy in 40 patients with unprovoked VTE was prospectively investigated[25]. All patients were initially screened for occult malignancy with a focused history, physical, and laboratory evaluation. Patients underwent whole-body PET/CT and were followed for up to 2 years. PET/CT imaging identified abnormal findings requiring additional assessment in 62.5% of patients (25 out of 40 patients). Among them, only one patient had confirmed cancer. No patients with a negative PET/CT were diagnosed with malignancy during the follow-up.

In the Alfonso *et al.* study,[25] patients ≥50 years old with a first unprovoked VTE episode underwent screening for occult cancer with PET/CT, which was performed 3 – 4 weeks after the VTE event. Clinical follow-up continued for 2 years. The PET/CT was negative in 68 out of 99 patients (68.7%), while suspicious uptake was detected in 31 out of 99 patients (31.3%). Additional diagnostic work-up confirmed a malignancy in 7 out of 31 patients (22.6%), with six at an early stage. During follow-up, two patients with negative PET/CT were diagnosed with cancer. The sensitivity, positive predictive value, and negative predictive value of PET/CT were 77.8%, 22.6%, and 97.1%, respectively.

In these studies, 3.6% (35 out of 977) of patients were diagnosed with cancer at initial screening, and 36 additional cases (3.7%) were detected during follow-up. In the analysis of the studies with available data, cancer-related mortality was 2.7% (25 out of 926), and the overall mortality rate was 8.4% (69 out of 819).

Regarding the risk of bias, only the study by Van Doormaal *et al.*[23] included a cohort of unexposed patients (limited tests). The studies by Jara-Palomares *et al.*[20] and Rieu *et al.*[22] were considered unrepresentative of the case sample as they included only patients with PE and aged over 70 years, respectively. Therefore, all studies were considered to have a moderate risk of bias, except the study by Van Doormaal *et al.*,[23] which was regarded as low risk (Table 5).

4. Discussion

This systematic review compiled four RCTs.[16-19] and one prospective open-label study without randomization[23] that compared extensive versus limited screening for occult malignancy after an unprovoked VTE, as well as five observational prospective studies that evaluated a comprehensive strategy lacking a control group.[20-22,24,25]

Among 2621 patients included, we estimated the 24-month prevalence of occult cancer detection to be 8.2%. About two-thirds of these cases were detected by screening tests, whereas the remaining one-third became clinically overt during follow-up. The pooled analysis of the RCTs demonstrated that the probability of a cancer diagnosis was strongly associated with extensive screening. At initial screening, a comprehensive strategy detected more than twice as many occult cancer cases compared to a limited test. Moreover, there was a strong trend towards a diagnosis of early-stage cancer in the extensive screening group when data from RCTs was analyzed separately. However, no statistically significant difference was observed in cancer- and overall-related mortality.

Early detection of an occult malignancy after a diagnosis of unprovoked VTE might be translated to a better prognosis for these patients; nevertheless, the optimal screening strategy remains unclear. At present, there is no evidence that testing for undiagnosed cancer in patients with a first episode of unprovoked VTE is effective in reducing cancer- and overall-related mortality or which tests for cancer are most helpful. On the other hand, two rules have been developed for occult cancer screening in patients with VTE: The RIETE and SOME scores. Jara-Palomares *et al.*[26] identified six independent predictors (RIETE score) of occult cancer between 30 days and 24 months after experiencing the VTE (provoked or unprovoked): male sex, age >70 years, chronic lung disease, anemia (hemoglobin levels <13 g/dL for men and <12 g/dL for women), elevated platelet count (≥350,000 platelets/mm^3), prior VTE, and recent surgery. Ihaddadene *et al.*[27] performed a post hoc analysis of the SOME trial. They found that age ≥60 years, previous provoked VTE, and current smoker were associated with occult cancer detection within 1 year of a diagnosis of unprovoked VTE. These rules are a potentially attractive strategy for occult cancer screening in patients with VTE; however, both scores' predictive performance was poor in external validation studies.[28-32]

The results of our study are in accordance with previous meta-analyses.[11,33,34] Klein *et al.*[33] and Robertson *et al.*[34] did not include the observational prospective studies in their meta-analysis, and in another meta-analysis of individual patient data,[11] the trial conducted by Piccioli *et al.*[16] was not included. Our study strengthens the results of previous meta-analyses, yet, it is more extensive and includes more studies and patients.

Table 5. Newcastle-Ottawa-scale tool for the quality assessment of included prospective studies

Study	Selection	Comparability	Outcome	Total stars	Risk of bias
Jara-Palomares *et al.*[20]	* *	—	* *	4	Moderate
Carrier *et al.*[21]	* * *	—	* *	5	Moderate
Rieu *et al.*[22]	* *	—	* *	4	Moderate
Van Doormaal *et al.*[23]	* * * *	*	* *	7	Low
Rondina *et al.*[24]	* * *	—	* *	5	Moderate
Alfonso *et al.*[25]	* * *	—	* *	5	Moderate

Note: * Indicates one point awarded in the corresponding domain of the Newcastle–Ottawa Scale.

Significant cancer management advances have been made over the past 10 years. Most are associated with developing new molecular diagnostics and more precise individual therapies, which result in extended survival due to personalized approaches for each tumor. Notably, the follow-up period of up to 24 months in most studies might be relatively short for evaluating all-cause and cancer-related mortality in the era of modern oncology. This is a plausible explanation for the lack of reduction in mortality in extensive screening compared to limited tests.

All studies were assessed as having a low risk of bias according to the Jadad scale. It should be noted that two trials were prematurely terminated.[16,18] In the Piccioli *et al.* study,[16] only five of the more than 40 anticipated participating centers could initially enroll subjects, and other centers could not proceed as the extensive screening proposed was considered unethical. Furthermore, the detection of cancer in its early stages in the extensively screened group prompted an increasing trend among investigators to start extensive screening of the control group for cancer. The study conducted by Prandoni *et al.*[18] was prematurely ended due to low recruitment. Robin *et al.*'s trial[19] was considered low risk, though the non-blinding of outcome assessors is noteworthy. According to NOS, except for the Van Doormaal *et al.* study,[23] all the prospective studies were considered to be at moderate risk of bias due to the lack of comparison.

Based on evidence, the International Society on Thrombosis and Haemostasis (ISTH) guidelines suggest a restricted approach to cancer screening for the first unprovoked VTE.[35] This includes conducting an in-depth medical history and physical examination alongside laboratory assessments (such as complete blood count, calcium levels, urinalysis, and liver function tests) and a chest X-ray. The guidelines also recommend age- and gender-specific cancer screenings (including those for cervical, prostate, lung, and colon cancers), and these recommendations remain unchanged. Similarly, the United Kingdom (UK) National Institute for Health and Care Excellence (NICE) guidelines for VTE only recommend routine screening (including a history, physical exam, and basic laboratory testing), unless patients have clinical symptoms or signs suspected of cancer.[36]

There seems to be a need for further research into hidden cancer in VTE. The Screening for Occult Malignancy in Patients with Unprovoked Venous Thromboembolism trial (MVTEP2/SOME2 trial; clinicaltrials.gov identifier: NCT04304651) seeks to determine whether, in patients ≥50 years old with a first unprovoked VTE, a cancer screening strategy incorporating 18F-fluorodeoxyglucose PET/CT can decrease the number of missed occult cancers detected over a 1-year follow-up period compared to limited screening.[37] The study's completion date is estimated to be September 2030. Recent findings have suggested a crucial role of biomarkers in predicting occult cancer in patients with unprovoked VTE. Several studies have revealed that soluble P-selectin might effectively differentiate patients with unprovoked VTE and occult cancer.[38,39] A recent cohort prospective study involving 19 patients with unprovoked VTE and cancer and 195 patients with VTE free from malignancies concluded that soluble P-selectin exceeded 62 ng/mL and D-dimer surpassed 10,000 μg/L, and the diagnosis of hidden neoplasm demonstrated a specificity of up to 91%.[39] The PLATO-VTE study, which is ongoing, investigates the sensitivity of various biomarkers, such as platelet mRNA sequencing, circulating tumor DNA, and proteomics analysis, for cancer detection compared with limited screening.[40] Notably, machine learning (ML) is emerging as a groundbreaking tool in medicine. This field might identify complex patterns associated with occult cancer in the thrombosis area. A recent systematic review analyzed the value of ML methods in predicting venous thrombosis in cancer patients.[41] The prediction ML models demonstrated good performance in predicting VTE. In another study conducted by Muñoz *et al.*,[42] a predictive model to assess the risk of VTE recurrence in these patients was developed using Natural Language Processing and ML, yielding encouraging outcomes. Hence, models

developed using ML techniques hold promise for accurately identifying VTE patients at elevated risk for occult cancer.

A few limitations to our analysis should be acknowledged. Differences were observed among the studies regarding patient characteristics, screening strategies, the time between the episode of VTE and screening, and the follow-up period. However, the conclusions are based on data collected from more than 2,000 patients. The study also did not measure VTE-related mortality, adverse effects of anticoagulation, adverse effects of cancer tests, participant satisfaction, or quality of life; consequently, the impact of these factors remains unknown.

5. Conclusion

This meta-analysis explores the usefulness of cancer screening in patients with unprovoked VTE. RCT data demonstrated that thorough screening enables earlier detection of cancer in patients with unprovoked VTE. However, this early detection does not improve overall patient survival or decrease cancer-related mortality. Consequently, current guidelines do not recommend extensive cancer screening for patients with unprovoked VTE, leaving the decision to screen at the discretion of individual clinicians on a case-by-case basis.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Anabel Franco-Moreno
Formal analysis: Anael Franco-Moreno, José Manuel Ruiz-Giardín
Methodology: All authors
Writing–original draft: All authors
Writing–review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not aplicable.

Availability of data

The datasets generated and analyzed for the current study are available from the corresponding author.

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REVIEW ARTICLE

Propranolol: Repurposing an old drug to modulate tumor growth, angiogenesis, and immunity in hepatocellular carcinoma

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Abstract

Background: Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the key risk determinants for hepatocellular carcinoma (HCC), which is a significant public health issue worldwide. Molecular mechanisms of HBV- and HCV-related hepatocarcinogenesis are reviewed here, together with the therapeutic potential of propranolol against HCC. HBV and HCV promote HCC development through chronic inflammation, oxidative stress, and dysregulation of signaling pathways involved in proliferation, apoptosis, and immunity. Propranolol demonstrates promise in inhibiting tumor growth, angiogenesis, and metastasis in HCC by modulating adrenergic receptors and the immune response. Evidence suggests propranolol reduces inflammatory cytokines, enhances natural killer cell activity, and decreases the expression of immune checkpoint proteins such as programmed cell death protein 1 and T cell immunoglobulin and mucin domain-containing protein-3 in HCC cells. Clinical studies indicate that propranolol may lower HCC incidence and improve survival in cirrhotic patients. However, optimal dosing, long-term safety, and efficacy require further research through large randomized controlled trials. **Aim:** This paper aims to review the potential of propranolol as an adjuvant therapy for HBV/HCV-induced HCC by examining its antitumor, anti-angiogenic, and immunomodulatory effects. **Conclusion:** Propranolol represents a prospective adjuvant therapy for HBV/HCV-induced HCC that warrants continued investigation to fully elucidate its therapeutic potential against this disease. **Relevance for patients:** Propranolol may improve outcomes in HBV/HCV-related HCC by reducing tumor growth, angiogenesis, and immune evasion, offering a potential adjunct therapy to enhance patient survival and prognosis.

Keywords: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Propranolol; Cirrhosis

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1. Introduction

1.1. General overview of hepatocellular carcinoma (HCC)

Globally, HCC ranks fifth in the frequency of new occurrences, with 500,000 – 1,000,000 cases reported yearly. Up to 80% of HCC cases are found in developing nations, with sub-Saharan Africa and Southeast Asia having the most significant incidence rates.[1,2] HCC has been the Taiwan region's primary source of cancer-related fatalities since 1984. In the Taiwan region, 8,000 new instances of HCC are discovered each year, and about 7,000 HCC deaths occur annually.[3] Around the world, hepatitis B virus (HBV) infections account for over half of all HCC cases, whereas hepatitis C virus (HCV) infections account for roughly one-third. However, the proportion of HCC caused by these viruses varies significantly by demographic, and there is evidence that the prevalence of HCV globally is significantly underreported.[4] Developed Western countries have a low prevalence of HCC, although there has been a rising trend over the past 20 years. These two viruses account for over 80% of all HCC cases worldwide, making them the two main risk factors for developing HCC. Non-viral variables, such as alcohol use as well as aflatoxin B1, and other chronic liver disorders, are less frequently associated with HCC risk.[2,5] Although several Asian and African nations are known to have a high incidence of HCC, there has been a more recent rise in the condition in nations such as the United States. Environmental pollutants, alcohol and drug abuse, autoimmune diseases, genetics, obesity, high hepatic iron levels, and infections with hepatotropic viruses have all been associated with HCC development.[5] Regardless of the condition's cause, patients with chronic viral infections are likely to develop liver cirrhosis, a significant risk factor for carcinogenesis.[6]

Finding effective HCC therapies is critical. Effective medical interventions can vastly enhance patient outcomes, increase survival rates, and improve the quality of life for patients suffering from this condition.[7] Furthermore, improvements in HCC treatment will lessen the strain on health-care systems by eliminating the requirement for costly interventions such as palliative care or liver transplantation. Finding successful HCC treatments will also give patients and their families hope and provide them with a chance to battle this terrible enemy. In addition, it would open the door for more investigation into the biology of liver cancer, allowing researchers to identify new therapeutic targets and create groundbreaking strategies to fight HCC.[8] In this paper, we aim to examine propranolol's potential as a treatment for HCC by thoroughly examining the available literature and research findings as part of our aspiration to contribute to the ongoing efforts of identifying novel therapeutics for this aggressive kind of liver cancer.

2. Molecular mechanisms of HBV in the development of HCC

2.1. HBV

HBV, a small pathogenic enveloped virus, belongs to the Hepadnaviridae family. Among DNA viruses, HBV is distinct since an RNA intermediate is utilized as an intermediate in its DNA genome replication.[9,10] The reverse transcriptase (the enzyme for DNA replication by reverse transcription), three envelope glycoproteins, and the C protein of the viral core (the chief component of the viral nucleocapsid) are all translated from the four open reading frames (ORFs) of the small, 3.2 kb HBV DNA genome. HBV is transmitted by contact with infected blood or body fluids in the same manner as HIV. In comparison to HIV, HBV is 50 – 100 times more infectious.[11] Malaise, exhaustion, jaundice, and skin and sclera discoloration are common symptoms of hepatitis, an inflammation of the liver which is brought on by liver malfunction and high blood bilirubin levels.[5] It is estimated that 350 million people globally have chronic HBV. Up to 40% of these people will experience problems from HCC and cirrhosis. Chronic HBV carriers have an annual risk of <1%, but individuals who also have cirrhosis have an annual risk of 2 – 3%. About 70 – 80% of HBV-related HCC develops in cirrhotic livers, whereas the remainder of HCC develops in livers that do not have cirrhosis. The formation of HCC in people with chronic hepatitis B is a multistage, complex process that involves the interaction of the host and the environment. HBV is not immediately cytopathic. Gender, age, cigarette smoking, alcohol use, chemical carcinogens, hormones, and genetic vulnerability are among the chronic HBV-associated risk factors for HCC.[6]

2.2. Overview of HBV replication

The Hepadnaviridae family of viruses, of which HBV is a part, can result in persistent liver infections. Each hepadnavirus appears to have a restricted host range dictated by intracellular signaling components and a cell-surface receptor.[5] The hepadnavirus known as HBV reproduces by use of RNA. The 3.2 kb partially double-stranded relaxed circular DNA (rcDNA) of HBV is converted into covalently closed circular DNA (cccDNA) after it enters hepatocytes. All viral mRNAs are transcriptionally transcribed from the cccDNA, which is organized as a minichromosome by both viral and cellular histone and non-histone proteins. Recurrence of the disease is possible even after successful treatment and HBsAg removal since HBV replication would still

continue after the intake of nucleoside analogs.[9] Upon entry into the nucleus of the partially double-stranded circular DNA, single-stranded DNA gaps are closed, and the HBV genome is transformed into a covalently closed circular, double-stranded DNA (cccDNA). cccDNA serves as a template for viral mRNA transcription and synthesis rather than replication. HBV genome is small and contains four overlapping ORFs encoding HBx protein, envelope (S antigen), reverse transcriptase/polymerase (Pol), and capsid (core). Pre-genomic RNA (pgRNA), the largest HBV transcript, is a terminally redundant viral replication intermediate. The viral reverse-transcriptase/polymerase of the infected hepatocyte cytoplasm replicates by reversing the pgRNA to the DNA genome. The HBsAg proteins either secrete the replicating, encapsulated viral genome to the nucleus to enhance the nuclear reservoir of cccDNA or sequester it as capsid buds within the endoplasmic reticulum. The encapsulated virion is released from the cell; numerous studies have demonstrated that HBV release from cells is regulated by multivesicular body components.[5] Three stages may be distinguished in the hepadnaviral genome replication process. First, the icosahedral core of the infectious virions contains rcDNA, which is a circular, partly double-stranded DNA molecule of around 3.2 kb. Second, within the host cell's nucleus, the rcDNA transforms into a plasmid-like cccDNA upon infection. Finally, pgRNA, which is preferentially packed into progeny capsids and reverse-transcribed by the co-packaged P protein into new rcDNA genomes, is one of the genomic and subgenomic RNAs produced by the cccDNA.[12]

2.3. Mechanisms of HBV-induced HCC

Prevention of HBV infection and early detection of HCC are crucial for reducing the disease's burden. Vaccination against HBV has proven to be highly effective at preventing new infections, and efforts should be made to increase global vaccination rates. Regular screening for HBV infection can assist in identifying individuals at risk for HCC, enabling early intervention and treatment.[13] Public health campaigns should emphasize the significance of vaccination and screening, especially among high-risk populations such as those with a family history of HBV or in areas with a high prevalence rate.[14] In HBV-related HCC, angiogenesis is critically regulated by vascular endothelial growth factor A (VEGFA), whose post-transcriptional control by HBV remains unclear. It has been revealed that HBV increases m6A methylation of VEGFA mRNA, thereby upregulating the RNA-binding protein IGF2BP3, stabilizing VEGFA in an m6A-dependent manner, and enhancing endothelial cell migration and tube formation. Knockdown of IGF2BP3 in an HBV-associated HCC xenograft model reduced VEGFA levels and tumor growth, identifying the IGF2BP3-VEGFA axis as a potential therapeutic target for antiangiogenic therapy in HBV-related HCC.[15]

Another major oncogenic mechanism involves HBV integration into the host genome, which subsequently disrupts essential cellular regulatory functions required for cell survival and proliferation. Interacting with different cellular proteins and regulating their functions, the HBV X protein (HBx) is a key molecule in promoting the development of HCC.[16] HBx can activate multiple signaling pathways involved in cell proliferation and survival, such as the PI3K/Akt and MAPK/ERK pathways. HBx can also disrupt DNA repair mechanisms, leading to genomic instability and mutation accumulation. Another essential mechanism is the induction of chronic inflammation by HBV infection, which creates a microenvironment conducive to the initiation and progression of tumors. During chronic inflammation, inflammatory cytokines stimulate cell proliferation, angiogenesis, and tissue remodeling.[17] By releasing pro-inflammatory mediators, HBV-induced immune responses can also contribute to liver injury and promote the development of HCC. These pro-inflammatory mediators exacerbate the inflammatory response, thereby perpetuating liver injury and promoting the development of HCC.[18] In addition, HBV infection can interfere directly with cellular processes involved in DNA repair and replication, resulting in genomic instability and an increased risk of mutations. These mutations contribute to the progression of HCC.[19] Furthermore, immune responses elicited by HBV are also capable of recruiting immune cells such as macrophages and lymphocytes, which produce other pro-inflammatory cytokines that lead to tissue damage and tumor development. This vicious cycle between chronic inflammation, immune response, genomic instability, and mutagenesis represents the complexity of HBV-induced HCC formation. All these processes should be unveiled to develop targeted therapies to prevent this complicated sequence and improve the prognosis of HBV-related HCC patients.[17]

3. Molecular mechanisms of HCV in the development of HCC

3.1. HCV

HCV is a member of the Flaviviridae family of enveloped RNA viruses. The 9 kb long, positive-strand, single-stranded RNA genome of HCV, similar to other flaviviruses, is translated as a polyprotein, which is processed by proteases into four functional proteins at the C-terminus. They comprise non-structural proteins involved in the replication of the virus, two envelope glycoproteins (E1

and E2), and a single capsid (C) protein. Like HBV, HCV is transmitted by blood and body fluids.[2,11] Despite having no known DNA form during its life cycle or latent stage, HCV commonly causes recurrent liver infections. Unlike HBV infections, the probability of developing a chronic HCV infection is between 55% and 85%, and it does not change much with age.[11] Worldwide, there are over 170 million HCV-infected people, and 20% of them will develop cirrhosis. Unlike patients with chronic hepatitis B, those with chronic hepatitis C nearly invariably develop HCC in the context of cirrhosis. Annually, 1 – 4% of chronic hepatitis C patients with cirrhosis are prone to developing HCC, whereas 1 – 3% of patients with a chronic HCV infection will do so within 30 years. Similar to HBV, the association between HCV and HCC likely involves both the indirect effect of cirrhosis and HCV's direct role in promoting hepatocarcinogenesis.[2] Because of the expression of the entry receptors needed for viral replication and host liver-enriched cellular factors (miRNA-122), HCV infects mainly hepatocytes. Extrahepatic manifestations were, however, observed in kidneys, peripheral nervous system, epithelial cells, and mononuclear blood cells.[20]

The livers of HCV-infected individuals have higher amounts of the hepatic low-density lipoprotein (LDL) receptor, which is crucial for assessing blood cholesterol levels. This suggests that in HCV patients, viral infection directly causes decreased LDL levels.[6] The combination of environmental, host, and viral variables is one of the processes behind HCV-induced HCC. HCV does not incorporate into the host genome such as HBV does. However, like HBV, HCV may cause chromosomal instability by directly affecting genes that control how centrosomal processes and mitotic spindles are arranged during the cell cycle.[21]

The attachment of HCV to cells marks the start of its life cycle. HCV particle entrance into hepatocytes is facilitated by a variety of cellular components, including proteins, lipids, and glycans. HCV first binds to surface proteoglycans, including the tetraspanin CD81 and the scavenger receptor class B type I. Following their relocation to tight junctions, occluding proteins and claudin-1 become essential for HCV penetration.[22]

3.2. Mechanisms of HCV-induced HCC

The mechanisms of HCV-induced HCC involve a complex interplay of numerous factors. Through its persistent infection, the virus causes chronic inflammation in the liver. This inflammatory response activates multiple signaling pathways, including the JAK/STAT and NF-κB pathways, which promote cell survival and proliferation.[23] Key factors include genetic mutations (e.g., TP53 and CTNNB1), dysregulated signaling pathways (e.g., Wnt/β-catenin and PI3K/AKT/mTOR), and epigenetic modifications that drive uncontrolled hepatocyte proliferation. The tumor microenvironment, comprising immune cells, fibroblasts, and extracellular matrix components, further promotes HCC by fostering immune evasion, angiogenesis, and metastasis. Chronic inflammation due to hepatitis B/C infection, metabolic disorders (e.g., non-alcoholic fatty liver disease), and oxidative stress also contribute to HCC pathogenesis by sustaining pro-tumorigenic conditions.[24] In addition, HCV proteins, notably core and NS5A, directly contribute to hepatocarcinogenesis by interfering with essential cellular processes. NS5A enhances cell proliferation and inhibits apoptosis,[25] whereas the core protein disrupts cell cycle regulation and promotes genomic instability. Moreover, HCV-induced oxidative stress and DNA damage play essential roles in the progression of HCC. The virus induces the production of reactive oxygen species and impairs antioxidant defense mechanisms, resulting in DNA lesions and genomic modifications.[26] These alterations can activate oncogenes and inactivate tumor suppressor genes, further promoting hepatocarcinogenesis. Furthermore, persistent inflammation in the liver brought on by HCV infection creates an environment that is favorable for tumor development and progression. Pro-inflammatory cytokines and chemokines are released by the virus, drawing immune cells that produce growth factors and encourage angiogenesis.[27] This sustained inflammatory response also contributes to the activation of cell proliferation and survival signaling pathways. In addition, HCV proteins interact with host factors to dysregulate cellular signaling pathways, such as those associated with cell proliferation, apoptosis, and immune responses. These interactions disrupt normal cellular functions and foster the growth of cancer. Individuals infected with HCV develop hepatocarcinogenesis due to the complex effects of HCV on critical cellular processes, including oxidative stress-induced DNA damage, chronic inflammation, and dysregulation of signaling pathways.[28] Moreover, HCV infection affects hepatocytes and the microenvironment surrounding the liver. Chronic inflammation triggered by HCV recruits immune cells, such as macrophages and lymphocytes, which release pro-inflammatory cytokines and chemokines.[29] This disruption permits unrestrained cell proliferation and survival, thereby fostering the development of HCC. Taken together, the complex interplay between chronic inflammation, oxidative stress-induced DNA damage, and dysregulated signaling pathways underlie the pathogenesis of HCV-associated hepatocarcinogenesis. Understanding these

mechanisms is crucial for developing targeted therapies to prevent or treat HCC in patients with chronic HCV infection.[30]

4. Propranolol

4.1. Properties of propranolol

Propranolol (Figure 1) is a non-selective beta-adrenergic receptor antagonist first developed by Sir James Black in 1962 and approved for clinical use in 1964.[31] It blocks both β1 and β2 receptors, leading to reduced heart rate, myocardial contractility, and blood pressure.[32] Beyond its cardiovascular applications, propranolol demonstrates anti-angiogenic, anti-inflammatory, and immunomodulatory properties. These characteristics form the basis of its emerging role in oncology, including HCC.[33,34]

Due to its high lipophilicity, propranolol readily crosses the blood-brain barrier and undergoes extensive first-pass hepatic metabolism, with approximately 25% bioavailability.[35] While it binds to plasma proteins at a high rate (>90%), propranolol's distribution across tissues, including the liver, supports its potential therapeutic use in hepatic malignancies.[35]

Research has shown that propranolol can inhibit β-adrenergic receptor-mediated signaling pathways, which are implicated in tumor cell proliferation, migration, and angiogenesis.[36] The drug suppresses the production of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 alpha (HIF-1α), key regulators of angiogenesis in tumors.[37,38] It also induces apoptosis in endothelial cells, thereby disrupting tumor vascularization.[39]

Propranolol's immunomodulatory effects are also noteworthy. It reduces the release of pro-inflammatory cytokines and enhances the activity of natural killer cells, which play a vital role in antitumor immunity.[40,41] These combined effects suggest that propranolol may be effective in preventing tumor progression and recurrence in HBV- and HCV-associated HCC.[42]

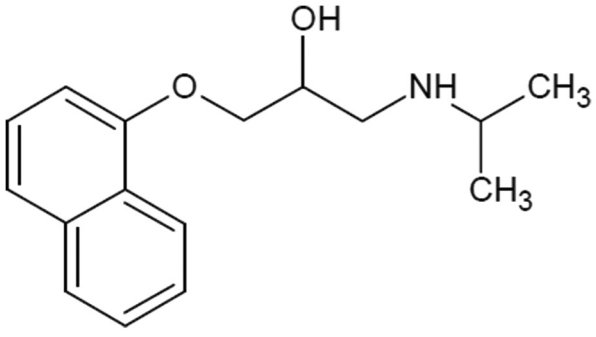

Figure 1. Chemical structure of propranolol

5. Role of propranolol in the prevention of HCC

Propranolol has been found to suppress the growth and proliferation of HCC cells by blocking beta-adrenergic receptors, which were found to be a key regulator for cancer development.[36] In addition, propranolol was said to regulate the immune system by blocking the secretion of pro-inflammatory cytokines and inducing anti-tumor immunity.[43] This dual mechanism of action makes propranolol an attractive treatment option for HCC patients infected with HBV and HCV. In addition, clinical investigations have shown that propranolol can increase overall survival rates and decrease tumor recurrence in these patients.[42] Furthermore, propranolol's anti-angiogenic effects contribute to its anticancer activity. By suppressing VEGF and HIF-1α expression, it effectively reduces neovascularization required for tumor growth and metastasis.[38,44,45] These properties have shown promise not only in HCC but also in other malignancies such as breast, lung, and colorectal cancers.[41] Propranolol effectively starves cancer cells and inhibits their ability to proliferate by targeting this critical phase of tumor development. This anti-angiogenic effect has been observed in breast, lung, and colorectal cancers. Propranolol has demonstrated immunomodulatory effects that extend beyond cytokine suppression. Notably, it has been shown to enhance the activity of natural killer cells – key components of the innate immune system responsible for targeting and eliminating cancer cells. By activating natural killer cells and boosting their cytotoxic function, propranolol strengthens the body's antitumor response, offering a promising mechanism for its potential efficacy in cancer therapy.[40,41,46] This discovery sheds light on the potential of propranolol as an adjunct therapy for various cancers, including breast, lung, and colorectal cancers. With its multiple effects on cytokine production and immune cell activity, propranolol bears promise as a cancer-fighting agent.[47] Propranolol can reduce the expression of programmed cell death protein 1 (PD-1) and T cell immunoglobulin and mucin domain-containing protein-3 (TIM-3) in liver cancer cells. PD-1 and TIM-3 are proteins that help cancer cells evade the immune system. By reducing the expression of these proteins, propranolol can make liver cancer cells more susceptible to attack by the immune system.[48] It has also been found that propranolol can increase the expression of granzyme B and interferon-gamma (IFN-γ) in liver cancer cells. Granzyme B is a protein that helps to kill cancer cells, and IFN-γ is a cytokine that helps to activate the immune system. By increasing the expression of these proteins,

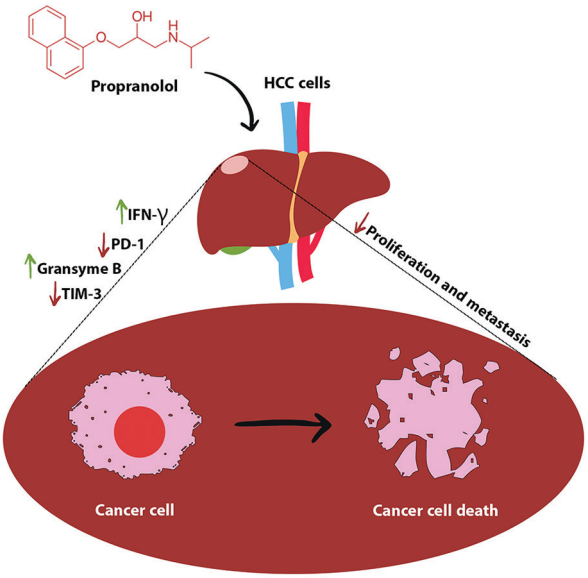

Figure 2. Schematic mechanism of the effect of propranolol on liver cancer cells. The use of propranolol reduces PD-1 and TIM-3, which increases granzyme B and IFN-γ, which ultimately reduces the proliferation and metastasis of cancer cells.
Abbreviations: HCC: Hepatocellular carcinoma; IFN-γ: Interferon-gamma; PD-1: Programmed cell death protein 1; TIM-3: T cell immunoglobulin and mucin domain-containing protein-3.

propranolol can help kill liver cancer cells and boost the immune response against cancer (Figure 2).[49]

Propranolol has been shown to inhibit the formation of new blood vessels essential for tumor growth. This is one potential mechanism for its antitumor activity. Observational studies have yielded promising results; however, randomized controlled trials are required to establish the drug's efficacy and safety profile definitively. This multifaceted mechanism of action makes propranolol a compelling adjunct therapy for HBV- and HCV-associated HCC. However, further studies, including randomized clinical trials, are needed to confirm its efficacy, determine optimal dosing, and evaluate its long-term safety in cancer patients.[42,50]

6. Clinical studies on propranolol's efficacy against HBV/HCV-induced HCC

Several studies have investigated the effect of propranolol on HCC. In 2023, a study by Wu *et al.*[51] showed that propranolol reduced the risk of HCC development in patients with cirrhosis by up to 40% and also improved survival in patients with HCC, with a median survival of 20 months in patients who received propranolol compared to 12 months in patients who did not receive propranolol, but the optimal dose and duration of propranolol treatment for HCC remains unknown. Cheng *et al.*[52] found that propranolol was associated with a decreased risk of HCC death in patients with cirrhosis; however, a significant difference in overall survival between patients who took propranolol and those who did not was not detected. Another study by Nkontchou *et al.*[53] found that long-term propranolol treatment was associated with a significant reduction in the incidence of HCC in patients with HCV-associated cirrhosis. In addition, London and McGlynn showed that patients who took propranolol for an average of 3.5 years were significantly less likely to develop HCC than patients who did not take propranolol, and the risk of HCC was reduced by about 50% in those taking propranolol.[54]

At present, there are four clinical trials on ClinicalTrials.gov investigating the effects of propranolol on HCC. At the time of writing this article, one of these trials was in phase II, while patient enrollment was still ongoing for the remainder of the trials. To determine whether propranolol is a safe and effective treatment for HCC, the outcomes of these trials will be crucial. The status of these trials may alter over time; therefore, it is always advisable to consult the ClinicalTrials.gov website for the most recent information, as summarized in Table 1.

7. Comparison of propranolol with other HCC prevention strategies

7.1. Antiviral therapies

The goals of antiviral treatments for persistent HBV infection are to inhibit viral replication, lessen hepatic inflammation, and stop the development of cirrhosis and HCC. Nucleos(t)ide analogs and IFN are the two main families of antiviral drugs that have shown promise in reducing HBV infection.[55] By inhibiting HBV DNA polymerase, nucleos(t)ide analogs such as entecavir (ETV), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide provide strong viral suppression while lowering hepatic inflammation and fibrosis.[56] Patients with persistent HBV infection have shown a considerable decrease in their likelihood of developing HCC while receiving long-term nucleos(t)ide analogs.[57] Nonetheless, there is variation in HCC risk reduction between various nucleos(t)ide analogs; research indicates that TDF users could have a lower incidence of HCC than ETV users. However, because these results are observational in nature, they should be interpreted with caution. Furthermore, compared to individuals who were not treated, IFN treatment has demonstrated a 34 – 41% reduced incidence of HCC, indicating its potential advantages despite restrictions on patient eligibility and adverse effects.[58] Direct-acting antivirals (DAAs), which provide sustained virologic response (SVR) rates of 95% across all genotypes,

Table 1. Summarized clinical trials of propranolol therapy for hepatocellular carcinoma

No.	NCT number	Study status	Interventions	Characteristics	Population	Years	Country/ Region
1	NCT01298284	Unknown	Propranolol	Interventional/Phase IV	No.: 60 Age: 18 – 80 years	2009 – 2011	Taiwan region
2	NCT01451658	Unknown	Propranolol	Interventional/Phase IV	No.: 100 Age: 18 – 80 years	2009 – 2020	Taiwan region
3	NCT01970748	Recruiting	Propranolol	Interventional/Phase IV	No.: 200 Age: 20 – 80 years	2009 – 2025	Taiwan region
4	NCT05451043	Not yet recruiting	Propranolol/Cisplatin/ Durvalumab/Tremelimumab Gemcitabine/Nab-paclitaxel	Interventional/Phase II	No.: 62 Age: 18 – 80 years	2023 – 2028	Canada

Abbreviations: HCC: Hepatocellular carcinoma; NCT: National Clinical Trial.

thus corresponding to a virological cure, have completely changed the therapy landscape for chronic HCV infection.[59] Reaching SVR lowers the chance of developing HCC by dramatically reducing hepatic inflammation and stopping the growth of fibrosis.[60] Studies have shown that compared to individuals with an active infection, those who achieve SVR had a much-decreased risk of HCC.[61] Liver stiffness decreased from 12.3 kPa at baseline to 6.6 kPa over 5 years, with the most significant improvement occurring in the 1st year post-treatment.[61] Early concerns about increased HCC incidence and recurrence following DAA-induced SVR, potentially due to disrupted immune surveillance, have been alleviated by recent meta-analyses, which found no evidence of differential HCC risk between DAA and IFN-based therapies.[62]

In addition to antiviral therapies, pharmacological interventions offer promising strategies for HCC prevention, particularly in populations with chronic liver disease, metabolic dysfunction, or other risk factors. Aspirin, for instance, demonstrates chemopreventive effects through the inhibition of cyclooxygenase-2, reducing pro-inflammatory prostaglandins and disrupting platelet–tumor cell interactions that facilitate tumor growth and metastasis.[63] Meta-analyses consistently show that regular aspirin use lowers HCC risk, particularly in individuals with chronic liver disease, though gastrointestinal bleeding and hemorrhagic stroke risks necessitate careful risk–benefit assessments.[64,65] By inhibiting hydroxymethylglutaryl-CoA reductase, statins reduce oncogenic signaling pathways such as Ras/Raf/MEK/ERK and modify the mevalonate pathway, which has anti-inflammatory, immunomodulatory, and antiproliferative effects.[66] Although myopathy or rhabdomyolysis is still a problem, especially in patients with decompensated cirrhosis, lipophilic statins, such as simvastatin and atorvastatin, have stronger protective benefits against HCC than hydrophilic versions.[67]

By activating AMP-activated protein kinase and blocking the mammalian target of the rapamycin pathway, metformin, a medication often used to treat type 2 diabetes, has also shown chemopreventive promise against HCC by reducing the development and proliferation of tumor cells.[68] It also lowers levels of insulin and insulin-like growth factor, both of which are linked to the development of hepatocarcinogenesis.[69] Although lactic acidosis in individuals with renal impairment highlights the need for careful patient selection, clinical data indicates that diabetic patients treated with metformin have a much lower risk of HCC.[70] Similarly, glucagon-like peptide-1 (GLP-1) agonists decrease the risk of HCC by improving insulin sensitivity, reducing hyperinsulinemia, and exhibiting anti-inflammatory qualities.[71] A large cohort study by Wang *et al.*[72] found that GLP-1 agonists were associated with a markedly reduced HCC risk compared to other antidiabetic therapies, though gastrointestinal disturbances and pancreatitis risks warrant careful monitoring.

By enhancing indicators of steatosis and fibrosis, sodium-glucose cotransporter 2 inhibitors, which are mainly used for glycemic management, have the potential to prevent HCC.[73] Angiotensin receptor blockers and angiotensin-converting enzyme inhibitors have anti-inflammatory and anti-fibrotic effects, which lessen liver fibrosis, a significant risk factor for HCC.[74] Known for its immunomodulatory and anti-inflammatory properties, Vitamin D supplements have also been associated with a lower risk of HCC, with a deficit markedly raising the risk of liver cancer.[75] Finally, nutraceuticals and herbal supplements, including curcumin, resveratrol, and silymarin, have shown promise in preclinical studies due to their antioxidant, anti-inflammatory, and anti-proliferative properties.[76] However, cases of drug-induced liver injury and variability in quality and potency highlight the need for regulation and standardization.[77] Collectively, these pharmacological interventions offer

valuable tools for HCC prevention, but their use must be tailored to individual patient profiles to balance efficacy and safety.

8. Potential limitations or challenges in using propranolol for HCC prevention/ treatment

While propranolol is potentially a therapeutic agent in reducing HBV/HCV-induced HCC, several limitations and challenges must be considered. The optimal dosage and treatment duration of propranolol for HCC prevention or treatment remain unclear. Drug interactions with other medications should be carefully evaluated, as propranolol may affect the hepatic metabolism of certain drugs.[78] Individual patient characteristics, such as age, liver function status, and comorbidities, may influence the efficacy and safety profile of propranolol treatment. The effectiveness of propranolol may vary among individuals due to differences in metabolism and genetic factors. Long-term use of propranolol might lead to adverse effects such as fatigue, dizziness, and gastrointestinal disturbances, potentially impacting patient compliance and overall treatment outcomes.[53]

9. Conclusion and future perspectives

HCC caused by HBV and HCV is a significant global health burden. Chronic viral infection results in various molecular alterations that promote the development of liver tumors. Due to its ability to inhibit critical processes involved in hepatocarcinogenesis caused by these viruses, propranolol has emerged as a potential therapeutic option. In conclusion, the prospective benefits of adding propranolol to HCC treatment are encouraging. Studies have demonstrated that it can inhibit tumor growth, angiogenesis, and metastasis, suggesting it could be a valuable addition to existing treatment strategies. However, some constraints and obstacles must be addressed. One such limitation is the requirement for dosage optimization, as the optimal dose of propranolol for treating HCC has yet to be determined. In addition, potential adverse effects associated with the use of propranolol must be monitored and managed with care. Future research should refine the delivery regimen and identify the patient populations most likely to benefit from propranolol therapy. In addition, investigating combination therapies that combine propranolol with other targeted agents may result in even greater efficacy in treating HCC. It is essential to address the current need for more well-defined approaches to enhance the treatment of HCC. In addition, thorough monitoring and effective management of potential side effects associated with propranolol are required.

Future research should prioritize fine-tuning dosing regimens and identify specific patient populations that would experience the most significant benefits from propranolol therapy. We can aim for more effective and individualized strategies to combat this problematic disease by pursuing these avenues. Investigating the use of propranolol in combination with immunotherapeutic agents is a prospective future research direction for exploring the full potential of propranolol in HCC treatment. Combining propranolol with immunotherapies such as immune checkpoint inhibitors or adoptive cell therapies may result in synergistic effects, enhancing treatment outcomes and patient survival rates. In addition, future research could target identifying specific biomarkers or genetic profiles that could predict a patient's response to propranolol therapy. This personalized approach would enable more targeted treatment strategies and maximize the use of propranolol in patients who are most likely to benefit. In addition, it is essential to investigate the optimal dosage and treatment duration of propranolol for HCC. Researchers can identify specific biomarkers or genetic profiles that predict a patient's response to propranolol therapy by conducting additional research. There is a need for large-scale, multicenter clinical trials to provide solid evidence on the long-term outcomes and potential adverse effects of propranolol use in this context. If proven effective, propranolol could be an accessible and cost-effective treatment option for those at high risk for or diagnosed with HBV/HCV-associated HCC.

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Conflict of interest

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Effect of physical exercise on functional capacity and dyspnea in patients with chronic obstructive pulmonary disease: A systematic review and meta-analysis

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Abstract

Introduction: Functional capacity is significantly impaired in patients with chronic obstructive pulmonary disease (COPD), as they tend to lead a less active lifestyle compared to healthy controls. Physical activity offers several benefits for COPD patients, including a reduction in mortality risk, fewer hospitalizations due to illness, and improved functional capacity. **Objective:** To review the effects of physical exercise on the functional capacity of COPD patients. **Methods:** The databases PubMed, Web of Science, OVID, Cochrane Central Register of Controlled Trials, Latin American and Caribbean Health Sciences Literature, and Scientific Electronic Library Online were searched without language restrictions. Randomized controlled trials investigating the effects of physical exercise on COPD patients were included. Study selection, data extraction, and risk of bias assessment were conducted independently. RevMan software (version 5.3) was used for the meta-analysis. **Results:** Five studies were identified from the database search. Compared to the control group, the 6-min walking distance improved in the experimental group (mean difference [MD]: 43.12 m; 95% confidence interval [CI]: −0.70 – 86.94). Notably, Borg scale scores (MD: −0.24 points; 95% CI: −0.72 – 0.25) did not differ significantly between groups. **Conclusion:** Exercise may improve the functional capacity of patients with COPD. **Relevance for patients:** Exercise interventions may enhance walking ability in COPD patients, potentially improving daily function and quality of life.

Keywords: Chronic obstructive pulmonary disease; Dyspnea; Exercise capacity; Pulmonary rehabilitation

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive respiratory condition characterized by persistent airflow limitation, usually associated with a chronic inflammatory response in the airways and lungs.[1] The impact of this disease on the functional capacity of patients is significant, as the progression of COPD leads to a gradual decline in quality of life, with limitations in daily activities and a reduced ability to perform physical exertion.[1,2] Dyspnea, one of the main symptoms of COPD, often worsens as functional capacity declines, making everyday tasks increasingly challenging.[2]

In this context, physical exercise has proven to be an effective intervention for enhancing functional capacity and alleviating dyspnea, offering cardiovascular and respiratory benefits, in addition to promoting functional independence and improving the quality of life of COPD patients.[3]

The progression of COPD leads to the deterioration of physiological functions across various systems, resulting in a significant loss of functional capacity. Chronic airway obstruction reduces alveolar ventilation, which impairs gas exchange and increases the sensation of breathlessness.[4] In addition, muscle dysfunction, particularly in the respiratory and peripheral muscles, is one of the main contributors to the functional limitations observed in these patients.[5] Studies indicate that sarcopenia, or the loss of muscle mass, is common among individuals with COPD, contributing to difficulties in performing physical activities and increasing sensations of fatigue and dyspnea.[5,6] The increased respiratory effort associated with chronic inflammation, along with the imbalance between oxygen supply and demand, also exacerbates the condition.

Physical exercise plays a key role in the management of COPD, as it can contribute to the partial reversal of functional decline. Regular exercise, especially aerobic and resistance training, has been shown to improve aerobic capacity, reduce dyspnea, and strengthen both respiratory and peripheral muscles, particularly those in the lower limbs.[7,8] Studies demonstrate that pulmonary rehabilitation programs, which include physical training, not only improve exercise tolerance but also reduce dyspnea symptoms and enhance the quality of life for COPD patients. Furthermore, exercise can improve respiratory efficiency by reducing the workload on the respiratory muscles and enhancing ventilation and oxygenation.[7-10]

Given the importance of this topic and its increasing prevalence over the years, the objective of this study is to synthesize currently available literature on the effects of physical exercise on the functional capacity and dyspnea of patients with COPD.

2. Methods

2.1. Protocol and registration

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.[11] It is registered in the International Prospective Register of Systematic Reviews under the number CRD42025634983.

2.2. Eligibility criteria

The Population, Intervention, Comparison, Outcomes, and Study (PICOS) strategy[12] was used to determine the eligibility criteria for this systematic review. The "population" consisted of patients with COPD, and the "intervention" was physical exercise, with a comparison to patients who did not undergo physical exercise. The "outcomes" were related to functional capacity and dyspnea. Only randomized controlled trials were included, with no restrictions on language or year of publication. Due to the nature of the study and in pursuit of a more precise response, only randomized clinical trials were included based on the PICOS strategy.

2.3. Information sources

A search was conducted in the following databases: PubMed, Web of Science, OVID, Cochrane Central Register of Controlled Trials, Latin American and Caribbean Health Sciences Literature, and Scientific Electronic Library Online. In addition, the reference lists of previous systematic reviews and eligible clinical trials were screened. The search for articles was completed in August 2024.

2.4. Search strategy

The search was based on the previously described PICOS framework, using the Boolean operators "AND" and "OR." The descriptors used for the population were "COPD," "chronic obstructive pulmonary disease," "chronic airway obstruction," and "chronic respiratory flow obstruction." For the intervention, terms such as "physical exercise," "physical activity," "aerobic exercise," "isometric exercise," and "physical training" were employed. The outcomes considered were "functional capacity" and "dyspnea." To identify relevant study designs, the descriptors "randomized controlled trials," "clinical trials," and "controlled trials" were used. The complete search strategy is presented in the Appendix 1.

2.5. Study selection

Randomized clinical trials involving patients with COPD were included in this systematic review. Only clinical trials that assigned patients with COPD to a physical exercise intervention were considered eligible for inclusion. Studies involving adults aged 18 years and older, regardless of sex, were included. Physical exercise was defined as a planned, structured, and repetitive activity, with a defined rhythm and duration. Studies were excluded if they involved other associated comorbidities or any combined intervention with physical exercise.

2.6. Data collection process

In the data extraction process, article titles were screened in the first stage, abstracts in the second stage, and full texts in the third stage. An exploratory reading of the selected

studies was then conducted, followed by a selective and analytical reading. Data extracted from the articles were summarized with information, such as authors, journal, year, title, and conclusions, to obtain relevant information for the research.

Two independent reviewers assessed the methodological quality of the studies. In cases of disagreement, the article was re-evaluated through full-text reading. If the disagreement persisted, a third reviewer was consulted to assess the article and make the final decision.

2.7. Data items

Two authors (VKMFJ and LML) independently extracted data from the published reports using a standardized data extraction process, considering the following items: (i) Patients of both sexes, aged between 18 and 80 years, (ii) sample size, (iii) follow-up duration, (iv) loss to follow-up, (v) outcome measures, and (vi) presentation of results.

2.8. Study quality

The methodological quality of the studies was assessed using the Physiotherapy Evidence Database (PEDro) scale,[13] which evaluates 11 criteria: (i) Eligibility criteria, (ii) random allocation, (iii) concealed allocation, (iv) baseline comparison, (v) blinding of participants, (vi) blinding of therapists, (vii) blinding of assessors, (viii) adequate follow-up, (ix) intention-to-treat analysis, (x) comparisons between groups, and (xi) reporting of point estimates and variability. Each item is scored as present (1) or absent (0), with a maximum total score of 10 points (excluding the first item, which is not included in the final score).

2.9. Synthesis of results

Heterogeneity among the studies was evaluated using the Chi-square test and the I^2 statistic. The I^2 statistic indicates the percentage of variability in effect estimates that is due to heterogeneity rather than sampling error.

2.10. Statistical assessment

The mean difference (MD) between groups and the corresponding 95% confidence intervals (CIs) were calculated to quantify the effect of continuous outcomes. For the meta-analyses in which studies used the same scales, the results were presented as MD with 95% CIs. When different scales were used, the effects were calculated using standardized MD (SMD) and 95% CIs. The effect size of the interventions was defined as small (MD <10% of the scale or SMD <0.4), moderate (MD = 10 – 20% of the scale or SMD = 0.41 – 0.7), or large (MD >20% of the scale or SMD >0.7).

3. Results

According to the data presented in the article selection flowchart (Figure 1), the database search yielded a total of 6,550 articles, with 2,010 initially excluded due to duplication. Of the remaining articles, 2,198 were excluded based on title screening. Subsequently, 1,345 articles were assessed by abstract reading, from which 897 were excluded for not being directly related to the theme of this study. Thus, 100 articles were selected for full-text reading. Of these, 10 did not present the necessary data, 67 did not classify the severity of airflow limitation, and 18 were pilot studies; all were excluded. Therefore, five articles met the eligibility criteria and were included in this systematic review.

3.1. Methodological quality results

According to the PEDro scale, two studies[14,15] were classified as having moderate methodological quality, and three as having high methodological quality.[16-18] The criteria evaluated by the PEDro scale and the scores obtained by each study are presented in detail in Table 1.

3.2. Participants

A total of 576 patients received the intervention across the studies included in this review, with ages ranging from 41 to 76 years. Additional data are presented in Tables 2 and 3.

3.3. Functional capacity

Five studies[14,16-18] analyzed the impact of exercise on functional capacity. For the meta-analysis of this comparison, a random-effects model was used (I^2 = 80%, degree of freedom [df] = 4, p=0.0006). A statistically significant difference was observed between the exercise and control groups (MD: 43.12 m; 95% CI: −0.70 – 86.94; Figure 2).

3.4. Dyspnea

Two studies[15,17] analyzed the impact of exercise on dyspnea. For the meta-analysis of this comparison, a random-effects model was used (I^2 = 0%, df = 1, p=0.84). No statistically significant difference was found between the exercise and the control groups (MD: −0.24; 95% CI: −0.72 – 0.25; Figure 3).

4. Discussion

The effects of physical exercise on the functional capacity of patients with COPD have been extensively investigated, with studies suggesting significant benefits. However, the variability in observed results, particularly regarding exercise intensity, type, and duration of rehabilitation programs, highlights the complexity of COPD treatment.

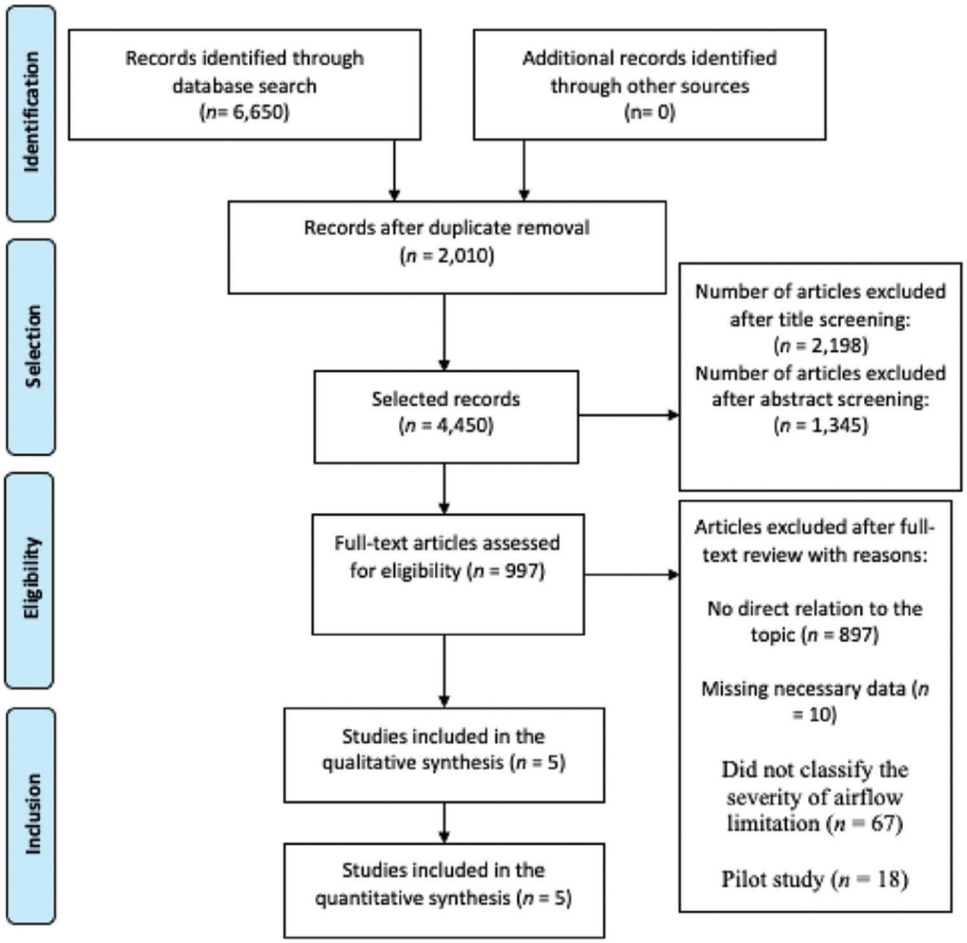

Figure 1. Flowchart of the search strategy

The studies included in this analysis provide evidence that physical exercise can have a positive impact on the functional capacity of patients with COPD. However, the magnitude and consistency of these effects vary depending on the type of intervention, program duration, and assessment methods used.

Although five studies were included in the analysis, it is important to highlight that not all employed the same exercise testing protocol, which limits the direct comparability of the results. While one study adopted Nordic walking, another used the 6-min walk test, and a third employed the incremental shuttle walk test (ISWT). These tests assess functional capacity through different methodologies and contexts, and their results, especially in terms of distance covered, are not directly comparable. To minimize the effect of heterogeneity among the exercise tests used, data analysis was based on MD only for studies that used the same unit of measurement (meters walked). In addition, statistical heterogeneity was assessed using the I^2 index, allowing the identification of consistency in effects across studies.

The study by Zwerink *et al.*[16] observed that participation in a community-based physiotherapy exercise program (COPE-active) resulted in significant improvements in functional capacity, as evidenced by increases in the distances achieved in the ISWT and endurance shuttle walk test (ESWT). The correlations between increases in daily step count and performance on the ISWT and ESWT (r = 0.47 and r = 0.38, respectively) suggested that an increase in daily physical activity may be a useful predictor of improvements in functional capacity. This finding supports the idea that engaging in regular, low-impact physical activities, such as walking, can significantly benefit patients with COPD, as these activities increase muscular endurance and improve cardiovascular efficiency without overloading the lungs.

Gea *et al.*,[19] in a study on the "less aerobic phenotype" of patients with COPD, pointed out that the reduction

in oxidative muscle fibers, along with decreased vascularization and oxygen transport capacity, is one of the main causes of the decline in functional capacity in these patients. In this context, aerobic exercise, as proposed by Zwerink *et al.*,[16] improves muscle perfusion and oxygenation, partially reversing this "phenotype" and, consequently, increasing exercise capacity.

However, in the study by Breyer *et al.*,[14] which used outdoor Nordic walking training, a significant improvement in the 6-min walk distance was observed, but there was no change in the perception of dyspnea. This suggests that, despite improvements in physical endurance, Nordic walking may not be sufficiently effective in improving the sensation of shortness of breath, a common symptom in COPD. This finding is important, as dyspnea is one of the main limiting factors of functional capacity in these patients and one of the primary targets of pulmonary rehabilitation training.

Table 1. Article classification on the Physiotherapy Evidence Database scale

Study	1[a]	2	3	4	5	6	7	8	9	10	11	Total
Zwerink *et al.*[16]	-	1	1	1	0	0	1	1	0	1	1	7
Breyer *et al.*[14]	-	1	0	1	0	0	0	1	0	1	1	5
Wootton *et al.*[17]	-	1	1	1	0	0	1	1	1	1	1	8
Behnke *et al.*[15]	-	1	0	1	0	0	0	1	0	1	1	5
Wootton *et al.*[18]	-	1	1	1	0	0	1	1	1	1	1	8

Notes: Items on the Physiotherapy Evidence Database (PEDro) Scale: (1) Eligibility criteria were specified ([a]This item is not used to calculate the PEDro score); (2) Subjects were randomly allocated to groups; (3) Allocation of subjects was concealed; (4) Groups were initially similar regarding the most important prognostic indicators; (5) All subjects were blinded in the study; (6) All therapists who administered the therapy were blinded; (7) All assessors who measured at least one key outcome were blinded; (8) Measurements of at least one key outcome were obtained for more than 85% of the subjects initially allocated to groups; (9) All subjects from whom outcome measurements were presented received the treatment or control condition according to allocation or, if this was not the case, data were analyzed for at least one outcome by intention-to-treat; (10) Statistical comparisons between groups were reported for at least one key outcome; (11) The study presented both precision and variability measures for at least one key outcome.
Abbreviations: 1:Item present; 0: Item not present.

According to Lange *et al.*,[20] the effectiveness of exercise in improving functional capacity is not only related to the intensity or type of exercise but also to the psychological and behavioral effects of exercise, which can reduce the perception of effort and, consequently, dyspnea. Perceived effort is a complex psychophysiological factor influenced by the interaction between respiratory and muscular capacities. Often, improvements in cardiovascular or muscular endurance do not directly lead to a reduction in the sensation of dyspnea without a more specific approach to respiratory symptom management.

Another relevant aspect to consider is the duration of the training and its long-term effects. The study by Behnke *et al.*,[15] which implemented a 10-day training program, showed a significant improvement in functional capacity, but the observed effects were limited to the duration of the program. Although the increase in physical activity over 24 h was more pronounced in the intervention group, the short duration of the study limits conclusions about the sustainability of long-term gains.

Similarly, the study by Breyer *et al.*[14] showed improvements in walking distances after 3 months of

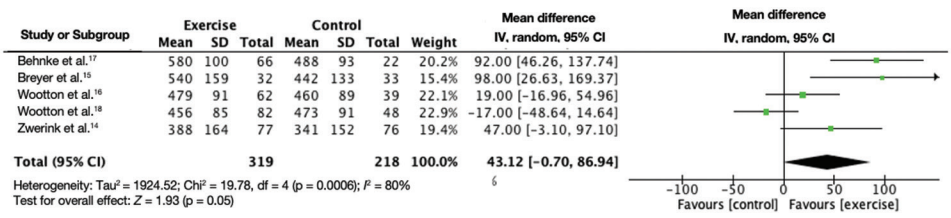

Figure 2. Forest plot of the results of the meta-analysis
Abbreviations: CI: Confidence interval; df: Degree of freedom; IV: Inverse variance; SD: Standard deviation.

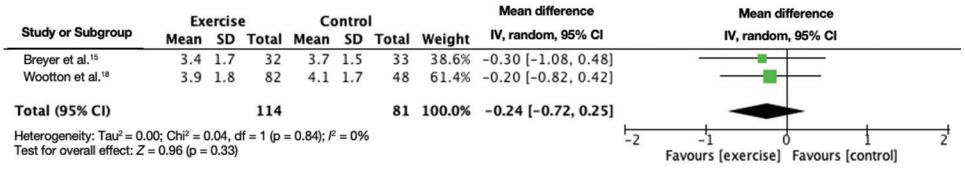

Figure 3. Forest plot of the results of the meta-analysis
Abbreviations: CI: Confidence interval; df: Degree of freedom; IV: Inverse variance; SD: Standard deviation.

Table 2. General characteristics of the included articles

Authors	Country	Sample	Participants	Control	Intervention	Measure	Results
Zwerink *et al.*[16]	Australia	153	Clinical diagnosis of COPD according to the GOLD criteria	No exercise	Participated in a community-based physiotherapeutic exercise program called COPE-active	Functional capacity	In the intervention group, correlations between the change in steps per day over 7 months and changes in ISWT (m), ESWT (m), and ESWT (s) were 0.47, 0.41, and 0.38, respectively (all $p<0.01$)
Breyer *et al.*[14]	Austria	60	COPD patients	No exercise	Outdoor Nordic walking exercise	6MWD, Borg scale	6MWD significantly increased compared to baseline (Δ 6MWD: +79±28 m) as well as compared to the control group (both $p<0.01$); no significant difference for dyspnea
Wootton *et al.* 2014[17]	Australia	143	Diagnosis of moderate, severe, or very severe COPD according to the GOLD spirometric classification	Did not participate in any exercise training and were not given any instructions regarding exercise	Ground-based walking training	Functional capacity	Compared to the control group, the walking group demonstrated greater improvements in endurance shuttle walk test time
Behnke *et al.*[15]	Germany	88	Patients with COPD and FEV_1 ≥75% of the predicted value	The control group did not participate in training but was otherwise treated in the same way as the training group with respect to medication and physiotherapy	A 10-day supervised training program was implemented, comprising a 6MTD and five walking sessions per day	Functional capacity	In both groups, there was a linear relationship ($r>0.84$, $p<0.0001$) between 6MTD and a 24-h activity, with the slope being 2.5-fold greater in the training group ($p<0.01$)
Wootton *et al.* 2017[18]	Australia	132	Participants with COPD were recruited from referrals to the outpatient pulmonary rehabilitation programs	Did not participate in any exercise training and were not given any instructions regarding exercise or daily physical activity	Walking training was conducted for 30–45 min/session, 2 – 3 times/week, for 8 – 10 weeks. Supervision was provided by experienced physiotherapists	Functional capacity	No between-group differences were found in any PA or ST measure (all $p>0.05$)

Abbreviations: COPE: Community-based physiotherapy exercise; COPD: Chronic obstructive pulmonary disease; ESWT: Endurance shuttle walk test; FEV_1: Forced expiratory volume in one second; GOLD: Global Initiative for Chronic Obstructive Lung Disease; ISWT: Incremental shuttle walk test; PA: Physical activity; ST: Sedentary time; 6MTD: 6-min treadmill distance; 6MWD: 6-min walk distance; 6MWT: 6 min-walk test.

doi: 10.36922/jctr.25.00004

Table 3. Parameters used in each study included in this review

Study (year)	Frequency	Intensity	Duration	Type
Zwerink *et al.*[16]	In the first period, patients trained 3 times/week, and in the second period, twice per week	Intensity of the program was tailored to the individual patient's performance level by the physiotherapist	A compulsory 6-month period, followed by an optional but recommended 5-month training period	Training sessions consisted of cycling, walking, climbing stairs, and lifting weights
Breyer *et al.*[14]	3 times/week	75% of the initial maximum heart rate	3 months	Nordic walking
Wootton *et al.*[17]	3 times/week	Ground-based walking training commenced at 30 min duration, speed set at 80% of the average speed achieved during the 6-min walk test	8 weeks	Walking training was performed on a flat indoor track within the participating hospitals
Behnke *et al.*[15]	Not informed	Not informed	10 days	Supervised walks on aisle, separated by at least an hour and spread over the whole day
Wootton *et al.*[18]	2 or 3 times/week	Walking training commenced at 30 min; pace adjusted to elicit a dyspnea score of 3 – 4 on a modified 0 – 10 point category-ratio dyspnea scale	8 – 10 weeks	Supervised, ground-based walking training

training, but stagnation in progress between months 3 and 9. These results are consistent with those of Jehn *et al.*,[21] who suggested that the training response may be greater in the early stages of a program, with a "learning" effect initially, followed by a plateau in further gains. Bourbeau *et al.*[22] explained that while adaptation to exercise may occur quickly, psychosocial factors, such as adherence to the exercise program and patient behavior, can influence the maintenance of long-term benefits.

The stagnation in progress observed by Zwerink *et al.*[16] between 7 and 12 months into the intervention is another important consideration. The authors suggested that this may be related to a lack of patient adherence and motivation over time. Sullivan *et al.*[23] also pointed out that after pulmonary rehabilitation programs end, many COPD patients return to previous levels of inactivity, which contributes to the loss of gains achieved.

Puhan *et al.*[24] noted that a combination of aerobic and resistance exercise may be more effective than aerobic exercise alone in improving functional capacity and quality of life in COPD patients. This is consistent with the findings of Breyer *et al.*,[14] who used Nordic walking training, and Zwerink *et al.*,[16] who incorporated a community physiotherapy program focused on walking. Lange *et al.*[20] also emphasized that combining aerobic training with muscle-strengthening exercises aimed at improving the endurance of type I and type II muscle fibers can result in more lasting benefits, as these modalities address the peripheral muscle deficits observed in COPD.

Borg scale scores may not show significant changes due to several factors, such as the type of exercise performed, the intensity applied, the short duration of interventions, the patients' physiological adaptation to the effort, or individual characteristics like an altered effort perception threshold in people with COPD.

The analysis of the studies reinforces the idea that, in order to maximize the benefits of exercise in COPD, the approach must be individualized, taking into account the severity of the disease, the patient's physical condition, motivation, and the presence of comorbidities. Exercise should be adjusted in terms of intensity, duration, and type to ensure that patients not only improve their functional capacity but also maintain these gains over time.

With such a small number of studies, the ability to detect true and consistent effects across them is reduced, increasing the risk of type II errors (false negatives). Furthermore, the variability among studies may have a disproportionate influence on the overall results, making it difficult to assess heterogeneity and generalize the findings. The wide CI observed in the distance walked during the 6-min walk test may be attributed to factors such as small sample sizes, high variability among participants, and inconsistencies in test protocols, all of which hinder the accuracy of clinical interpretation. Heterogeneity among the studies was assessed using the I^2 index, which revealed moderate to high variability. Furthermore, differences in exercise protocols across the included studies contributed to this variability, highlighting the importance of considering these aspects when interpreting the results.

5. Conclusion

Although studies showed that physical exercise is effective in improving the functional capacity of patients with COPD, the results were influenced by several factors, including the type of exercise, the intensity of the program, the duration of the training, and patient adherence. Improvements were more evident in the short and medium terms, with stagnation observed in the long term in some cases. This suggests the need for more sustainable exercise programs that combine aerobic and resistance exercises, tailored to the individual needs of each patient and supported by continuous follow-up to ensure adherence and maximize benefits. The implementation of combined and individualized approaches, along with greater attention to the psychosocial aspects influencing adherence, is essential to optimize the outcomes of pulmonary rehabilitation in COPD patients.

The findings suggest that incorporating exercise programs into COPD management can be an effective strategy to improve patients' functional capacity, providing healthcare professionals with a basis for prescribing more targeted and evidence-based interventions. Despite the demonstrated benefits, gaps remain in the literature regarding the optimal duration, intensity, and type of exercise most effective for different COPD patient profiles. This highlights the need for future research to explore these variables with greater precision.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

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Appendix 1

Search strategy

((“pulmonary disease, chronic obstructive”[MeSH Terms] OR (“pulmonary”[All Fields] AND “disease”[All Fields] AND “chronic”[All Fields] AND “obstructive”[All Fields]) OR “chronic obstructive pulmonary disease”[All Fields] OR (“chronic”[All Fields] AND “obstructive”[All Fields] AND “pulmonary”[All Fields] AND “diseases”[All Fields]) OR “chronic obstructive pulmonary diseases”[All Fields] OR (“pulmonary disease, chronic obstructive”[MeSH Terms] OR (“pulmonary”[All Fields] AND “disease”[All Fields] AND “chronic”[All Fields] AND “obstructive”[All Fields]) OR “chronic obstructive pulmonary disease”[All Fields] OR “copd”[All Fields]) OR (“pulmonary disease, chronic obstructive”[MeSH Terms] OR (“pulmonary”[All Fields] AND “disease”[All Fields] AND “chronic”[All Fields] AND “obstructive”[All Fields]) OR “chronic obstructive pulmonary disease”[All Fields] OR (“chronic”[All Fields] AND “obstructive”[All Fields] AND “lung”[All Fields] AND “disease”[All Fields]) OR “chronic obstructive lung 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Gender differences in otoacoustic emissions test pass rates: Evidence from a general population and twin study

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Abstract

Background: Evoked otoacoustic emissions are one of the most widely employed techniques in assessing neonatal hearing. However, several factors may influence the outcomes of this test. One such factor is gender, as previous research has shown that females tend to exhibit a greater otoacoustic response than males. **Aim:** This study evaluates whether gender influences the pass rate of the otoacoustic emissions test in neonatal hearing screening, using data from both the general population and twin pairs. **Methods:** Data from the Newborn Hearing Screening (NHS) program and infant gender were collected between 2002 and 2023 from 22,825 healthy newborns in the maternity or neonatal ward. Of these, 586 cases were from twin pregnancies. A separate analysis was conducted for twins, distinguishing between same-sex and opposite-sex pairs. **Results:** A highly significant difference ($p<0.0001$) in NHS pass rates was observed in favor of females, who demonstrated better responses to the test. In the twin subgroup, analysis of the 112 discordant-sex pairs with differing test outcomes revealed a statistically significant result ($p<0.023$), further supporting the positive effect of female sex on successfully passing the hearing screening test. **Conclusion:** Female newborns exhibit stronger otoacoustic emission responses and higher pass rates in the NHS test compared to male newborns. These gender-based variations in otoacoustic emission responses may have important implications for the NHS, as otoacoustic emissions are a commonly used screening tool in neonatal hearing assessment. **Relevance for patients:** Recognizing and accounting for these gender-based differences in otoacoustic emission responses may inform modifications to screening program protocols, potentially improving the identification of hearing impairment in newborns.

Keywords: Newborn hearing screening; Gender; Otoacoustic emissions; Twins

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1. Introduction

Evoked otoacoustic emissions (EOAEs) are faint sounds generated within the inner ear by the cochlea's outer hair cells in response to acoustic stimulation. Their

detection serves as a marker of cochlear integrity and function.

Among the widely used methods for newborn hearing screening (NHS) is the transient EOAE (TEOAE) test. However, research has suggested that there may be gender-based differences in the strength of these OAE responses, with female newborns exhibiting stronger reactions compared to their male counterparts.[1,2]

Studies have found significantly higher signal-to-noise ratios and response levels in females across various frequencies.[3-5] These differences are more pronounced at higher frequencies.[3] In addition to gender-based differences, interaural asymmetries have been observed. Both sexes tend to demonstrate a right-ear advantage, with higher reproducibility and response levels compared to the left ear.[4-6] These inter-aural differences are more pronounced in males.[6] The observed sex-based differences in OAE responses and ear asymmetry persist from birth through at least the 1st month of life.[5]

The superior responses in EOAE tests observed in females are likely attributed to a combination of hormonal, anatomical, genetic, and possibly evolutionary factors. Female sex hormones, especially estrogens, play a crucial role in enhancing the function of outer hair cells, resulting in stronger and more detectable OAEs. These differences are present from birth, indicating a biological basis in the development of the auditory system.

Due to its speed, affordability, and proven reliability, OAE detection serves as the foundation for many NHS programs. While most studies have focused on the difference in response intensity, which is usually around 1 – 1.5 dB in favor of females,[2,7] an important clinical question remains: Do these physiological differences influence actual pass rates in neonatal hearing screening? If so, this could have significant implications for refining current screening protocols, potentially incorporating sex-specific considerations.

The objective of this study is to evaluate whether gender-based differences exist in the pass rate of the OAE test during neonatal hearing screening, both in the general population and among twin pairs.

2. Materials and methods

Data were collected between 2002 and 2023 from healthy newborns in the maternity or neonatal ward of the Francesc de Borja Hospital. A separate analysis was conducted for same-sex and different-sex twins. This retrospective study was approved by the ethical committee of the hospital on July 15, 2019, with the code 12/2019.

2.1. Inclusion criteria

The study focused on newborns who did not require intensive care. Most participants were from the Maternity Ward, with a few from the Intermediate Neonatal Unit.

2.2. Hearing screening protocol

For newborns delivered vaginally, bilateral TEOAE measurements were typically conducted around 48 h after birth. In some cases, an initial screening was earlier, coinciding with the availability of the infant and the screener, regardless of whether the infant was a little irritable. If this initial screening resulted in a "refer" outcome, it was repeated a few hours later, just before discharge. In most such cases, the repeated screening yielded a "pass," and that result was recorded in the dataset. Infants who passed the first screening did not undergo further testing. For infants born through cesarean section, screening was postponed until approximately 72 h post-delivery, in accordance with the longer postpartum stay.

All nurses on the unit were trained to conduct hearing screenings and performed them during each shift, throughout the week, whenever workload allowed. Tests were conducted in the newborn room with background noise kept to a minimum. Parental verbal consent was obtained prior to testing. Whenever possible, measurements were taken shortly after feeding to ensure the infant was calm and to reduce noise interference. No pharmacological sedation was used.

2.3. Recording equipment and protocol

All recordings were conducted using versions of the EchoCheck screener, based on the Intelligent Laser Otoscope (ILO) 88 system (Otodynamics, United Kingdom), coupled to an ILO Ear Coupler Probe designed for neonatal use. The device presents a standard 1 ms nonlinear click at 84 ± 3 dB sound pressure level, delivered 80 times/s, and averages cochlear responses across the 1 – 4 kHz frequency range. The principal analysis focused on the 1.6 – 3.2 kHz band, with the 1.6 kHz region filtered to suppress external noise. The instrument was compact and automatically adjusted to the diameter of the external auditory canal, while light-emitting diode indicators verified adequate stimulus delivery and acceptable background noise (<47.3 dB sound pressure level on average). A "pass" indicated the presence of TEOAEs. The Otodynamics standard default for TEOAE screening with the ILO EchoCheck uses four frequency bands (roughly centered around 1.5, 2, 3, and 4 kHz). The usual pass criterion is an adequate signal-to-noise ratio (commonly ≥6 dB) in at least three of those four bands with a minimum of 512 valid responses for at least 5 s. The duration of the test usually oscillates between 45 s and 5 min.

A newborn with a normal bilateral response was accepted as a "pass." Otherwise, the result was recorded as a "fail".[8,9]

2.4. Statistical analysis

The dependent variable is the TEOAE result before discharge (pass/refer). The independent explanatory variable was the infant's sex as documented in the medical notes. After computing basic frequency distributions, we examined the association between TEOAE outcome and each study variable with a Chisquare test. Only cases with complete data for the variable in question (sex or twin birth) and the corresponding TEOAE result were included. Statistical significance was defined as $p<0.05$. The data were analyzed using 2016 Microsoft Excel and the Statistical Package for Social Sciences version 22.

3. Results

The total sample consisted of 22,825 newborns, of which 11,265 were female and 11,560 were male (50.6%). Only 0.4% had an Apgar score <5 at 1 min, and only four cases (0.01%) had an Apgar score <5 at 5 min. Gestational age ranged from a minimum of 33 weeks (only 20 [0.09%] cases below 34 weeks) – 42 weeks. When stratified by sex, no significant differences were observed between male and female newborns in terms of minimum gestational age (33 weeks) or maximum gestational age (42 weeks). The mean gestational age was 39.19 weeks for both groups. The standard deviation was 1.35 for males and 1.32 for females. The third percentile was 36 weeks for both, and the 97th percentile was 41 weeks for both.

Birth weights ranged from 1,500 g to 6,150 g, with 4% of the samples weighing <2,500 g. When analyzed by sex, there were also no significant differences in birth weight, with a minimum value of 1,500 g for females and 1,630 g for males. The maximum value was 6,150 g for females and 5,510 g for males. The mean birth weight was 3,361.71 g for males and 3,238.56 g for females. The third percentile of birth weight was 2,410 g for females and 2,465 g for males.

There were also no significant differences in the type of delivery, with 28% cesarean sections among males and 26.9% among females. In 7.8% of cases, altered OAEs were detected during their stay in the maternity unit, while 92.2% showed normal results at discharge. The global study group included 22,623 newborns with known gender and OAE outcomes. A highly significant difference ($p<0.0001$) was observed in favor of females, who demonstrated better responses to the neonatal hearing screening test (Table 1).

A total of 28.2% were fed with artificial formula, while 71.8% received maternal or mixed breastfeeding. Maternal breastfeeding is a well-established factor associated with higher response rates in the OAE test; in this subgroup, the association was highly significant (χ^2, $p<0.0001$). To control for this confounding variable, a separate analysis was conducted on the group of exclusively formula-fed infants (6,313 cases), which also demonstrated a statistically significant association between sex and test outcome ($p<0.041$). These findings confirm that the observed sex-based difference in OAE responses remains significant, with females demonstrating better responses to the test regardless of feeding types.

Table 1. Otoacoustic emissions of the general population

Transient evoked otoacoustic emissions	Gender		Total
	Female	Male	
Altered otoacoustic emissions	792	970	1,762
Normal otoacoustic emissions	10,379	10,482	20,861
Total	11,171	11,452	22,623
Pearson's Chi-square	Value	Log	Significance (two-sided)
	15.002	1	0.000

3.1. Twin study

Among the global data of 22,825 newborns, 586 cases were twin pregnancies (2.6%). Data were obtained from 254 pairs of twin newborns (n=508), of which 256 were female (50.4%) and 252 were males (49.6%). A total of 73.7% were born through cesarean section. The minimum Apgar score at 1 min was two (only 2 cases=0.4%), with the remainder scoring above five. At 5 min, the minimum Apgar score was eight (in one case), with the rest scoring nine or 10. Maternal breastfeeding was given to 51.9% of the twins. Altered OAEs were detected in 7.5% of cases (n=38) during their stay in the maternity unit.

The cross-tabulation between gender and OAE outcomes in the global twin group did not yield statistically significant results in any of its variations ($p>0.05$), questioning the effect of gender in this group of newborns (Table 2).

3.2. Analysis of formula-fed twins

When analyzing only newborns exclusively fed with formula (to eliminate the influence of breastfeeding), a group of 244 neonates was obtained. The results were not statistically significant, with a Chi-square p=0.308 and a Fisher's exact test p=0.415 when comparing gender and OAE outcomes.

3.3. Further analysis of twins

When differentiating between same-sex and different-sex twin pairs, two separate groups can be analyzed as shown in Figure 1. However, in neither group was a significant difference evident.

Table 2. Transient evoked otoacoustic emissions in twins

Gender	Transient evoked otoacoustic emissions in twins		Total	
	Altered otoacoustic emissions	Normal otoacoustic emissions		
Female	16	240	256	
Male	22	230	252	
Total	38	470	508	
Chi square	**Value**	**Log**	**Asymptomatic significance (two-sided)**	**Exact significance (two-sided)**
Pearson's Chi-square	1.129	1	0.288	
Fisher's exact test				0.315

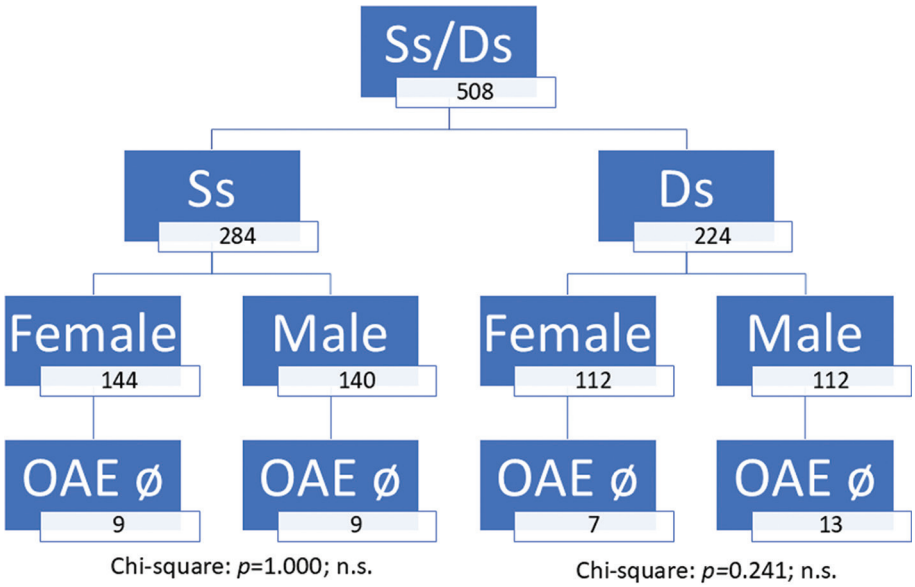

Figure 1. Analysis of twins
Abbreviations: Ds: Different-sex; n.s.: Not significant; OAE: Otoacoustic emission; Ss: Same-sex.

These results were unexpected in light of prior findings and published literature. To better understand the real effect of gender in twins, the analysis was limited to only twin pairs of different sexes, totaling 112 pairs. Among these pairs, 10 showed discordant OAE results. In eight of these pairs, the female passed the test while the male did not, and in two pairs, the opposite occurred. All pairs received the same type of feeding.

When performing a Chi-square test with Fisher's exact test, the results were statistically significant ($p<0.023$), supporting the positive effect of the female sex on OAE outcomes. This finding reinforces the gender effect in NHS performance.

4. Discussion

4.1. Global study

In our study of the global group, the positive effect of the female sex on the results of TEOAE is significant. This finding aligns with previously documented differences in response intensity and amplitude. This difference remains even when other positive factors, such as breastfeeding or the method of performing the test, are excluded.[6,10,11]

Previous research investigating the auditory response of newborns found that girls had stronger EOAEs than boys. This suggests that sex differences in auditory response are present from birth, reinforcing the hypothesis of the impact of prenatal and hormonal factors on auditory function.

This superior response appears to manifest in the 1st days of life, as premature female infants showed more robust OAEs than males, concluding that sex differences in OAEs are present from early stages of development, indicating a biological basis.[12-14] This greater response occurs in both spontaneous and EOAEs.[15] The causes of this superior response in females may be attributed to several factors.

4.1.1. Direct hormonal factors

Female sex hormones, especially estrogens, have a positive effect on the function of the outer hair cells. Estrogens may increase auditory sensitivity and enhance the amplitude of OAEs. A study analyzed how hormonal fluctuations during the menstrual cycle affected EOAEs in women, studying the relationship between sex hormone levels and their responses in OAE tests during different phases of the cycle.[16] The study found that during phases with higher estrogen levels, otoacoustic responses were stronger, reinforcing the link between hormonal activity and auditory sensitivity, suggesting a hormonal modulation of cochlear function.

In contrast, testosterone, predominant in males, may have an inhibitory effect on cochlear function, resulting in OAEs of lower amplitude. Thus, women, on average, present stronger OAEs than men.[17] It was also observed that men with a history of prenatal exposure to high testosterone levels (e.g., in multiple pregnancies) had weaker auditory responses. This supported the notion that sex hormones play a significant role in differentiating auditory responses between sexes. In summary, sex differences in OAEs suggest a hormonal and genetic influence on the development of the auditory system.[18]

4.1.2. Prenatal development and hormonal exposure

During fetal development, differences in exposure to sex hormones can influence the maturation of the auditory system. As mentioned earlier, exposure to higher levels of androgens in male fetuses may affect the formation and functionality of the outer hair cells. Auditory differences between the sexes may begin to develop in the uterus. Evidence suggests that prenatal exposure to sex hormones, such as testosterone, can influence auditory system development. Male fetuses are typically exposed to higher testosterone levels, which could affect the maturation of outer hair cells and weaken OAEs compared to females.[18,19]

4.1.3. Anatomical and physiological differences

Anatomical studies have found small structural differences in the cochlea between males and females, which could explain functional differences. Women have a slightly shorter cochlea, which might make it more efficient for certain types of auditory responses. In addition, some research suggests that women might have a greater number or better functionality of outer hair cells.[20]

In the context of differential functioning of the medial olivocochlear efferent system, human studies reveal parallel earside and sex-related differences in hearing thresholds and in the prevalence of spontaneous OAEs. One hypothesis attributes these disparities to variations in the strength of efferent neural suppression delivered to the individual cochleae, suggesting that efferent inhibition is less pronounced in right ears and in women compared with left ears and in men.[6,21,22]

4.1.4. Genetic factors

There could be X-linked genes influencing auditory function that are more effectively expressed in women. Earlier investigations have consistently shown that women with normal hearing present lower average auditory thresholds than men. Some datasets echoed this finding, demonstrating a clear and uniform tendency for females to record lower mean thresholds. These findings support a link between the observed greater male variability and the mosaic pattern of X-activation in females, which is not present in males.[23]

4.1.5. Evolutionary and adaptive effects

Some hypotheses suggest that women may have developed greater auditory sensitivity for evolutionary reasons, such as the need to better perceive soft or low-intensity sounds and detect high-frequency sounds that are important for communication and child-rearing. Although this idea remains speculative, it is one of the theories proposed to explain why women may have a more sensitive auditory system, which in turn develops better neural processing. This finding may be connected to the welldocumented asymmetries within the cerebral cortex that are thought to support speech perception, speech production, and other distinctly human capacities.[24,25]

4.2. Implications for NHS

The sexrelated disparities detected in OAE outcomes are of considerable significance for neonatal hearingscreening protocols, suggesting the need to consider gender and ear-specific factors when interpreting results and establishing pass/fail criteria.[4,5,26]

Given that almost 40% of babies who fail the first newborn hearing screen are never brought back for the followup assessment required to confirm their auditory status, it is crucial to explore ways to improve the identification of hearing loss in this population, reducing the number of newborns needing a referral. The lower sensitivity and prevalence of OAEs in male newborns suggest that they may have a higher likelihood of false negatives in hearing screening tests. On the contrary, this means that female newborns are more likely to pass the initial hearing screening, reducing the need for follow-up tests and associated parental anxiety.

Given the evidence of sex-based differences in OAEs and auditory sensitivity, modifying neonatal hearing

screening protocols to account for gender differences may enhance the accuracy and effectiveness of early auditory assessments. Below are potential considerations and suggestions for adapting screening protocols.

4.2.1. Adjustment of thresholds for screening results

Setting the same pass/fail thresholds for both sexes may lead to a higher rate of false positives in males and false negatives in females. Changes in protocols can include developing sex-specific thresholds for OAE amplitude and response times or establishing normative data for OAE responses in male and female neonates to guide individualized assessments.

4.2.2. Improvement in the test performance in males

For instance, a higher threshold for passing might be set for male newborns to account for their lower sensitivity, thereby reducing false positives and unnecessary follow-ups. Another option would be to increase the TEOAE stimulus in males to compensate for gender differences, as females have OAEs of larger amplitude than males. With an increment in the click stimulus rate, the significance of this difference would be reduced.[27,28]

These two measures can be easily included to automatically modify screening devices currently in use with the introduction of the neonate's sex. However, it can be difficult to implement due to the characteristics of the cochlear response.[15] Hence, more studies are needed before this implementation.

An alternative approach would be to perform the test as late as possible for males to facilitate a better response on the first attempt, while performing the screening earlier for females.

4.2.3. Consider anatomical and functional differences in screening design

Structural differences in cochlea length and outer hair cell functionality suggest that females might have a natural advantage in detecting high-frequency sounds. One could adjust screening frequencies to account for these differences. For example, slightly lower frequencies may be emphasized in male neonates to improve detection rates.

4.2.4. Tailor follow-up recommendations

Males might be at a higher risk of failing initial screenings without having true auditory dysfunction due to potentially weaker OAEs influenced by hormonal and prenatal factors. Hence, protocols may implement a repeat screening at discharge before recommending additional diagnostic procedures for male neonates with borderline test results. For female neonates, consider higher sensitivity as early signs of subtle auditory deficits, as better responses may mask mild dysfunctions.

4.2.5. Risk stratification based on prenatal and hormonal factors

Prenatal exposure to hormones like testosterone can influence auditory development. Male neonates, especially those from multiple pregnancies or with indicators of high androgen exposure, may require closer monitoring. It is necessary to integrate prenatal history into screening protocols to identify neonates at higher risk of auditory issues. It will be necessary to include questions about prenatal androgen exposure (e.g., multiple pregnancies or maternal hormonal treatments).

4.2.6. Gender-specific recommendations for early interventions

By incorporating these considerations into neonatal hearing screening protocols, healthcare providers can achieve a more nuanced and effective approach, ensuring better outcomes for both male and female neonates.

In the future, and following the trend of personalized medicine, with the aid of artificial intelligence, other known factors that modify the response to OAEs should also be taken into account. These factors could be included in the newborn's medical record and used to adjust the "pass" criteria of the devices utilized for the test. For instance, breastfeeding is a highly positive factor that should be compensated for in neonates who are formula-fed. Similarly, a cesarean delivery allows the test to be performed later on the newborn (as they tend to stay in hospital longer), which can result in a better response.[29]

It would be interesting to conduct longitudinal studies following twins over time to evaluate how these initial differences in EOAEs may influence later auditory and linguistic development. For example, as highlighted in Nolan's study,[30] taking sex into account as a biological factor is essential for studying the etiology of agerelated auditory decline. Ageingrelated loss of hearing is selective across frequencies and varies according to sex. Evidence further suggests that male–female distinctions in cochlear function are evident from neonatal life. Clarifying the molecular underpinnings of these sex differences may accelerate the development of targeted therapeutic approaches in precision medicine.

4.3. Twins study

In our study on twins, when the group is analyzed as a whole, the effect of gender is not significant. This may be due to the presence of a stronger factor: The day of the discharge examination. The incidence of caesarean

deliveries is higher in the twin group compared to the general population, and their discharge day is later (>72 h) compared to vaginal births (48 h). It has been demonstrated that the maximum intensity of OAEs is reached starting from days 3 to 4.[31,32] In addition, twins received a lower proportion of breastfeeding, which is another positive factor for OAEs.[29,33]

This makes it particularly interesting to analyze pairs of different-sex twins with discordant OAE results since they are discharged on the same day. In this subgroup, the differences are significant, favoring females, although the groups are small. This confirms the superior response of females under equal conditions.

Several studies on twins also demonstrate a positive effect of the female gender. Studies evaluating premature twins for auditory differences by sex found that females had higher OAE amplitudes than males, even during early developmental stages. These differences persisted regardless of factors such as birth weight or gestational age.

Furthermore, Johansson *et al.*[7] reported that, in both samesex and differentsex twin pairs, TEOAE amplitudes were appreciably greater in right ears and females than in left ears and males - a pattern previously observed in young adult twins and large cohorts of neonates.

However, the relationship in twin pregnancies may be more complex due to the influence of the co-twin's sex. Specifically, females with male twins may have less intense OAEs than females with female twins, likely due to exposure to male hormones *in utero*. This was described by McFadden and Loehlin,[34] who studied monozygotic (identical) and dizygotic (fraternal) twins to evaluate the heritability of spontaneous OAEs. They found that females had stronger emissions than males. In addition, female twins showed greater similarity in OAEs than male or mixed-sex twins. They concluded that heritability plays a significant role in OAEs, and sex differences suggest a prenatal hormonal influence.

Moreover, within the context of twin studies, McFadden[35] also investigated women who had a male twin and found that these women had less intense OAEs than women with a female twin. It was suggested that prenatal exposure to testosterone from a male twin could masculinize the female auditory system. The presence of a male twin may influence the auditory development of the female twin through shared hormonal exposure in utero. This hormonal exposure may lead to partial masculinization of the auditory system, resulting in lower amplitude OAEs in girls with male twins.

Moreover, the relationship between monozygotic and dizygotic twins may be more complex than is commonly believed. Being a monozygotic twin is not the same as being dizygotic, as demonstrated by McFadden and Loehlin[34] when evaluating the genetic influence on spontaneous OAEs in identical and fraternal twins. They found greater concordance in the auditory responses of monozygotic twins compared to dizygotic twins, although sex differences remained significant in both types. They concluded that both genetics and the intrauterine environment influence spontaneous OAEs, with sex differences suggesting a complex interaction of factors. The similarity in spontaneous OAEs among monozygotic twins indicates a strong genetic influence on auditory development. There may be specific genes that affect auditory function and are expressed differently depending on sex.

Another factor to consider could be nutritional competition, as the availability of nutrients and hormones may vary in twins, differentially affecting auditory development.

4.4. Study limitations

The EchoCheck screener does not output absolute TEOAE amplitudes. The test without normal results merely indicates whether the response corresponds to a hearing loss greater than 30 dB hearing loss. Future work should employ instruments that provide amplitude data so that the magnitude of any intergroup differences can be quantified.

The present investigation used the nonlinear protocol that dominates routine TEOAE recording.[8,32] This sequence delivers three clicks of identical polarity followed by a single click of the opposite polarity at triple intensity, enabling cochlear activity to be detected despite linear artifacts. Nevertheless, because all linear components of the waveform are subtracted, a portion of the genuine OAE signal is inevitably discarded, yielding a generally modest signaltonoise ratio. Including a linear recording condition alongside the nonlinear protocol may therefore be advisable in subsequent studies.

Finally, the EchoCheck device analyzes emissions only within 0 – 4 kHz. Work employing equipment that extends to higher frequencies is required to establish whether clinically relevant sex- or earrelated effects arise outside this band.

Given that healthy newborns were examined for this study, it remains unknown if gender increases the susceptibility to other neonatal hearing loss factors. In addition, perhaps other unknown perinatal factors can vary the response in newborns. More studies in this area are needed.

In this study, no distinction was made between monozygotic and dizygotic twins, which could also be of

interest. However, due to the statistical need for sufficiently large study groups, this could only be achieved with large cohorts of twins collected through multicenter studies.

Another limitation arises from the retrospective nature of this study, which prevents us from investigating other factors that may affect male newborns and have not yet been considered. A prospective study with modifications to the pass criteria based on sex could shed more light on hidden factors that might influence the results.

5. Conclusion

The existing literature indicates that female newborns tend to have stronger OAE responses compared to male newborns. Studies suggest a complex interaction between genetic and hormonal factors in auditory development, with both contributing to the differences observed in OAEs.

This gender-based variation in OAE responses could have important implications for NHS programs, as these tests are commonly used to identify hearing impairments in infants. The data in this study suggest that female newborns exhibit stronger OAE responses and pass rates on the test compared to male newborns.

The stronger OAE responses observed in female newborns lead to higher pass rates on initial NHSs. Conversely, male newborns may be more likely to fail the initial screening, even in the absence of true hearing impairment.

Changes to National Hearing Screening protocols should be evaluated to improve the reliability and specificity of neonatal hearing screening.

By acknowledging and accounting for these gender-based differences in OAE responses, screening programs may be able to improve the identification of hearing impairments, particularly milder forms, and ensure that affected children receive the early intervention services they need to support their language and cognitive development.

Among other measures, the "pass" criteria could be made more flexible to account for the observed differences. For instance:

- Sex-specific pass thresholds: Adjusting the amplitude or response time thresholds for OAEs based on the neonate's sex. Increasing the stimulus could also be considered in males to overcome these gender differences. This would help reduce false positives in male neonates and false negatives in females.
- Contextual considerations: Incorporating additional factors such as birth method (e.g., cesarean versus vaginal delivery), discharge day, and breastfeeding status when interpreting OAE results. These contextual factors can influence the robustness of OAE responses and pass rate.
- Follow-up adjustments: For borderline results, especially in male neonates, implement a second screening before labeling a potential issue. Conversely, a stricter follow-up protocol could be applied for females with borderline results to ensure the subtle issues are not overlooked.

Such measures would allow for a more personalized approach. They may improve the results of NHS, reducing the number of neonates who need to be rescheduled for a retest, as well as the associated anxiety and the possibility of losing patients during follow-up. By understanding and incorporating these gender differences, healthcare providers can enhance the effectiveness of NHS programs, ensuring better outcomes for all infants.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Ethics approval and consent to participate

This retrospective study was approved by the ethical committee of the Francesc de Borja Hospital in Gandia on July 15, 2019, with the code 12/2019. Parental verbal consent was obtained prior to screening.

Consent for publication

Datasets were anonymized before analysis, and parents gave consent to publication the data.

Availability of data

The database belongs to the public health service and is accessible upon obtaining the appropriate authorization.

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ACCSCIENCE PUBLISHING

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ORIGINAL ARTICLE

Three-dimensional printing-guided coaxiality assessment in transcatheter aortic valve replacement for aortic regurgitation

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Abstract

Background: Transcatheter aortic valve replacement (TAVR) using the J-Valve system provides a solution for patients with aortic regurgitation (AR). However, it remains unclear whether its coaxiality performance is related to procedural complications. **Aim:** Our goal was to improve the efficacy and accuracy of TAVR in patients with AR by adjusting J-Valve coaxiality using three-dimensional printing (3DP). **Methods:** This multicenter, prospective study included 612 patients with AR who underwent transapical TAVR. Of these, 228 patients were assigned to the 3DP group, in which the insertion angle and implantation depth were pre-determined. The coaxiality index was calculated using the distances from the bottom of the bioprosthesis to the base of the three cusps. **Results:** Compared to the non-3DP group, the 3DP group demonstrated better coaxiality performance (coaxiality index: 3.4 ± 1.7 vs. 4.0 ± 2.1; coaxiality angle: 10.5 ± 3.7° vs. 12 ± 4.2°; both $p<0.001$). Post-operative coaxiality index showed a strong correlation with the coaxiality angle (correlation coefficients: 0.85 in the 3DP group and 0.88 in the non-3DP group). The procedural success rate was higher in the 3DP group (100% vs. 96.4%; $p=0.008$). Paravalvular leakage (PVL) occurred less frequently in the 3DP group (mild PVL: 3.51% vs. 18.2%; $p<0.001$; moderate PVL: 0% vs. 1.04%; $p<0.001$). Multivariable analysis identified the coaxiality index, coaxiality angle, and horizocardia as independent predictors of PVL. **Conclusion:** PVL incidence after TAVR can be reduced through pre-operative simulations that adjust coaxiality using 3DP. **Relevance for patients:** The high incidence of PVL in transapical TAVR with the J-Valve is associated with coaxiality after bioprosthesis implantation. This study suggests that adjusting coaxiality using pre-procedural 3DP simulations may effectively reduce PVL incidence and other procedural complications during

transapical TAVR. In the future, randomized clinical trials will be needed to evaluate the efficacy and accuracy of pre-procedural 3DP simulations and the coaxiality index in treating patients with pure AR undergoing transapical TAVR.

Keywords: Aortic regurgitation; Transcatheter aortic valve replacement; Three-dimensional printing; Coaxiality; Clinical outcome

1. Introduction

Aortic regurgitation (AR) is the fourth most common type of valvular heart disease globally,[1] with a prevalence of 4.9%.[2] The indications for transcatheter aortic valve replacement (TAVR) continue to expand, and its safety and efficacy have been demonstrated even in patients with low-surgical risk aortic stenosis (AS).[3,4] However, TAVR presents unique anatomical challenges in patients with AR, including a larger annulus, lack of calcification, and dilation of the ascending aorta.[5] As a result, off-label implantation using a conventional transcatheter heart valve (THV) is more difficult, leading to a higher incidence of paravalvular leakage (PVL), THV displacement, and conduction block.[6]

In its early stages, the J-Valve (Jiecheng Medical Co., LTD., China) was developed specifically for the treatment of pure AR and has demonstrated therapeutic effectiveness in China.[7] The distinctive design features of the THV include three U-shaped positioning keys and a movable connection between the keys and the THV.[7] The inward force of the positioning keys and the outward radial force of the THV clamp the native leaflets between them like a paper clip. To ensure maximum fixation of the THV, it is critical to place the U-shaped keys accurately and expand them fully to achieve THV coaxiality, thereby reducing the incidence of PVL and other procedural complications.[7,8]

Conventionally, computed tomography angiography (CTA) has provided essential information for sizing THVs in patients with AR, as it does for those with AS. However, the size and orientation of the cusps vary among individuals, impacting how well the U-shaped keys fit into the cusps. In addition, CTA may be limited in accurately displaying THV orientation after implantation.[9,10] To address this, we used three-dimensional printing (3DP) to simulate the insertion of U-shaped keys into the sinuses and to optimize cusp fitting, thereby maximizing clamping force.

At present, no clinical studies have assessed the impact of coaxiality in transapical TAVR for patients with pure AR. Therefore, the goal of this study was to evaluate the feasibility of using 3DP to adjust the coaxiality of the J-Valve before TAVR, to improve implantation accuracy and clinical efficacy.

2. Materials and methods

2.1. Study design and population

A total of 694 high-risk patients with AR from six high-volume centers (Xijing Hospital; Beijing Fuwai Hospital; West China Hospital Affiliated with Sichuan University; Zhongshan Hospital Affiliated with Fudan University; Anzhen Hospital Affiliated to Capital Medical University; and Guangdong Provincial People's Hospital) were enrolled from January 2018 to March 2020. The inclusion criteria included (i) age ≥60 years; (ii) New York Heart Association functional class ≥II; (iii) ≥Moderate AR diagnosed by transthoracic echocardiography; and (iv) European System for Cardiac Operative Risk Evaluation score II >12% or Society of Thoracic Surgeons (STS) score >8%. The exclusion criteria were (i) <Moderate AR; (ii) myocardial infarction within the past month; (iii) history of endocarditis; (iv) hypertrophic cardiomyopathy; and (v) transient ischemic attack/stroke within the past 6 months. In addition, patients unsuitable for transapical TAVR were excluded from the study. Of the patients enrolled initially, 82 were excluded. The 3DP simulation was performed alternately in each subset of five consecutive patients (two cases assigned to the 3DP group and three cases to the non-3DP group) per center. Thus, the remaining 612 patients were assigned to the non-3DP group (n = 384) or the 3DP group (n = 228) (Figure 1). This study conformed to the Declaration of Helsinki and was approved by the Ethics Committee of Xijing Hospital (approval number: KY-20192138-C-1). All patients provided written informed consent for TAVR and follow-up data collection.

2.2. Pre-operative imaging assessment

All patients were required to complete an electrocardiograph-gated coronary CTA before undergoing the procedure. The standard Digital Imaging and Communications in Medicine format for CTA data was imported into 3Mensio software (Materialise, Belgium) to assess the aortic root, including the annulus and the left ventricular outflow tract, with particular attention to the size, location, and position of the sinuses of Valsalva and the angle of the aorta. Horizocardia is defined as an angle >48° between the long diameter of the heart and the longitudinal diameter of the chest.[11] Transthoracic echocardiography mainly

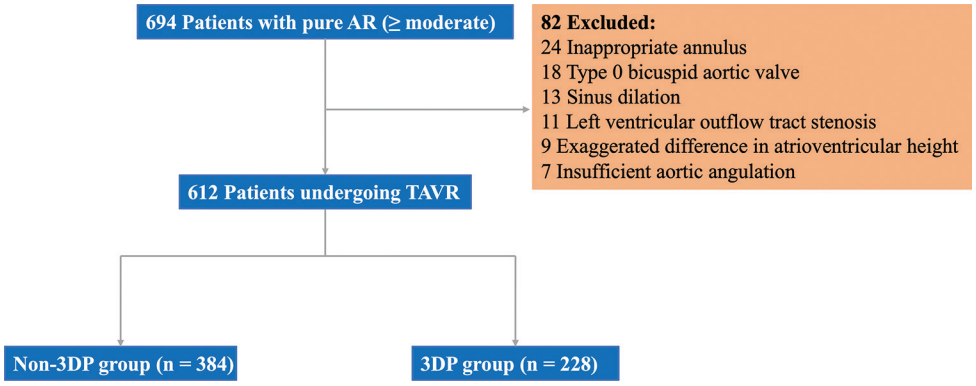

Figure 1. Study flow chart. Patients with pure AR of at least moderate severity who underwent TAVR with the J-Valve (Jiecheng Medical Co., LTD., China) were enrolled from January 2018 to March 2020.
Abbreviations: AR: Aortic regurgitation; TAVR: Transcatheter aortic valve replacement; 3DP: Three-dimensional printing.

assesses atrioventricular morphology and the degree of AR, pressure gradient, left ventricular ejection fraction, left ventricular fractional shortening, and other pre-existing cardiac complications (Figure 2A).

2.3. 3DP simulation and procedure

Based on the CTA measurements, the imaging data of all patients in the 3DP group were imported into Materialise Mimics version 21.0 (Materialise, Belgium) to perform 3D reconstructions of the aortic root. The 3D reconstructed models were exported in Standard Tessellation Language format and printed using a Stratasys PolyJet 850 multimaterial full-color 3D printer (Table S1). Based on the CTA assessment and the 3D-printed models, a J-Valve of a similar size was installed in the 3D transapical TAVR simulator to simulate the TAVR procedures (Figure 2B). Simulation details are provided in Table S2. Procedural details have been described previously.[7] For patients in the 3DP group, the implantation angle and the pre-shaped curvature of the delivery system were determined by pre-procedural measurements. The stent was then positioned at the aortic root and released (Figure 2C).

2.4. Definition of coaxiality

Coaxiality was first measured by the distances from the bottom of the THV to the bases of the three cusps. L, R, and N represent the distances from the bottom of the THV to the left-, right-, and non-coronary cusps, respectively. The coaxiality index was then used to evaluate the coaxiality of the implanted THV after the simulations and was calculated as follows:

$$\text{Coaxiality index} = \sqrt{(L-R)2 + (R-N)2 + (N-L)^2} \quad \text{(I)}$$

Furthermore, coaxiality was evaluated using post-procedural CTA data and the 3DP model. The coaxial angle (θ), the angle of the long axis of the THV and the vertical axis of the annular plane, was used to assess whether the prostheses were optimally located and served as a reference standard for the coaxiality index (Figure 2D).

2.5. Endpoints

The primary endpoint was the incidence and severity of the PVL before discharge. According to the Valvular Academic Research Consortium-3 criteria,[12] the secondary endpoints included (i) 3-year all-cause mortality; (ii) incidence of procedural- and device-related complications; and (iii) echocardiographic assessment of THV function (e.g., THV thrombosis and THV degeneration). Moreover, we evaluated procedural indicators (total operating time, digital subtraction angiography time, and radiation amounts) and coaxiality differences (coaxial angle and coaxiality index) between the two groups.

2.6. Statistical analysis

The results of this study are presented as the mean ± standard deviation for continuous variables with a normal distribution or as percentages for categorical data. The Student's *t*-test was used to compare normally distributed continuous variables between the two groups. The Fisher's exact test was used to compare categorical variables. Statistically significant variables with $p<0.10$ in the univariate analysis were included in the multivariate model. The final model was determined using forward and reverse elimination procedures, with a threshold of $p<0.05$. Logistic regression analysis results are expressed as odds ratios (ORs) and 95% confidence intervals (CIs). Survival curves for all-cause mortality were constructed using Kaplan–Meier estimates and compared using log-rank statistics. Proportional hazards regression models are used to compare mortality between groups at follow-up (non-3DP vs. 3DP groups), as well as to explore factors

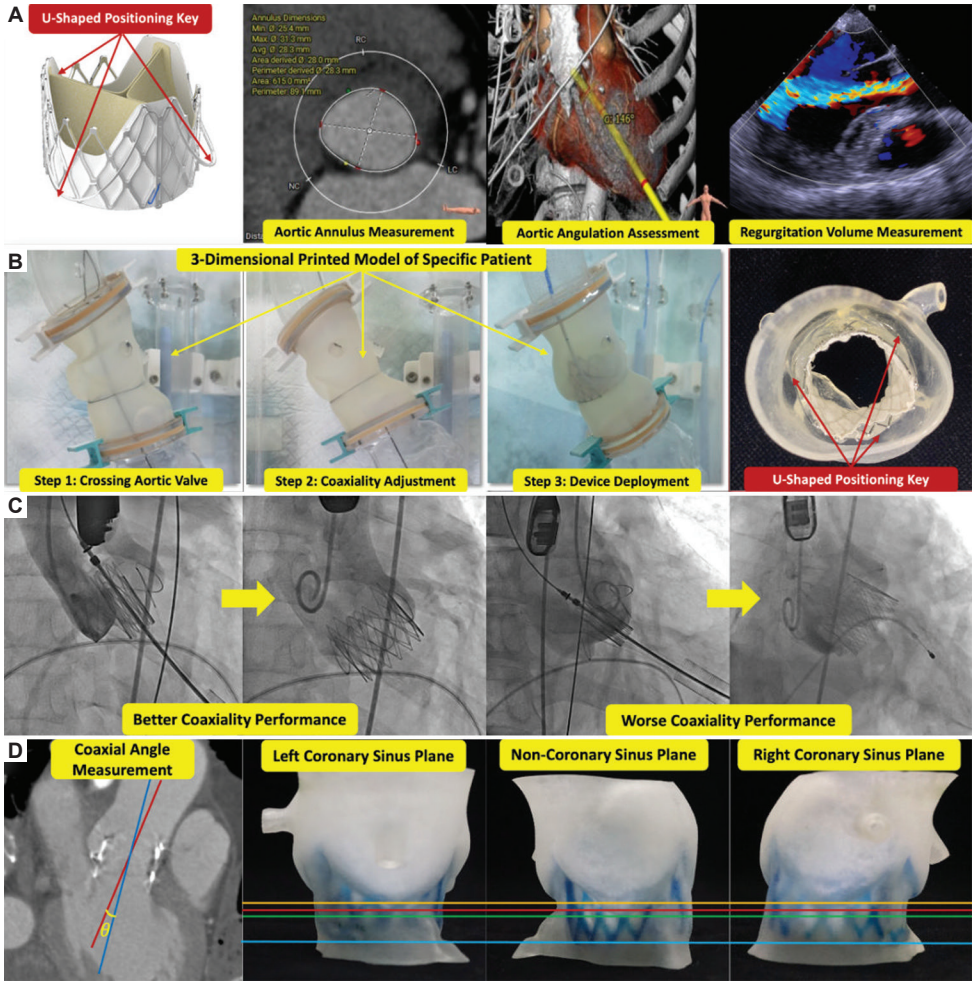

Figure 2. Advantages of coaxiality performance in the three-dimensional printing group based on accurate pre-procedural simulations. (A) J-Valve design and pre-operative two-dimensional imaging assessment of the aortic valve. Red arrows represent the three U-shaped positioning keys. (B) Pre-operative simulation using three-dimensional printed model and pulsatile simulator. Red arrows indicate the three U-shaped positioning keys; yellow arrows point to the three-dimensional-printed model. (C) Comparison of coaxiality performance during J-Valve deployment. Yellow arrows indicate the J-Valve from positioning to release. (D) Post-operative evaluation of coaxiality using the coaxial angle and coaxiality index. θ represents the coaxial angle. The dark red and the dark blue oblique lines represent the long axes of the aortic root and the J-Valve, respectively. The orange, red, green, and blue horizontal lines represent the heights of the non-coronary, left coronary, and right coronary sinuses, and the bottom of the J-Valve, respectively. The scale bar in the transthoracic echocardiography image was set to 50 – 70 cm/s.

affecting survival time. Hazard ratios (HRs) and 95% CIs are reported. In addition, linear regression is used to evaluate predictors of the coaxial index. All statistical analyses were considered statistically significant at two-sided $p<0.05$. The statistical analyses were performed using the R programming language version 4.2.2 (R Foundation for Statistical Computing, Austria).

3. Results

3.1. Baseline characteristics

Baseline characteristics of the 612 patients are summarized in Table 1. The mean age was 71.2 ± 5.3 years; 74.0% were male; and the surgical risk was moderate (the mean mortality risk scores predicted by the STS were 7.8 ± 3.4% and 8.3 ± 3.1% for patients in the 3DP and non-3DP groups, respectively). There were no significant differences between the two groups in terms of traditional prognostic risk factors, such as diabetes, peripheral artery disease, and atrial fibrillation. Table 2 shows the baseline echocardiographic and computed tomography features of the patients. No significant differences in echocardiographic variables were observed between the two groups, except that the pressure gradient was slightly lower in the 3DP group (6.9 ± 2.4 mmHg versus 7.5 ± 3.3 mmHg, $p=0.018$). In particular, there were no significant differences in the proportion of severe AR or moderate-or-higher mitral regurgitation ($p=0.361$ and $p=0.832$, respectively). CTA

Table 1. Baseline characteristics

Characteristics	Overall cohort (*n*=612)	Non-3DP group (*n*=384)	3DP group (*n*=228)	*p*-value
Demographics				
Age, years	71.2 (5.3)	71.2 (5.6)	71.3 (5.0)	0.715
Men	453 (74.0)	290 (75.5)	163 (71.5)	0.316
Body mass index, kg/m^2	22.6 (2.03)	22.8 (1.97)	22.3 (2.08)	0.173
Systolic pressure, mmHg	132 (19.9)	131 (21.4)	133 (17.1)	0.478
Diastolic pressure, mmHg	66.9 (12.0)	67.2 (12.1)	66.5 (12.0)	0.750
STS risk score	8.04 (3.35)	7.75 (3.39)	8.33 (3.14)	0.274
NYHA functional class≥III	598 (97.7)	374 (97.4)	224 (98.2)	0.511
Comorbidities				
Hypertension	390 (63.7)	244 (63.5)	151 (66.2)	0.671
Diabetes	111 (18.1)	70 (18.2)	47 (20.6)	0.598
Coronary artery disease	168 (27.5)	106 (27.6)	62 (27.2)	0.987
Previous percutaneous coronary intervention	18 (2.94)	11 (2.86)	7 (3.07)	0.973
Previous bypass graft surgery	7 (1.14)	5 (1.30)	2 (0.88)	0.476
Peripheral vascular disease	243 (39.7)	150 (39.1)	93 (40.8)	0.736
Atrial fibrillation	133 (21.7)	0 (20.8)	53 (23.2)	0.362
Permanent pacemaker	24 (3.92)	14 (3.65)	10 (4.39)	0.810
Stroke/transient ischemic attack	19 (3.10)	12 (3.12)	7 (3.07)	0.996
Chronic obstructive pulmonary disease	58 (9.48)	1 (10.7)	17 (7.46)	0.241
Dialysis	129 (21.1)	82 (21.4)	47 (20.6)	0.909

Notes: Continuous variables are expressed as mean and standard deviation, while categorical variables are presented as frequency and percentage. *p*-values represent the significance levels of the comparisons between the 3DP and non-3DP groups.
Abbreviations: NYHA: New York Heart Association; SD: Standard deviation; STS: Society of Thoracic Surgeons; 3DP: Three-dimensional printing.

results showed that the left ventricular longitudinal diameter was slightly shorter in the 3DP group (84.2 ± 9.9 mm vs. 88.7 ± 9.4 mm; p=0.032). A similar difference was observed in the left ventricular left–right diameter (61.9 ± 9.9 mm vs. 65.2 ± 9.1 mm; p=0.043). However, the groups did not demonstrate significant differences in aortic annular area or diameter (p=0.071 and p=0.266).

3.2. Procedural and clinical outcomes

Table 3 summarizes the procedural features and outcomes. The size of the annulus and the implanted valve was similar in both groups (p=0.693). According to the Valvular Academic Research Consortium-3, the operation success rate in the 3DP group was higher than that in the non-3DP group (100% vs. 96.4%; p=0.008). In terms of prosthesis selection, there was no correlation between the proportion of patients with an increased valve size in the 3DP group and those in the non-3DP group (p=0.553). Although there were no differences between the two groups in the incidence of major procedural complications (such as conversion to surgical aortic valve replacement [SAVR], THV displacement, tamponade, and coronary artery obstruction), no patients in the 3DP group developed related complications. Importantly, mild PVL occurred less frequently in the 3DP group (mild PVL: 3.51% vs. 18.2%; p<0.001; moderate PVL: 0% vs. 1.04%; p<0.001). Furthermore, the total operating time, digital subtracted angiography time, and radiation exposure in the non-3DP group were higher than those in the 3DP group (total operating time: 119.0 ± 15.2 min vs. 105.0 ± 16.1 min, p<0.001; digital subtracted angiography time: 8.4 ± 3.0 min vs. 6.0 ± 2.4 min, p<0.001; radiation exposure: 722 ± 113 mGy vs. 514 ± 48 mGy, p<0.001) (Figure S1). In addition, the average length of in-hospital stay was 8.7 ± 4.1 days. The incidence of in-hospital death, myocardial infarction, and stroke was 1.80%, 0.82%, and 0.49%, respectively, and the incidence of complete atrioventricular heart block was much higher in the non-3DP group than in the 3DP group (6.77% vs. 0%; p<0.001).

All patients were followed up for a median of 45.5 months (interquartile range: 39.2 – 50.7 months). No differences were observed between patients in the 3DP and non-3DP groups in terms of all-cause mortality, cardiovascular adverse events, stroke, and reintervention. A Kaplan–Meier survival curve is shown in Figure 3. In

Table 2. Summary of pre-operative imaging assessment

Characteristics	Overall cohort (*n*=612)	Non-3DP group (*n*=384)	3DP group (*n*=228)	*p*-value
Pre-operative transthoracic echocardiography				
Type 1 bicuspid aortic valve	40 (6.54%)	23 (5.99%)	17 (7.46%)	0.589
V_{max}, cm/s	1.70 (0.2)	1.71 (0.2)	1.69 (0.2)	0.847
MTVPG, mmHg	7.3 (3.0)	7.5 (3.3)	6.9 (2.4)	0.018*
Severe aortic regurgitation	500 (81.7)	309 (80.5)	191 (83.8)	0.361
LVEF, %	50.3 (7.6)	50.1 (8.1)	50.6 (6.6)	0.330
LVFS, %	26.1 (4.6)	25.9 (4.9)	26.3 (4.1)	0.296
Mitral regurgitation ≥moderate, %	133 (21.7)	85 (22.1)	48 (21.1)	0.832
Pre-operative computed tomography angiography				
LVLD, mm	86.8 (9.6)	88.7 (9.4)	84.2 (9.9)	0.032*
LVAPD, mm	63.4 (9.2)	64.1 (9.1)	62.4 (9.3)	0.294
LVLRD, mm	63.6 (9.5)	65.2 (9.1)	61.9 (9.9)	0.043*
Annulus area, mm^2	566 (81.3)	570 (74.9)	557 (88.9)	0.071
Annulus diameter, mm	27.3 (2.0)	27.5 (1.8)	26.9 (2.2)	0.266
LVOT diameter, mm	28.9 (2.3)	29.0 (2.1)	28.9 (2.7)	0.831
STJ diameter, mm	38.5 (3.3)	38.8 (2.9)	38.0 (3.9)	0.607
AA diameter, mm	40.9 (3.2)	40.6 (2.7)	41.5 (3.8)	0.701
LCH, mm	13.5 (3.7)	13.4 (3.6)	13.7 (3.9)	0.836
RCH, mm	17.6 (3.7)	17.8 (3.5)	17.3 (4.1)	0.719
Aorta angulation, °	55.3 (9.3)	55.1 (8.9)	56.4 (9.4)	0.101

Notes: Continuous variables are expressed as mean and standard deviation, while categorical variables are presented as frequency and percentage. *p*-values represent the significance levels of the comparisons between the 3DP and non-3DP groups. *$p<0.05$.
Abbreviations: AA: Ascending aorta; LCH: Left coronary artery height; LVAPD: Left ventricular anteroposterior diameter; LVEF: Left ventricle ejection fraction; LVFS: Left ventricular fraction shortening; LVLD: Left ventricular longitudinal diameter; LVLRD: Left ventricular left-right diameter; LVOT: Left ventricular outflow tract; MTVPG: Mean transvalvular pressure gradient; RCH: Right coronary artery height; SD: Standard deviation; STJ: Sinotubular junction; V_{max}: Peak flow velocity of aortic valve; 3DP: Three-dimensional printing.

multivariate Cox regression analysis, risk factors associated with increased 3-year mortality included: baseline STS score (HR: 1.30; 95% CI: 1.18 – 1.43; $p<0.001$), stroke history (HR: 2.14; 95% CI: 1.66 – 3.04; $p<0.001$), and pre-operative pacemaker implantation (HR: 1.39; 95% CI: 1.07 – 2.16; $p<0.001$). In addition, changes in the New York Heart Association functional class and incidence of PVL are shown in Figure 4. Left ventricular remodeling was observed in both groups by measuring its diameters (Figure S2).

3.3. Relationship among paravalvular leakage, coaxial angle, and coaxiality index

The univariate and multivariate logistic regression results of PVL are shown in Table 4. Patients with horizocardia had a higher risk of PVL (OR: 1.24; 95% CI: 1.16 – 1.33; $p<0.001$). As expected, additional risk factors included 3DP (OR: 0.18; 95% CI: 0.07 – 0.48; $p=0.001$), a larger coaxial angle (OR: 4.28; 95% CI: 3.06 – 6.00; $p<0.001$), and a higher coaxiality index (OR: 9.45; 95% CI: 4.40 – 20.28; $p<0.001$). The PVL densograms and coaxiality performance are displayed in Figure 5A and 5B. Meanwhile, coaxiality performance in the 3DP group was better than in the non-3DP group (coaxial angle: 10.5 ± 3.7° vs. 12 ± 4.2°; $p<0.001$; coaxiality index: 3.4 ± 1.7 vs. 4.0 ± 2.1; $p<0.001$) (Figure 5C and 5D). Furthermore, the post-operative coaxiality index showed a strong correlation with the coaxial angle (3DP group: correlation coefficient [R] = 0.85, $p<0.001$; non-3DP group: R = 0.88, $p<0.001$) (Figure 5E and 5F). The coaxiality index was analyzed by multiple linear regression. After adjustment, predictors of coaxiality index included horizocardia (coefficient: 0.03; 95% CI: 0.02 – 0.04; $p<0.001$), left coronary cusp depth (coefficient: 0.07; 95% CI: 0.03 – 0.11; $p<0.001$), and 3DP (coefficient: −0.41; 95% CI: −0.80 – −0.30; $p<0.001$) (Table 5).

4. Discussion

This is the first large-scale study of transapical TAVR using the J-Valve in patients with pure AR. The main findings are (i) transapical TAVR using the J-Valve is feasible for

Table 3. Procedural and hospitalization outcomes

Characteristics	Overall cohort (*n*=612)	Non-3DP group (*n*=384)	3DP group (*n*=228)	*p*-value
Procedural outcomes				
Bioprosthetic valve size	27.7 (1.7)	27.8 (1.5)	27.4 (1.9)	0.693
Implanted THV oversizing	41 (6.7)	28 (7.3)	13 (5.7)	0.553
Operating time, in	114 (16.8)	119 (15.2)	105 (16.1)	<0.001***
DSA time, min	7.5 (3.0)	8.4 (3.0)	6.0 (2.4)	<0.001***
Radiation amount, mL	645 (138)	722 (113)	514 (48.2)	<0.001***
Extubation in OR	230 (38.2)	104 (27.7)	126 (55.5)	<0.001***
Post-procedural outcomes				
Device success	598 (97.7)	370 (96.4)	228 (100.0)	0.008**
Conversion to open heart surgery	6 (1.0)	6 (1.6)	0 (0)	0.089
Transfer to ECMO	2 (0.3)	2 (0.5)	0 (0)	0.532
Cardiopulmonary bypass	3 (0.5)	3 (0.8)	0 (0)	0.298
THV displacement	6 (1.0)	6 (1.6)	0 (0)	0.089
Tamponade	6 (1.0)	6 (1.6)	0 (0)	0.089
Coronary obstruction	1 (0.2)	1 (0.3)	0 (0)	1.000
Valve-in-valve implant	2 (0.3)	2 (0.5)	0 (0)	0.532
Paravalvular leakage				
None	346 (56.5)	188 (49.0)	158 (69.3)	<0.001***
Trace	184 (30.1)	122 (31.8)	62 (27.2)	<0.001***
Mild	78 (12.7)	70 (18.2)	8 (3.5)	<0.001***
Moderate	4 (0.7)	4 (1.0)	0 (0)	<0.001***
Implanted depth				
LCC depth, mm	10.3 (3.0)	10.7 (3.0)	9.54 (2.9)	<0.001***
RCC depth, mm	8.1 (2.9)	8.4 (2.8)	7.5 (3.0)	<0.001***
NCC depth, mm	7.8 (3.1)	8.1 (3.0)	7.2 (3.2)	0.001**
Coaxial angle, °	11.5 (4.0)	12.0 (4.2)	10.5 (3.7)	<0.001***
Coaxiality index, %	3.8 (2.0)	4.0 (2.1)	3.4 (1.7)	<0.001***
ICU stay, days	1.6 (0.8)	1.8 (0.9)	1.2 (0.5)	<0.001***
Hospitalization stay, days	8.7 (4.1)	10.0 (4.2)	6.6 (2.7)	<0.001***
In-hospital outcomes				
Death	3 (0.5)	3 (0.8)	0 (0)	0.298
Myocardial infarction	5 (0.8)	5 (1.3)	0 (0)	0.163
Stroke	3 (0.5)	2 (0.5)	1 (0.4)	1.000
Bleeding (major or life-threatening)	2 (0.3)	2 (0.5)	0 (0)	0.532
Major vascular complications	3 (0.5)	3 (0.8)	0 (0)	0.298
Complete atrioventricular heart block	26 (4.3)	26 (6.8)	0 (0)	<0.001***
Acute kidney injury (stage 3)	33 (5.4)	29 (7.6)	4 (1.8)	0.004**

Notes: Continuous variables are expressed as mean and standard deviation, while categorical variables are presented as frequency and percentage. *p*-values represent the significance levels of the comparisons between the 3DP and non-3DP groups. **$p<0.01$, ***$p<0.001$.
Abbreviations: DSA: Digital subtracted angiography; ECMO: Extracorporeal membrane oxygenation; ICU: Intensive care unit; LCC: Left coronary cusp; NCC: Non-coronary cusp; OR: Operating room; RCC: Right coronary cusp; SD: Standard deviation; THV: Transcatheter heart valve; 3DP: Three-dimensional printing.

patients with pure AR, and the short- and mid-term clinical outcomes are satisfactory; (ii) the previously high incidence of PVL and other procedural complications after implantation appears to be reduced by pre-operative

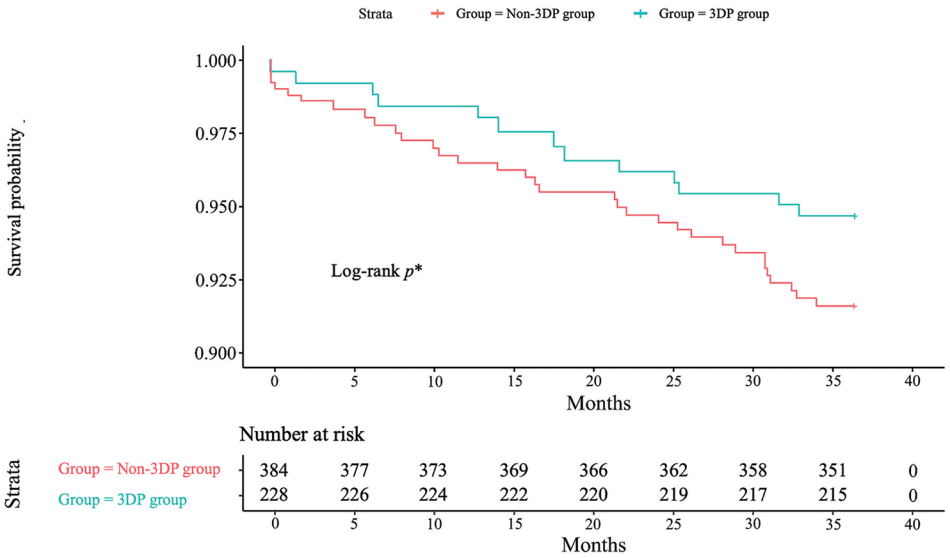

Figure 3. All-cause mortality after transcatheter aortic valve replacement at 3-year follow-up between groups. A total of 228 patients in the 3DP group and 384 patients in the non-3DP group were included in the analysis. Survival curves for all-cause mortality were generated using Kaplan–Meier estimates and compared using the log-rank statistics.
Note: ***p<0.001.
Abbreviation: 3DP: Three-dimensional printing.

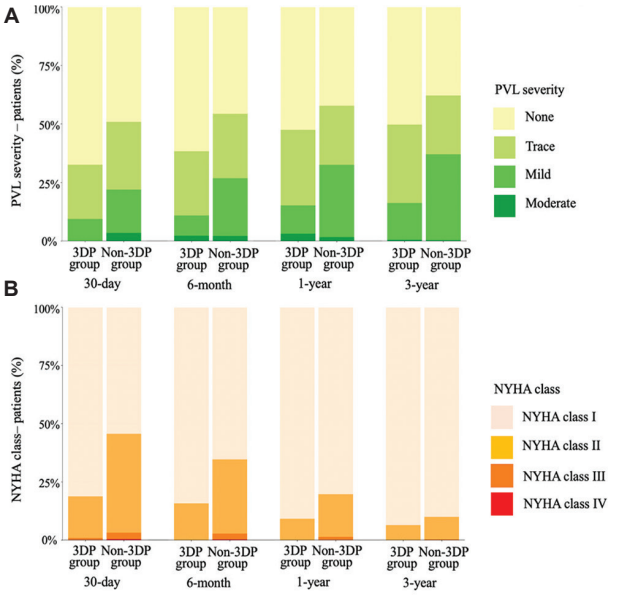

Figure 4. Comparison of PVL severity (A) and NYHA class (B) between groups. A total of 228 patients in the 3DP group and 384 patients in the non-3DP group were analyzed at the 30-day follow-up. At the 6-month follow-up, 225 patients in the 3DP group and 376 patients in the non-3DP group were analyzed. At the 1-year follow-up, 224 patients in the 3DP group and 370 patients in the non-3DP group were analyzed. At the 3-year follow-up, 215 patients in the 3DP group and 351 patients in the non-3DP group were analyzed. The Fisher exact test was used to compare categorical variables.
Abbreviations: NYHA: New York Heart Association; PVL: Paravalvular leakage; 3DP: Three-dimensional printing.

simulation based on 3DP; and (iii) PVL occurrence is closely related to coaxiality performance, which can be quantified using the coaxiality index.

In general, AR is more common than AS in the Chinese population. Studies have shown that in individuals over 75 years of age, the incidence of ≥ moderate AR is as high as 2.85%, and the proportion of severe AR is higher than that of severe AS.[13] The reason for this is that in the United States and European countries, AR is more common in the elderly and is often associated with degenerative aortic valve disease.[14,15] In contrast, in the Chinese population, the onset age of AR is earlier, and a larger proportion of cases are related to rheumatic heart disease or sequelae of infection.[16,17] In addition, the prevalence of hypertension in China is as high as 30%, but the control rate is low; long-term uncontrolled hypertension may accelerate the dilation of the aortic root and the progression of AR.[18]

Although patients may remain asymptomatic and maintain normal left ventricular systolic function for years after AR diagnosis,[19] the onset of symptoms indicates the possible presence of severe myocardial dysfunction. The annual mortality rate of patients with New York Heart Association functional class III is as high as 24.6%.[20]

The latest guidelines recommend that SAVR remain the first-line treatment for AR,[14,15] even though many patients with AR are unable to undergo SAVR due to advanced age or comorbidities and may benefit from TAVR.[21] However,

Table 4. Predictors of paravalvular leakage grade ≥mild

Characteristics	Univariate analysis			Multivariate analysis		
	OR	95% CI	*p*-value	OR	95% CI	*p*-value
BMI ≥30 kg/m^2	1.12	1.03 – 1.21	0.010*	-	-	-
STS ≥10%	1.31	0.73 – 2.28	0.028*	-	-	-
V_{max}	1.07	1.03 – 1.42	0.023*	-	-	-
Severe aortic regurgitation	0.68	0.45 – 1.04	0.037*	-	-	-
AA diameter ≥41 mm	0.96	0.91 – 1.14	0.012*	-	-	-
Horizocardia	1.23	0.83 – 1.83	<0.001***	1.24	1.16 – 1.33	<0.001***
LCH	1.11	1.06 – 1.16	<0.001***	-	-	-
Coaxial angle	3.55	2.82 – 4.46	<0.001***	4.28	3.06 – 6.00	<0.001***
LCC depth	1.20	1.13 – 1.27	<0.001***	-	-	-
Coaxiality index	3.22	2.66 – 3.9	<0.001***	9.45	4.40 – 20.28	<0.001***
3DP group versus non-3DP group	0.26	0.18 – 0.39	<0.001***	0.18	0.07 – 0.48	0.001**

Notes: Odd ratios and *p*-values are derived from logistic regression analyses. *p<0.05, **p<0.01, ***p<0.001.
Abbreviations: AA: Ascending aorta; BMI: Body mass index; CI: Confidence interval; LCC: Left coronary cusp; LCH: Left coronary artery height; OR: Odds ratio; STS: Society of Thoracic Surgeons; V_{max}: Peak flow velocity of aortic valve; 3DP: Three-dimensional printing.

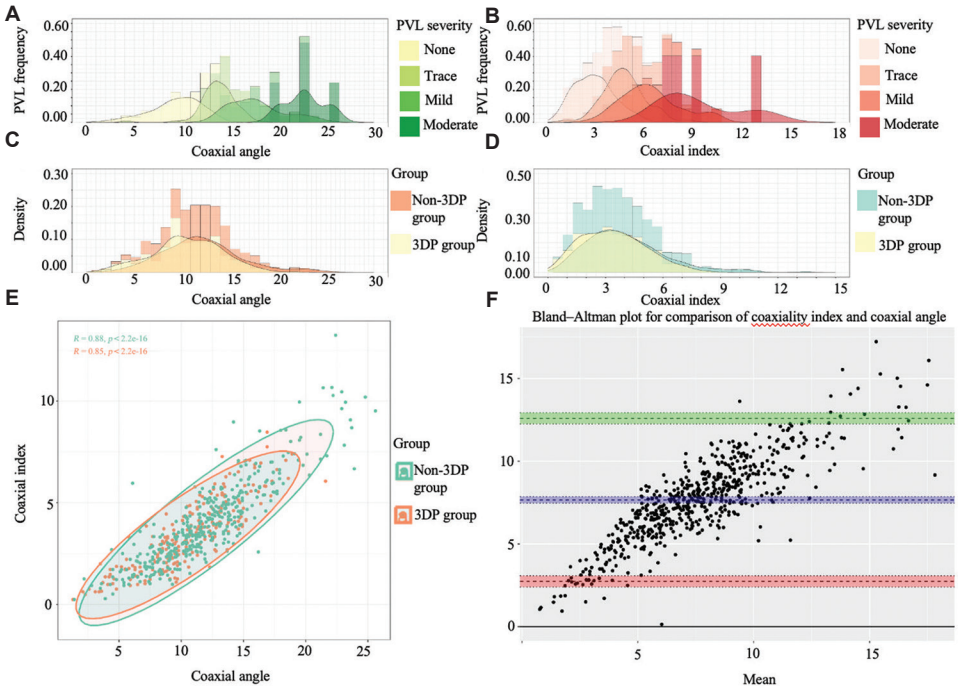

Figure 5. Relationship among PVL, Coaxiality Index, and Coaxial Angle in Groups. (A and B) Relationship of PVL incidence with coaxial angle and coaxiality index, respectively. (C and D) Relationship of PVL incidence between groups using coaxial angle and coaxiality index, respectively. (E) Correlation plot (*R* = Pearson correlation coefficient) displaying the correlation between coaxial angle and coaxiality index. (F) Bland–Altman plot assessing accuracy as the relative deviation from the mean values of the coaxial angle and coaxiality index. A total of 228 patients in the 3DP group and 384 patients in the non-3DP group were included in the analysis. Pearson correlation coefficient and Bland–Altman plot were used to analyze the correlations of variables. Accuracy was defined as a deviation of ≤10% from the mean.
Notes: *p<0.01; **p<0.001.
Abbreviations: PVL: Paravalvular leakage; 3DP: Three-dimensional printing.

performing TAVR in patients with AR presents unique anatomical and pathophysiological challenges.[14] A recent meta-analysis showed that the overall surgical success rate with first- and second-generation THV systems was 86.2%; the 30-day mortality rate was 11.9%, and the 1-year mortality rate was as high as 24.7%.[22] The J-Valve

Table 5. Predictors of coaxiality index

Characteristics	Univariate analysis			Multivariate analysis		
	Coefficients	95% CI	*p*-value	Coefficients	95% CI	*p*-value
BMI ≥30 kg/m^2	0.09	0.01 – 0.16	0.021*	-	-	-
STS ≥10%	0.04	0.01 – 0.07	0.011*	-	-	-
V_{max}	0.01	−0.01 – 0.02	<0.001***	-	-	-
MR ≥moderate	0.34	−0.03 – 0.71	0.017*	-	-	-
Annulus diameter	0.11	0.03 – 0.18	0.010*	-	-	-
AA diameter ≥41 mm	-0.01	−0.06 – 0.03	0.55	-	-	-
Horizocardia	0.02	0.01 – 0.04	<0.001***	0.03	0.02 – 0.04	<0.001***
LCC depth	0.20	0.15 – 0.24	<0.001***	0.07	0.03 – 0.11	<0.001***
3DP group vs non-3DP group	-0.60	−0.92 – −0.29	<0.001***	−0.41	−0.80 – −0.30	<0.001***

Notes: Coefficients and *p*-values are derived from logistic regression analyses. *p<0.05, ***p<0.001.
Abbreviations: AA: Ascending aorta; BMI: Body mass index; CI: Confidence interval; LCC: Left coronary cusp; MR: Mitral regurgitation; STS: Society of Thoracic Surgeons; V_{max}: Peak flow velocity of aortic valve; 3DP: Three-dimensional printing.

system features a three-pronged anchoring mechanism and a "two-stage implantation" design, wherein the native leaflets are captured by the buckle before the stent is released. This helps improve the accuracy of bioprosthetic positioning, enhances neocommissural alignment, and reduces THV displacement to some extent.[7] In our study, the overall cohort achieved a 3-year survival rate of 93.95% (n = 575), which is undoubtedly a considerable therapeutic achievement. In addition, data from this study showed that predictors of increased 3-year mortality in the overall cohort included the baseline STS score, history of stroke, and history of pacemaker implantation. Interestingly, the data also confirm that THV oversizing did not increase the risk of coronary artery obstruction or annular rupture. Regarding the lower incidence of coronary artery occlusion, we propose the following possible explanations: patients with severe AR often have a dilated aortic root, resulting in larger annular and sinus diameters. Selecting a THV with appropriate oversizing may better accommodate the dilated anatomy of the aortic root. The additional root space may buffer the direct compression of the coronary artery orifices by an oversized THV. Furthermore, subgroup analysis from the CHOICE-CLOSURE trial indicated that in AR patients, oversizing ≤20% was not significantly associated with coronary artery occlusion, which may be related to the mechanisms described above.[23] As for the lower incidence of annular rupture, our evaluation suggests the following factors: first, the J-Valve, as a self-expanding valve with a nitinol stent, exerts lower radial expansion force compared to balloon-expandable valves. Even when oversized, the local pressure distribution on the annulus is more evenly distributed, reducing the risk of annular rupture. Importantly, this THV achieves stable anchoring through three positioning keys, minimizing reliance on radial force and thus reducing the risks associated with oversizing. Second, unlike calcific AS, the annulus in pure AR has less calcification and greater tissue elasticity, making it more tolerant of mechanical stress. Finally, patients with pure AR have increased left ventricular volume load but a lower systolic pressure gradient, meaning the THV is subjected to less reverse pressure during diastole, further reducing sustained tension on the annulus.

In addition, previous studies have shown that the use of TAVR to treat pure AR is associated with a higher incidence of procedural complications, such as PVL, THV displacement, and the need for a second THV implant.[24] Rawasia *et al.*[22] reported that 9.2% of patients had residual PVL greater than mild in severity. PVL after TAVR significantly affects morbidity and mortality in these patients. Moderate-to-severe PVL has been independently associated with increased in-hospital and midterm deaths,[25] and even mild PVL has been shown to have adverse prognostic implications.[26] Therefore, accurate pre-procedural prediction and prevention of PVL are essential for optimizing clinical outcomes.

On the one hand, the prominent problem of valve positioning has limited the availability of dedicated devices for treating AR.[27] In addition to the J-Valve, the JenaValve system is another European conformity-certified device specifically designed for the treatment of AR. Featuring three radio-impervious positioning keys that anchor in the native leaflets, the Jena Valve allows fluoroscopic-guided positioning and employs a specialized clamping mechanism, enabling THV placement and fixation to be independent of atrioventricular calcification.[28] However, compared with severe AS, the anatomy of patients with severe AR presents greater challenges because of various

factors, resulting in a larger diameter profile in the planned anchoring area, and deviations between the measured and calculated diameters and the actual implanted valve size.[29] In addition, there remains an insufficient understanding of the impact of radial expansion with prosthetic sizing selection, making it difficult to achieve optimal therapeutic effects after implantation.[29]

It is worth noting that THV coaxiality has a significant influence on the occurrence of procedural complications in TAVR.[30] Pre-operative CTA or transthoracic echocardiography is typically required to assess the morphology and size of the aortic root to guide THV implantation planning and prosthesis size selection. However, unlike in SAVR, surgeons performing TAVR must rely solely on imaging as the procedure is conducted without direct surgical visualization. The advent of cardiovascular 3DP offers an innovative solution to this challenge. Previous studies have confirmed that pre-procedural planning enabled by 3DP may significantly improve anatomical visualization, provide more accurate THV sizing recommendations, and help determine the optimal THV implantation position while identifying potential complications through pre-procedural simulations.[31,32] Our preliminary data support these findings. The current outcome analysis showed a higher TAVR success rate in the 3DP group (100%) than in the non-3DP group (96.4%), along with shorter total operating time, digital subtraction angiography time, and reduced radiation exposure. Furthermore, no patients in the 3DP group experienced major procedural complications. In particular, the incidence of ≥ mild PVL was significantly lower in the 3DP group than in the non-3DP group, which yielded even more promising results than previous clinical studies.[24] Naturally, further research is needed to confirm these findings. Importantly, our data demonstrated that the post-procedural coaxiality index was strongly correlated with the coaxial angle. In addition to horizocardia, the coaxiality index was identified as an independent predictor of PVL. As expected, the 3DP group showed superior coaxiality performance compared to the non-3DP group. These results may have implications for future procedural planning in TAVR, with the potential to reduce the risk of PVL after implantation.

In addition, the incidence of the complete atrioventricular heart block group was lower in the 3DP group than in the non-3DP group (0% vs. 6.77%). This reduction may be attributed to the three positioning keys anchoring the base of the aortic sinus before valve deployment. Improper positioning, specifically deeper localization, can cause the device to compress the membranous interventricular septum, leading to conduction block.

5. Conclusion

In this prospective, observational, multicenter study, the results show that transapical TAVR using the J-Valve is feasible for patients with pure AR, with encouraging short- and midterm clinical outcomes. Importantly, the higher incidence of PVL and other procedural complications after implantation appears to be mitigated by pre-operative simulations based on the 3DP model. The occurrence of PVL is greatly reduced by evaluating the coaxiality index to achieve the desired position. Long-term follow-up of a larger number of patients is needed to more fully assess the effectiveness and durability of J-Valve implants in patients with AR.

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Conflict of interest

The authors declare they have no conflicts of interest.

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Ethics approval and consent to participate

The protocol was approved by the Ethics Committee of Xijing Hospital (approval number: KY-20192138-C-1). All

patients/participants provided written informed consent to participate in the study.

Consent for publication

Written informed consent was obtained from all individuals for the publication of any potentially identifiable images or data included in this article.

Availability of data

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

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ACCSCIENCE PUBLISHING

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ORIGINAL ARTICLE

Impact of treatment adherence on psoriasis severity: Insights from a multicenter cross-sectional study in Brazil

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Abstract

Background: Psoriasis is a chronic, non-contagious inflammatory skin disease with significant physical and quality-of-life impacts. In Brazil, its estimated incidence is 1.3%. Due to the complexity of the disease, effective management requires addressing multiple factors, with treatment adherence and persistence being critical challenges. **Aim:** To evaluate the correlation between psoriasis severity and treatment adherence and persistence among patients in Paraná, Brazil. **Methods:** This cross-sectional study included 133 psoriasis patients treated at the three primary specialized care centers in the state, between January 28, 2022, and December 9, 2022. **Results:** A negative correlation (–0.102, $rs^2 = 1.04\%$) was observed between psoriasis severity and treatment adherence. Conversely, there was a positive correlation (0.2444, $rs^2 = 5.97\%$) between psoriasis severity and treatment discontinuation history. **Conclusion:** Treatment interruptions were correlated with increased psoriasis severity, whereas higher adherence was associated with milder clinical manifestations. **Relevance for patients:** These findings underscore the critical role of consistent treatment adherence in managing psoriasis. Interruptions in treatment are linked to more severe forms of psoriasis, highlighting the detrimental effects of non-adherence. In contrast, patients who maintained high levels of adherence experienced less severe symptoms, emphasizing that consistent treatment is key to improved disease control. Strengthening patient-provider collaboration and adherence strategies can improve clinical outcomes and enhance quality of life.

Keywords: Psoriasis; Treatment adherence; Treatment persistence; Disease severity

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1. Introduction

Psoriasis is a chronic, immune-mediated inflammatory skin disease affecting 0.5 – 11.4% of adults and up to 1.4% of children, with no defined sex predilection.[1] In Brazil, the estimated prevalence is 1.3%.[2] Psoriasis can manifest at any age, but peak incidence occurs between 30 – 39 years and 50 – 69 years.[3] Despite available data, approximately 81% of countries require more comprehensive epidemiological information on the disease.[4]

Psoriasis onset is primarily triggered by a combination of genetic predisposition and environmental and behavioral factors, including obesity, smoking, excessive alcohol consumption, trauma, certain medications, and infections.[5] In the last few decades, studies on the pathogenesis of the disease have highlighted the great importance of cytokines, such as tumor necrosis factor-(TNF)-α, interleukin (IL)-17 and IL-23, in sustaining the inflammatory process.[6] Furthermore, oxidative stress is linked to the severity of psoriasis, with elevated markers such as malondialdehyde and nitric oxide observed in patients, alongside reduced antioxidant capacity and lower levels of vitamins A and E. Additionally, genetic polymorphisms in glutathione S-transferase genes are associated with susceptibility to psoriasis, potentially affecting antioxidant defenses and inflammatory responses.[7] After diagnosis, it is essential to determine the most appropriate therapy to improve clinical outcomes.

The ideal therapeutic goal for psoriasis patients is complete lesion clearance, indicated by a 100% response on the Psoriasis Area Severity Index (PASI). However, a 75% improvement (PASI75) from baseline is considered clinically significant.[8] This benchmark does not fully account for the detrimental effects of psoriasis on patients' physical, psychosocial, and emotional well-being, which reduces their quality of life. Therefore, treatment objectives should also focus on improving patients' overall well-being. Achieving this requires physicians to understand both the full impact of the disease and the outcomes that matter most to the patients.[9] To achieve therapeutic goals and improve the quality of life, treatment selection must be individualized, taking patient-specific factors into account, and ensuring adequate adherence and persistence.

Adherence refers to the extent to which a patient follows the prescribed dosing regimen in terms of timing and dosage, while persistence refers to the duration from treatment initiation to discontinuation—essentially, how long adherence is maintained. Therefore, any interruption in treatment is considered a failure in persistence.[10]

Low treatment adherence among psoriasis patients has been identified as a significant contributor to therapeutic failure and the inability to achieve treatment goals. The main reasons for poor adherence include frustration with treatment outcomes, perceived low efficacy, communication gaps between physician and patient, and fear of treatment-related adverse effects.[11,12] These adherence issues lead to unsuccessful clinical management, negatively impact overall health, and decrease quality of life. Additionally, they contribute to increased healthcare costs, as psoriasis therapy can be expensive.[13-15]

An Italian study on medication adherence among psoriasis patients provides valuable insights consistent with findings from other countries while also identifying key differences in treatment compliance patterns.[16] The overall adherence in Italy (48.7%) aligns with global reports, where failure to follow treatment recommendations may reach 30 – 40%, especially for high-cost and complex therapeutic regimens, such as those in North America. This comparison underscores the importance of understanding local healthcare contexts and patient demographics when addressing adherence challenges. The significance of this study lies in its comprehensive analysis of the factors affecting adherence, which not only provides crucial information for healthcare professionals in Italy but also offers a model adaptable to other countries facing similar obstacles in managing chronic diseases like psoriasis. Identifying barriers to adherence and evaluating the impact of treatment modifications are essential steps toward developing targeted interventions that improve patient outcomes on a global scale.[16,17] Therefore, it is of great relevance to assess adherence and persistence to psoriasis treatment in patients from southern Brazil (Paraná state). Data from different populations provide critical insights into how environmental, socioeconomic, and cultural factors influence disease severity. Collecting such localized information can improve patient outcomes and healthcare efficiency in the region.

The state of Paraná serves as the starting point for the Pso.BRA study, a pharmacoepidemiological investigation of psoriasis aimed at identifying key patient, disease, and treatment characteristics across Brazil. Preliminary results, including the prevalence of comorbidities among patients in Curitiba—an important factor in the clinical course of the disease—have been previously published.[18] Limited information exists on adherence patterns within the Brazilian healthcare system, which faces unique challenges, including socioeconomic disparities and varying levels of access to specialized treatments such as biologics. Studies analyzing the association between treatment adherence and disease severity through a multicenter approach can provide real-world insights into an understudied population and contribute important data to the global understanding of psoriasis management. By addressing this research gap, this study—part of the Pso.BRA project—aims to evaluate correlations between psoriasis severity and treatment-related factors, such as adherence and history of treatment interruption or discontinuation, in patients from the state of Paraná.

2. Materials and methods

A cross-sectional study was conducted using data from voluntary patients of both sexes who consented to the study by signing the informed consent form (ICF). The study period spanned from January 28, 2022, to

December 9, 2022, with participants receiving treatment at the Santa Casa de Curitiba Hospital (Dermatology Department), the Dermatology Service of Mackenzie Evangelical University Hospital, and the Specialty Outpatient Clinic of the State University of Londrina University Hospital (AEHU).

The study included 133 participants, recruited randomly and indirectly through posters advertising the research, in accordance with ethical guidelines. The inclusion criteria for the study required patients with psoriasis at any level of activity, diagnosed either clinically or confirmed through pathological laboratory reports. Individuals under 18 years of age and those of indigenous descent were excluded from the study. Data were collected by dermatology residents through interviews after routine consultations, using a pre-tested and validated form,[18] at the participating study centers.

The project was approved by the Human Research Ethics Committee of the Health Sciences Sector at the Federal University of Paraná (SCS/UFPR) under approval number 4.294.864, issued in 2021.

Authorization for data collection at the participating centers—Santa Casa de Curitiba Hospital, Mackenzie Evangelical University Hospital, and the University Hospital of the State University of Londrina—was granted following a preliminary project review assessing both methodological and ethical aspects. Approval was obtained from the respective hospital superintendents and ethics committees.

To assess participant profiles, sociodemographic information was collected, including sex, age, race or ethnicity, current marital status, education level, and household income.

Psoriasis severity was assessed through both clinical evaluation and patient self-assessment using a 0 – 10 scale. Clinically, severity was measured using the PASI, which considers three criteria: Erythema, infiltration, and scaling. Each criterion is rated on a 0 – 4 intensity scale across four body regions: Head, upper limbs, trunk, and lower limbs. In addition to these criteria, the total affected area is graded on a 0 – 6 scale. The final PASI score is calculated by summing the intensity ratings for erythema, infiltration, and scaling, multiplied by the area grade for each body region. The score ranges from 0 to 72, with higher scores indicating more severe psoriasis.[15]

Treatment adherence was assessed using the Morisky-Green[19] questionnaire, which includes the following questions to evaluate adherence: (1) Do you sometimes have trouble remembering to take your medication?; (2) Do you sometimes neglect to take your medication?; (3) When you feel better, do you sometimes stop taking your medication?; (4) If you feel worse while taking the medication, do you sometimes stop taking it?

Adherence was classified as follows: High adherence for no positive responses, moderate adherence for 1 or 2 positive responses, and low adherence for 3 or 4 positive responses.

The questionnaire also inquired whether the patient had interrupted or discontinued treatment. If so, the patient was further questioned about the reasons for the interruption or discontinuation.

The dermatology life quality index (DLQI) was used to assess the impact of psoriasis on patient quality of life. The DLQI consists of 10 questions, with total scores ranging from 0 to 30. Based on the sum of the points, the impact on quality of life was categorized as follows: 0 – 1 for no impact, 2 – 5 for a small impact, 6 – 10 for a moderate impact, 11 – 20 for a very large impact, and 21 – 30 for an extremely large impact.[20]

Initially, a descriptive statistical analysis was performed. This was followed by the Shapiro-Wilk and Shapiro-Francia tests to assess the distribution of the dependent variable (psoriasis severity). Spearman's correlation analysis was then conducted to evaluate the correlations between variables, presented as rs^2 values.

3. Results

Table 1 presents the sociodemographic profile of the participants. The mean age of participants was 55.09 ± 12.54 years, ranging from 25 to 83 years. Table 2 shows the psoriasis classification results. Based on the PASI assessment, 65.41% (87/133) of participants had mild psoriasis, 24.06% (32/133) had moderate psoriasis, and 10.53% (14/133) had severe psoriasis. The average self-assessed psoriasis severity on a 0 – 10 scale was 4.06 ± 3.09. Notably, 62.41% (83/133) of participants had never experienced an asymptomatic period, while only 37.59% (50/133) reported periods without lesions.

Most patients were using topical treatment with corticosteroids (60.90%), while 30.82% used calcipotriol (Table A1). Among systemic medications, methotrexate (21.05%) and acitretin (10.52%) were the most commonly used (Table A2), while 17.29% of participants were treated with secukinumab, and 16.54% with adalimumab (Table A3). Table 3 presents the results related to treatment adherence among participants. The majority, 75.94% (101/133), reported never interrupting their psoriasis treatment, while 23.31% (31/133) had started and later discontinued treatment. One participant could not recall whether they had ever discontinued treatment. The main

Table 1. Sociodemographic characteristics of the participants

Characteristic	*n*	%
Sex		
Male	71	53.38
Female (non-pregnant)	58	43.61
Female (pregnant)	4	3.01
Age		
Adults*	80	60.15
Elderly**	53	39.85
Race or ethnicity		
White	88	66.17
Mixed race	38	28.57
Black	7	5.26
Marital status		
Married	84	63.16
Legally married	18	13.53
Single	12	9.02
In a stable union for more than 6 months	12	9.02
Widowed	7	5.26
Education level		
Primary school	5	3.76
Junior high or middle school	4	3.01
1st grade, elementary, or 1st grade equivalent	38	28.57
2nd grade, high school, technical, normal, scientific, or 2nd grade equivalent	57	42.86
Higher education or college degree	22	16.54
Postgraduate: specialization, master's, doctorate	2	1.50
Never studied	3	2.26
Did not know how to respond	2	1.50
Household income		
<1 minimum wage	2	1.50
1 minimum wage	25	18.80
2 minimum wages	46	34.59
3 minimum wages	22	16.54
4 minimum wages	19	14.29
5 – 10 minimum wages	12	9.02

Notes: * Indicates individuals aged 18 – 64; ** Indicates individuals aged ≥65.

Table 2. Clinical variants of psoriasis among participants

Psoriasis variant	*n*	%
Plaque	124	93.23
Guttate	2	1.50
Erythrodermic	2	1.50
Plaque, inverse	2	1.50
Pustular	1	0.75
Inverse	1	0.75
Plaque, pustular	1	0.75

Table 3. Treatment adherence among participants

Treatment adherence	*n*	%
High adherence	66	49.62
Moderate adherence	42	31.58
Moderate adherence	22	16.54
Low adherence	3	2.26

Table 4. Reasons for treatment discontinuation or interruption among participants

Reason	*n*	%
Experienced discomfort or side effects	14	45.16
Perceived lack of treatment effectiveness	13	41.94
Felt exhausted from continuous treatment	4	12.90
Lost faith in psoriasis treatment	3	9.68
Uncertain about the reason	3	9.68
Financial constraints	2	6.45

Table 5. Dermatology life quality index of participants

Impact on quality of life	*n*	%
No impact	64	48.12
Small impact	34	25.56
Moderate impact	12	9.02
Very large impact	18	13.53
Extremely large impact	5	3.76

reasons for treatment discontinuation included discomfort and lack of belief in treatment effectiveness (Table 4).

The DLQI results reveal that most participants perceived their skin condition as having little impact on their quality of life. Nearly half (48.12%) reported "no impact" at all, and about a quarter (25.56%) experienced only a "small impact." A smaller proportion reported more noticeable effects such as "moderate impact" (9.02%), "very large impact" (13.53%), and "extremely large impact" (3.76%), as detailed in Table 5.

Figure 1 illustrates a negative correlation between PASI and treatment adherence, with a correlation coefficient of −0.102 (rs^2 = 1.04%). Conversely, there was a positive correlation between PASI and the history of treatment interruption (0.2444, rs^2 = 5.97%). This suggests that 1.04% of the PASI variation may be attributed to treatment adherence failure, while 5.97% may be related to the history of treatment interruptions.

	PASI	Satisfaction with treatment	Adherence	Interruption of treatment
PASI	1			
Satisfaction with treatment	-0.0208	1		
Adherence	-0.102	0.1208	1	
Interruption of treatment	0.2444	-0.0967	-0.3207	1

Figure 1. Correlation matrix of psoriasis severity, satisfaction with treatment, adherence to treatment, and treatment interruption. The values in the matrix represent the correlation coefficients between these variables, indicating the strength and direction of their relationships. Positive correlations are highlighted in green, while negative correlations are highlighted in red, with the intensity of the color reflecting the magnitude of the correlation. The psoriasis area severity index score is used as an indicator of psoriasis severity.

4. Discussion

Adherence and persistence to pharmacotherapy are critical determinants of psoriasis treatment success, particularly in chronic conditions requiring long-term management. High levels of adherence and persistence are associated with better therapeutic outcomes, including symptom control, prevention of disease progression, and improved quality of life. Conversely, poor adherence and frequent discontinuation can lead to suboptimal treatment outcomes, exacerbation of disease, and increased healthcare costs due to the need for more intensive interventions.[21] Therefore, enhancing adherence and persistence through patient education, regular follow-up, and addressing barriers to consistent medication use is vital for achieving therapeutic goals and ensuring effective long-term disease management.

Our results demonstrated a relationship between psoriasis severity, as measured by the PASI, and patient adherence to treatment protocols. Specifically, a negative correlation was observed between PASI scores and treatment adherence, with a correlation coefficient of −0.102, indicating that lower adherence is associated with slightly higher PASI scores, though this relationship accounted for only 1.04% of PASI variance. Conversely, a positive correlation was found between PASI scores and the history of treatment interruption, with a correlation coefficient of 0.2444, suggesting that patients with a history of treatment discontinuation had higher PASI scores, accounting for 5.97% of the variance. These findings imply that while both adherence and treatment interruption influence psoriasis severity, the latter has a more pronounced impact on disease worsening.

The sample showed a similar representation of men and women, aligning with previous findings that psoriasis has no sex predilection.[3] The patient-assessed disease severity differed from the PASI score, underscoring the importance of incorporating patient perceptions into assessments of both disease burden and quality of life. Integrating subjective patient-reported outcomes with objective clinical measures is essential for a comprehensive evaluation of therapeutic efficacy.[22]

Literature on treatment adherence in psoriasis is heterogeneous. Our findings are consistent with those of Avazeh *et al.*,[23] who observed that among 575 psoriasis patients, a significant majority (70.7%) reported low medication adherence, while 24.1% had moderate adherence and only 5.2% achieved high adherence. Factors such as age, comorbidities, treatment type, adverse effects, treatment satisfaction, and health literacy were significantly associated with medication adherence. Notably, 57.6% of participants cited forgetfulness as a primary reason for non-adherence, while 54.6% discontinued treatment without consulting their doctor due to adverse effects.[23]

Adherence to topical treatments is generally poor, with real-world application rates between 50% and 60% of prescribed doses, and patients applying only 35 – 72% of the recommended dose. Key barriers to adherence include perceived low efficacy, time-consuming application, and unfavorable cosmetic properties. These findings highlight the need for improved patient education and tailored strategies to enhance adherence.[24]

A systematic review and meta-analysis of 62 studies involving 169,371 psoriasis patients found an overall adherence rate of 61% for biological therapies.[25] Factors influencing adherence included insurance coverage, patient satisfaction, treatment efficacy, socioeconomic status, dosing frequency, and physician-patient relationship. Non-adherence can lead to increased healthcare costs, as patients with treatment failures incur higher overall medical expenses.[26]

Adherence to methotrexate—a widely available, low-cost treatment in underdeveloped countries —was lower than that of biological drugs.[27] A study comparing adherence rates among new users of biologics found significantly higher adherence to adalimumab (odds ratio [OR] = 2.24), etanercept (OR = 1.77), and ustekinumab (OR = 2.54) had greater adherence rates compared to methotrexate; Acitretin (OR = 0.57) had the lowest adherence.[28]

The disparity in adherence between topical treatments and biological agents highlights important factors influencing patient behavior and decision-making. Topical therapies often fall short of full adherence because patients struggle with their perceived inefficacy, the time and effort required for application, and the cosmetic drawbacks of some treatments. In contrast, biological treatments tend to see better adherence, likely due to their convenience and more noticeable results. However, even biologics are not exempt from adherence challenges. This suggests that factors like medication cost, accessibility, and patient understanding of their condition and treatment continue to play a significant role. Addressing these issues is essential for developing targeted interventions that improve adherence across all treatment modalities.

In this study, a significant portion of participants never experienced asymptomatic periods, highlighting the challenge of achieving ideal treatment outcomes in practice. Failures in adherence and persistence were identified as key contributors to disease severity. The primary reasons for treatment discontinuation included therapy-related discomfort, dissatisfaction with the lack of visible improvement, and skepticism about treatment efficacy. These factors emphasize the need to enhance patient comfort, manage expectations, and reinforce treatment effectiveness to improve adherence. As demonstrated in this study, adherence issues are well-recognized contributors to therapeutic failure, preventing patients from achieving optimal clinical outcomes and significantly affecting their quality of life.[13-15]

Beyond clinical consequences, poor adherence also imposes a substantial economic burden. Inadequate disease control leads to increased direct and indirect costs, including more frequent follow-up visits, higher absenteeism from work or school, and the eventual need for costlier therapeutic alternatives. Thus, improving adherence is directly linked to better psoriasis management and reduced healthcare expenditures.[28]

Non-adherence to treatment can result from factors related to the patient, the patient-physician relationship, and the treatment itself.[29] Patient-related risk factors include being male sex, single marital status, unemployment, and excessive alcohol and tobacco use.[30] A poor physician-patient relationship is also a critical determinant; loss of trust during consultations and lack of patient involvement in therapeutic decision-making negatively impact adherence.[31] Treatment-related barriers to adherence are multifactorial, encompassing intolerance or dissatisfaction with therapy, discomfort with pharmaceutical formulations, the time-consuming application of topical medications, fear of adverse effects, and unmet patient expectations.[31-33]

The adverse effects of pharmacological treatments for psoriasis vary by drug class. Systemic immunosuppressants like methotrexate and cyclosporine carry risks of hepatotoxicity, nephrotoxicity, and increased susceptibility to infections. Biologic therapies, including TNF-α inhibitors (e.g., etanercept, adalimumab) and IL-17 inhibitors, have been associated with serious infections, injection site-related reactions, and exacerbation of preexisting conditions such as congestive heart failure or demyelinating diseases. Newer agents, such as phosphodiesterase-4 inhibitors (e.g., apremilast), are known to cause gastrointestinal disturbances, headaches, and weight loss.[34] Improving adherence in psoriasis patients can be achieved through simple health education interventions, which can significantly enhance clinical outcomes, improve patient quality of life, and reduce healthcare costs.[14]

Beyond patient education, ensuring treatment adherence requires a multidisciplinary approach, including collaborative patient care, cessation of alcohol and tobacco use, psychotherapy, and strengthening the physician-patient relationship. Effective communication, shared decision-making, and aligning treatment choices with patient expectations are essential for improving

adherence. Selecting a therapeutic option that is convenient, effective, cost-effecient, and well-tolerated—along with pharmacotherapeutic monitoring—can significantly enhance adherence and optimize psoriasis management. These measures can improve the patient's quality of life and reduce costs for both the patient and the healthcare system.[29]

This study has several limitations. Firstly, its cross-sectional design prevents the establishment of causal relationships between the severity of psoriasis and the associated sociodemographic and clinical factors. Data collected were restricted to selected dermatology centers, which may limit the generalizability of the findings, particularly for patients who do not seek specialized care. In addition, variables such as treatment adherence and lifestyle habits were self-reported, which could introduce recall bias or inaccuracies. The sample size of 133 participants, while sufficient for this analysis, limited the statistical power, preventing the execution of the originally planned nonparametric tests. Finally, although the study collected extensive data on clinical, sociodemographic, and treatment-related factors, it did not thoroughly assess psychological aspects, which are known to influence both psoriasis severity and quality of life. Future research should address these gaps by employing longitudinal designs, increasing sample size, and incorporating detailed psychological assessments.

5. Conclusion

Our study shows a connection between treatment adherence and psoriasis severity, emphasizing that choosing the right therapy alone is not enough to achieve the best results. Treatment interruptions were associated with increased psoriasis severity. In contrast, patients with higher treatment adherence had lower psoriasis severity. These findings highlight the critical role of consistent adherence in effective disease management. To improve patient outcomes, healthcare providers should prioritize regular follow-ups and patient education—simple yet powerful tools that can enhance treatment success, improve quality of life, and reduce healthcare costs. Our findings suggest that supporting better treatment adherence should be a key priority in psoriasis management strategies.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Ethics approval and consent to participate

This study was approved by the Human Research Ethics Committee of the Health Sciences Sector, Federal University of Paraná (SCS/UFPR) under approval number 4.294.864, issued in 2021. All participants consented to be part of the study via the ICF.

Consent for publication

Patients consented on the publication of their data.

Availability of data

Data is available from the corresponding author upon reasonable request.

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Appendix

Table A1. Topical treatments used by study participants

Treatment	*n*	%
Corticosteroid	81	60.90
Calcipotriol	41	30.82
Salicylic acid	15	11.27
Common moisturizer	10	7.51
Coal tar or liquor carbonis detergens	6	4.51
Urea	5	3.75
Tacrolimus or pimecrolimus	1	0.75
Anti-dandruff shampoo	1	0.75
Manipulated formula (silicone+osu+ceramides)	1	0.75

Table A2. Systemic medications used by study participants

Treatment	*n*	%
Methotrexate	28	21.05
Acitretin	14	10.52
Cyclosporine	4	3.00
Corticosteroid	3	2.25
Leflunomide	2	1.50
8-methoxypsoralen	1	0.75

Table A3. Immunobiologicals used by study participants

Treatment	*n*	%
Secukinumab	23	17.29
Adalimumab	22	16.54
Ustekinumab	11	8.27
Etanercept	2	1.50
Risankizumab	2	1.50
Infliximab	1	0.75
Ixekizumab	1	0.75

ACCSCIENCE PUBLISHING

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ORIGINAL ARTICLE

Evaluation of the therapeutic effects of nicotinamide adenine dinucleotide phosphate oxidase inhibition in a rodent model of transient ischaemic stroke

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Abstract

Background: Ischaemic stroke, a leading cause of mortality and disability, induces oxidative stress (OS), largely driven by overactive nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Targeting this enzyme system may offer therapeutic benefits by mitigating cerebrovascular damage. **Aim:** This study investigated whether suppressing NADPH oxidase through VAS2870 reduces ischaemic brain injury and functional deficits in a rodent stroke model. **Methods:** Male Sprague Dawley rats underwent 45-min middle cerebral artery occlusion (MCAO), followed by intravenous VAS2870 or vehicle administration 30 min post-reperfusion. Infarct volume was measured at 48 h and day 11 post-MCAO using magnetic resonance imaging or Nissl staining. At day 11 post-MCAO, brains and blood samples were collected to analyse OS, inflammation and cellular changes. Behavioural tests were used to evaluate cognitive and functional outcomes. **Results:** VAS2870 significantly improved survival outcome following MCAO. However, no significant differences in infarct volume were observed between the control and VAS2870-treated groups. In addition, no significant alterations were detected in total antioxidant capacity, interleukin-1 beta, tissue inhibitor of metalloproteinases-1, or vascular endothelial growth factor levels. Assessment of post-MCAO functional and cognitive deficits revealed a significant worsening of neurological function following VAS2870 treatment on day 2, whereas no significant effect of NADPH oxidase inhibition was found at day 11 post-MCAO. In addition, cellular analysis showed no effect of NADPH oxidase inhibition on neuronal counts, neurogenesis, or angiogenesis in MCAO-affected brain regions. **Conclusion:** Although post-MCAO targeting of NADPH oxidase significantly improved acute survival, it did not significantly reduce ischaemic injury or improve functional outcome. These findings suggest that although NADPH oxidase inhibition holds promise as a therapeutic strategy, its effectiveness may be limited, particularly when administered during early phases of cerebral reperfusion. **Relevance for patients:** Although inhibition of NADPH oxidase alone did not improve cognition and neurovascular recovery, it may be beneficial in post-stroke recanalisation therapy.

Keywords: Blood–brain barrier; Ischaemic stroke; Nicotinamide adenine dinucleotide phosphate oxidase; VAS2870; Oxidative stress

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1. Introduction

Although stroke research has led to improvements in projected life expectancy following stroke,[1] it remains the third leading cause of mortality, after heart disease and COVID-19, and a leading cause of disease burden.[1] Ischaemic stroke, resulting from cerebral blood flow (CBF) interruption, accounts for approximately 87% of all stroke cases[2] and is a major source of disability adjusted life years, morbidity, and mortality.[3,4] Without the implementation of primary, risk-factor-focused prevention strategies, stroke-related mortality/burden will continue to rise globally, particularly in low-income countries.[5] Thus, there is a continued need for targeted approaches to understand and treat the acute cellular impacts of ischaemic stroke.

Following ischaemic stroke, CBF restoration is the present primary clinical treatment strategy to improve patient outcomes. At present, tissue plasminogen activator is the only pharmacological treatment used.[6] In addition, the use and availability of direct clot removal using endovascular thrombectomy is increasingly prevalent.[7] However, restrictive eligibility criteria—including a limited therapeutic time window (4.5 h from symptom onset), low global availability/administration rates, low recanalisation rates and haemorrhagic transformation risk—result in fewer than 5% of stroke patients being treated worldwide.[2,8,9] Although many pharmaceutical therapeutics have shown pre-clinical successes, these have largely failed to progress beyond clinical trials.[10,11] Consequently, they have failed to advance the present status quo of ischaemic stroke treatment. Despite this, there remains a critical need to improve clinical outcomes using novel neuroprotective treatments to address the unmet need for standalone or combined effective therapeutic treatments in the clinic.

One pathway to consider is oxidative stress (OS), induced by the surge in reactive oxygen species (ROS) produced during ischaemic stroke. While ROS are normally present at low levels and essential for physiological signalling, cellular homeostasis and vascular tone, redox homeostasis is disrupted under ischaemic conditions, triggering a substantial overproduction of ROS (e.g., superoxide/hydrogen peroxide).[12] This surge overwhelms endogenous antioxidant defence mechanisms, resulting in the pathophysiological effects of ROS and OS. OS compromises the integrity of the blood–brain barrier (BBB), leading to its dysfunction and increased permeability through mechanisms, including endothelial cell damage, inflammatory gene expression and DNA fragmentation.[6,13,14] The resultant BBB compromise facilitates neuroinflammation and contributes significantly to the formation of cerebral oedema, a major factor in early mortality after stroke.[15] Targeting excessive ROS using antioxidant treatment has shown pre-clinical promise in attenuating stroke damage, but these approaches failed to progress to clinical success.[16,17] Therefore, effective translation of pre-clinical findings to clinical therapies that mitigate OS-driven BBB dysfunction by targeting specific mechanisms implicated in ROS production and OS exacerbation, without disrupting essential ROS functions, remains a critical unmet need.

A source of ischaemia-induced pathophysiological levels of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major enzymatic source of ROS within the cerebrovasculature and cerebral cells.[18-22] NADPH oxidases, a family of enzymes dedicated to ROS production, have the capacity to produce large amounts of ROS. To date, seven NADPH oxidase isoforms (NOXs) have been identified (NOX 1–5 and dual oxidase 1–2).[23] Vascular NADPH oxidase isoforms contribute to vascular tone through low-level ROS production[24,25] and physiological redox signalling under basal conditions—distinct from the inducible phagocytic form[24]—but become key drivers of pathology upon overactivation during ischaemia. Specifically, NOX2 and NOX4 have been reported to be the predominant isoforms within the brain, with NOX4 being the most abundant vascular isoform.[26-28] The NOX4 isoform exhibits higher cerebrovascular expression than within the periphery,[29,30] and both NOX2 and NOX4 have been implicated in stroke injury.[29,31-34] Under ischaemic conditions, NADPH oxidase is a primary source of increased superoxide production.[18,32,35-38] Furthermore, NOX4-generated superoxide is rapidly converted to hydrogen peroxide and mediates glutamate neurotoxicity,[39-41] a significant pathophysiological ischaemic cascade mechanism. Increased NADPH oxidase activity coincides with both increased NADPH oxidase enzymes' microRNA and protein expressions within the peri-infarct region 2–48 h after cerebral ischaemia injury.[29,31-34] Inhibition and reduced expression of NADPH oxidase have been reported to result in neuroprotection and reduced functional deficits,[32,33,42] suggesting that the targeted inhibition of NADPH oxidase is a potential therapeutic avenue. Targeted ROS inhibition is required to promote continued vascular function[20,22] and reduce pro-inflammatory ROS functions without impeding anti-inflammatory or functional immune responses.[43] The pivotal role NADPH oxidase plays in OS in laboratory conditions[44] combined with the limited success of broad-spectrum general antioxidants at clinical trial, such as NXY-059 (SAINT I/II trials)[16,17] and the established role of NADPH oxidase-derived OS in post-stroke pathophysiology, including BBB disruption,[6,12-15] further support the rationale of targeting specific enzymatic sources of OS through NADPH oxidase inhibition.

The NADPH oxidase inhibitor VAS2870, a triazolopyrimidine derivative, has been shown to inhibit a selection of NADPH oxidase isoforms, including NOX2 (inhibitory concentration [IC]50: 1.1μM) and NOX4 (IC50: 12.3μM),[45-47] both of which are isoforms present within the cerebrovasculature and ischaemic brain regions.[26-28] Kleinschnitz *et al.*[32] reported a neuroprotective outcome comparable to that observed in NOX4$^{-/-}$ knockout mice at 24 h following cerebral ischaemia when intrathecal VAS2870 was administered at 2 h and 12 h post-middle cerebral artery occlusion (MCAO),[32] suggesting that VAS2870 is effective in attenuating NOX4-driven injury progression during the early hours of stroke injury. More recently, VAS2870—when administered before reperfusion—reduced post-ischaemia injury and mortality, potentially through miRNA autoregulation of the NOX2/4 isoforms.[48] In addition, pre-reperfusion intravenous administration of VAS2870 suppressed OS, neuronal apoptosis and NOX2 and NOX4 upregulation, attenuated brain oedema, reduced BBB permeability and improved neurological function in a rat model of acute ischaemic stroke with haemorrhagic transformation.[49] Furthermore, VAS2870 suppression of OS *in vitro* has shown enhanced tubulogenic and BBB-forming capacities of endothelial progenitor cells.[44,50]

However, the post-stroke role of NADPH oxidase remains unclear. It has the potential to induce injuries during the acute phase of ischaemic injury while potentially contributing to recovery during the sub-acute phase through mechanisms, such as angiogenesis.[51] Previous studies using VAS2870 have investigated the contribution of NADPH oxidase to ischaemic injury in the short term, typically at 24 h, and other acute post-stroke time points, but information regarding sub-acute neuroprotective outcomes and downstream cellular changes remains scarce. Furthermore, a previous work has highlighted that NADPH oxidase suppression in the ischaemic setting may generate opposing effects when combined with other treatments, such as endothelial stem cell therapies[44]. Consequently, we utilised VAS2870, a covalent inhibitor of NOX2 and NOX4,[45,46,52] to assess the therapeutic potential of modulating these key isoforms during early post-stroke reperfusion and determine the downstream acute and sub-acute effects on brain injury and functional deficits using a multimodal approach within an ischaemic stroke rodent model.

2. Materials and methods

2.1. Study design

This study was conducted in accordance with the United Kingdom (UK) Animals (Scientific Procedures) Act, 1986 (Project Licence No. PP9645035), following ethical approval by the University of Nottingham Animal Welfare Ethical Review Body (AWERB) and reported in line with Animal Research Reporting of *in vivo* Experiments guidelines.[53] Male Sprague Dawley rats (Envigo, UK) aged between 52 and 59 days on arrival were used. Animals, randomly allocated into groups of three, were housed according to UK standards of care regulations.[54] All animals underwent 45 min of transient intraluminal filament MCAO with recovery at 78–85 days old. Animals were randomly allocated (n = 18; vehicle = 9, VAS2870 = 9XX) to receive 1 mg/kg of intravenous VAS2870 (SML0273 Sigma Aldrich, UK) or vehicle (10% dimethyl sulfoxide [DMSO] in 0.9% sodium chloride [NaCl] solution) at 30 min post-reperfusion, by tail vein injection under general anaesthesia (GA). Sterile VAS2870 stock solution (4 mg/mL in 100% DMSO; aliquoted and stored at −80°C) was diluted at the point of injection using 0.9% NaCl solution to obtain a 0.4 mg/mL solution in 10% DMSO. Both VAS2870 and vehicle injection volumes were <5 μL/g total volume. At 48 h post-MCAO, a subset of animals underwent T2-weighted (T2w) magnetic resonance imaging (MRI; n: vehicle = 4, VAS2870 = 5). All animals underwent sensorimotor and cognitive testing at various time points (Figure 1). A total of 18 animals were used within this study: one animal died within the first 20 h following MCAO, three animals were humanely killed by overdose of anaesthesia followed by femoral artery severance due to severity limits within the first 48 h following MCAO, and one animal was excluded during surgery due to technical complications and humanely killed under anaesthesia. On day 11 post-MCAO, all remaining animals (n = 11) underwent terminal perfusion fixation under GA before tissue removal. Sample sizes were calculated using the resource equation (Equation I)[55] to determine a biologically relevant effect size for a pilot design. For data analysis, only animals that survived to the end of the study were included, unless otherwise stated.

Error (E) degrees of freedom (dof) = Total dof −Treatment dof −(blocks−1); 10<E<20 (I)

2.2. Blinding and randomisation

To ensure consistent environmental conditions across treatment groups, each cage was allocated one vehicle and one VAS2870 treatment animal, with the remaining vehicle or VAS2870 allocation randomly assigned across cages (automated randomiser, random.org). Animals were randomly allocated to cages upon arrival by the facility staff, who were blinded to the allocations. Animals underwent MCAO and behaviour assessment in random order (automated list randomiser, random.org). The experimenter was blinded to the treatment group at the

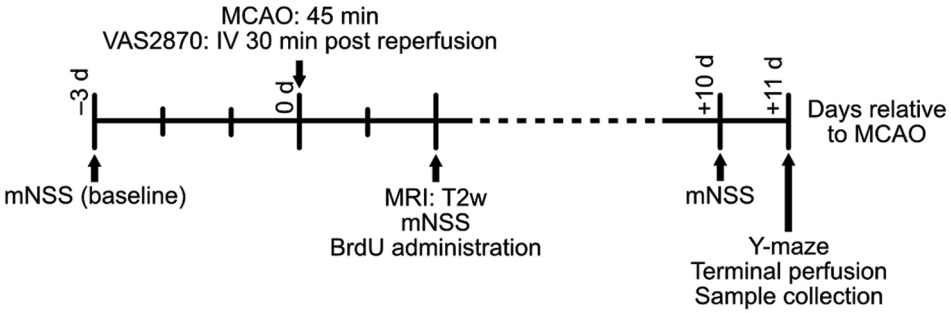

Figure 1. Longitudinal experimental timeline, expressed as days (d) relative to MCAO procedure. All rats were handled and acclimatized to housing before undergoing assessments and surgical procedures. All rats underwent 45 min of MCAO, followed by treatment with VAS2870 or vehicle at 30 min following MCA reperfusion at day 0. A subset of animals underwent MRI imaging (n = 9), due to technical issues. All animals received BrdU, a cell proliferation marker, by intraperitoneal injection at day 2 post-MCAO. Rats were assessed for sensorimotor deficits using modified neurological severity scoring (mNSS) at various time points (baseline −3 days, +2 days, and +10 days relative to MCAO) and for cognitive deficits using the Y-maze (11 days post-MCAO). All animals underwent terminal perfusion with sample collection at day 11 post-MCAO.
Abbreviations: BrdU: 5-bromo-2'-deoxyuridine; MCAO: Middle cerebral artery occlusion; MRI: Magnetic resonance imaging.

time of MCAO, behavioural assessment, tissue/sample processing, and image analysis. Tissue samples were given a randomised ID at processing to add a further layer of blinding for post-mortem analysis (random string generator, random.org). The experimenter was blinded to treatment and hemisphere awareness using randomised ID codes for all immunofluorescence images (automated random string generation, random.org).

2.3. Analgesia and welfare monitoring

Animals received subcutaneous (SC) buprenorphine (0.04 mg/kg; Vetergesic 0.3 mg/mL) immediately before MCAO and SC bupivacaine hydrochloride directly at the incision site (2 mg/kg; Marcain 0.5%; 1:2 dilution in 0.9% NaCl) following incision. After surgery, animals received SC meloxicam (1 mg/kg; Metacam 2 mg/mL; 5–6 h post-recovery) and were monitored using rat grimace score (RGS)[56] to determine additional analgesia needs. No animals received additional analgesia. To support fluid levels, animals were administered 0.9% NaCl solution pre-operatively through intraperitoneal (IP) injection (2 mL), immediately post-operatively (SC; 4 mL), and at 24 h post-MCAO (SC; 2 mL). Following MCAO, animals were weighed daily until the end of the study and monitored for overall health and well-being.

2.4. MCAO surgery and treatment administration

Animals underwent transient MCAO as previously described,[57] with modifications. Briefly, animals were anaesthetised using isoflurane (induction 5% in O_2; maintenance 2% in N_2O/O_2 70/30%). During all surgery and GA, a homeothermic monitoring and heat system (Harvard Apparatus, UK) maintained body temperature at 37 ± 1°C. MCA territory CBF was monitored using a laser Doppler flowmetry (Moor Instruments, UK) attached to the superior position of the temporal bone. MCAO was achieved using an intraluminal coated monofilament (Doccol, United States; United States Pharmacopoeia 4–0, length 4–5 mm and diameter 0.37–0.41 mm, following the manufacturer's recommendations), advanced to occlude the right MCA origin beyond the internal carotid artery bifurcation. MCAO was confirmed by a reduction in CBF signal, at which point the filament was secured and the incision was sutured. Animals recovered during the MCAO period within a pre-warmed recovery cage. Immediately before the end of the 45-min MCAO period, the animal was re-anaesthetised and the filament was removed. The artery was ligated using silk sutures, and the incision was sutured. Animals remained under GA. At 30 min post-MCA reperfusion, vehicle or VAS2870 treatment was administered intravenously by tail vein injection. Isoflurane was reduced to 0% and animals were provided oxygen until paw reflex response, at which point they were moved to a pre-warmed recovery cage.

2.5. Magnetic resonance imaging image acquisition and analysis

At 48 h post-MCAO, nine animals underwent MRI imaging for infarct identification. High-resolution T2w sequence MRI scans were performed on a 7T small animal MRI scanner with a 72 mm radiofrequency volume transmit coil and BGA12 gradient coil system, utilising a four-channel surface phased array receiving coil (Bruker BioSpin, Germany). MRI scans were performed under GA (Isoflurane in O_2: induction 5%, maintenance 2%). Animals were secured using a head holder with a bite bar and ear inserts. Animals were physiologically monitored using an MRI-compatible physiological monitoring system (SA

Instruments, United States) with a respiration pillow and rectal thermometer to monitor temperature. Temperature was maintained at 37 ± 1°C using a water-heated MRI bed. To locate the cortical region, coronal scout imaging was performed. T2w images were acquired using a turboRARE sequence with the following parameters: a repetition time/echo time of 5,000/50 ms, a field of view of 28 × 28 mm (256 × 256 matrix), 18 × 1 mm cortical coronal slices, four signal averages and a total scan duration of 10 min and 40 s. Region of interest analysis of infarct volume was performed using T2w images in FIJI ImageJ (v1.54f; NIH USA) software, using Bio-formats Plug-in[58] for Neuroimaging Informatics Technology Initiative file visualisation. For all slices, three repeat measurements of the contralateral hemisphere area, ipsilateral hemisphere area, and, if identified, infarct area (denoted by regions of hyperintense T2 signal) were delimitated manually by an experienced operator, who was blinded to the treatment group and animal ID. To calculate volume, the area means were multiplied by the slice thickness. Total infarct volume was adjusted for hemispheric swelling per slice (Equation II).

Infarct volume/(ipsilateral as a % of contralateral) (II)

2.6. Neurogenesis marker administration

Cell proliferation at 48 h post-MCAO was assessed using the cell proliferation marker 5-bromo-2'deoxyuridine (BrdU). At 48 h post-MCAO, animals received 50 mg/kg BrdU solution (IP; 10 mg/mL in 10% DMSO and 0.9% NaCl solution) under GA (isoflurane in O_2: induction 5%; maintenance 2%). Animals that underwent MRI received the BrdU treatment after removal from the MRI scanner, and animals that did not undergo MRI received GA and BrdU at the same time point. Following injection, animals were observed and were left to recover within a pre-warmed recovery cage, before being returned to the home cage.

2.7. Behavioural assessment

Sensorimotor impairment was assessed using a modified neurological severity score (mNSS) adapted from Ord *et al.*[59] to incorporate assessments relevant to sensorimotor function. The score has a total range of 0–28, where lower scores indicate greater deficits (Figure A1). Baseline assessment was conducted three days before MCAO, with post-MCAO assessment on days 2 and 10. Cognitive dysfunction, particularly spatial working memory, was assessed using spontaneous alternations during an 8-min exploratory activity observation within a Y-maze[60] on post-MCAO day 11. During the Y-maze assessment, animals were remotely tracked using a ceiling-mounted camera (VCB-310SP, Sanyo Panasonic, Japan) with the EthoVision (version 16) software and were randomly allocated to a start arm before the trial.

2.8. Terminal perfusion, blood sampling and tissue collection

On post-MCAO day 11, animals underwent transcardial terminal perfusion following GA overdose (induction: isoflurane 5% in O_2; overdose: IP pentobarbital). After confirming pedal reflex loss, animals were perfused with a 0.9% NaCl solution followed by 4% paraformaldehyde solution. Cortices were removed and stored in 4% paraformaldehyde solution overnight at 4°C, then dehydrated in 30% sucrose and phosphate buffer saline and stored at 4°C until the tissue sank (~24–48 h). Cortices were optimal cutting temperature-embedded and stored at −80°C. For plasma samples, blood was collected from the atrium blood pool at the start of transcardial perfusion and placed in ethylenediaminetetraacetic acid-coated 1.3 mL vials (ID 41.1395.005, Starsdet, UK). The samples were left to rest at room temperature for an hour, and then spun at 2,000 × g for 15 min. Plasma supernatant was aliquoted and stored at −80°C.

2.9. Biomarker analysis

Total antioxidant capacity, a measure of OS reducing capacity, was measured using the Trolox equivalent antioxidant capacity assay kit (MAK187-1KT; Merck, UK), according to the manufacturer's instructions, with samples run in triplicate. An estimation of total antioxidant capacity was assessed using a copper (II) ion-buffered reagent compared against the Trolox (a water-soluble vitamin E analogue) antioxidant standard through colourimetric detection at 570 nm (A_{570}).

Cytokine analytes interleukin (IL) 1β, tissue inhibitor of metalloproteinase (TIMP) 1 and vascular endothelin growth factor (VEGF) levels were measured, in triplicate, from plasma using a multiplex assay (Luminex Discovery Assay [Rat A Standards], R&D Systems, UK) with a Bio-Rad® Bio-Plex 200® system (Bio-Rad Laboratories Ltd, UK), according to the manufacturer's instructions. Standard curves for each analyte were used to calculate protein concentrations (pg/mg). Initial fluorescence analysis was conducted using the Bio-Rad Bio-Plex Manager (v.6.2.0.175), with subsequent comparative analysis using GraphPad PRISM (v10.3.1).

2.10. Tissue slicing

Five randomly selected brains per treatment group underwent coronal sectioning using a cryostat (Microm HM505e) at −16°C. Brains were serially sectioned using interlocking intermittent series for free-floating double-label immunofluorescence (40 μM; 1:10 series) and Nissl

staining (20 μM; 1:20 series). Slices were obtained from striatal (morphology from approximately +2 mm to −0.2mm relative to the anterior bregma) and hippocampal (morphology from approximately −1.45mm to −3.5mm relative to the anterior bregma) regions, stored in 0.02% sodium azide and Tris-buffered saline (TBS) solution, and sealed at 4°C until required. For Nissl staining, 20 μM slices were taken throughout the cortex and mounted onto glass slides (10149870, Fisher, UK), air-dried and stored at −80°C until required.

2.11. Immunofluorescence staining and imaging

Immunofluorescence staining was performed using a free-floating method to identify neuronal nuclei (NeuN; neuronal cell marker), BrdU (cell proliferation marker), and cluster of differentiation marker 31 (CD31; vascular endothelial cell marker). 4',6-diamidino-2-phenylindole nuclear DNA stain (DAPI; cell nucleus marker) was also used. See Table A1 for antibody details. For $NeuN^+$ DAPI staining, six randomly selected brains (three per treatment group) were stained, and for both CD31 and $NeuN^+$ BrdU staining, 10 randomly selected brains (five per treatment group) were stained. All immunofluorescence staining steps were performed at room temperature unless otherwise stated. For $NeuN^+$ DAPI and CD31 staining, sections were washed in TBS (3 times for 5 min each), blocked and permeabilised in blocking buffer (1 h; 5% normal goat serum + 0.3% Triton X-100 and TBS). Sections were then incubated overnight at 4°C with primary antibodies. After washing with TBS (3 times for 5 min each), sections were incubated with secondary antibodies for 2 h, followed by washing with TBS (3 times for 5 min each). Sections were mounted and fluorescence preserved using ProLong Gold reagent (11539306, Fisher Scientific, UK) and stored at 4°C until imaging. For $NeuN^+$ BrdU staining, acid antigen retrieval was performed. Sections were washed in TBS (3 times for 5 min each), incubated at 37°C in pre-warmed two normal (2N) solutions of hydrochloric acid (30 min), and then neutralised using 0.1 M borate buffer (pH 8.5; 10 min). Sections were washed with TBS (3 times for 5 min each), blocked for an hour and washed again, as above. Before primary antibody application, residual endogenous immunoglobulin G was blocked for 2 h with anti-rat immunoglobulin G antibody and blocking buffer, and sections were washed in TBS (3 times for 5 min each). Subsequently, the sections were incubated in primary antibody solution overnight at 4°C. The secondary antibody staining was performed according to the steps above.

$Neuronal\ nuclei^+$ DAPI immunofluorescent images were acquired using fluorescence microscopy (20× air objective lens with 0.75 numerical aperture; Zeiss LSM Exciter Wide Field Microscope, Zeiss, Germany), and analysed using the MicroManager v1.4.21 software with an attached charge-coupled device camera (Retiga R1, QImaging, USA), utilising filter cubes suitable for 400 nM (blue) and 546 nM (red) emission imaging. Region-specific images were obtained from both ipsilateral and contralateral hemispheres with three images/slice/hemisphere from the striatum (STR), sensorimotor cortex (SC), and hippocampal CA1 (CA1) regions, and one image/slice/hemisphere from the dentate gyrus hilus (DG) region. $NeuN^+$ cells were manually counted within the central diagonal of a 4 × 4 grid overlaid on each image. Counts from replicate images were averaged per slice. Slice averages were summed across all slices to represent the total $NeuN^+$ cell count per region.

$Neuronal\ nuclei^+$ BrdU and $CD31^+$ DAPI immunofluorescent images were acquired using laser scanning confocal microscopy (Zeiss LSM880C, Zeiss, Germany) and analysed using the Zen Imaging software (Black v2.3). $NeuN^+$ BrdU images were obtained from the DG region at 10× and 63× magnification. Whole DG images were taken at 10× for both the ipsilateral and contralateral hemispheres, one image/slice/hemisphere. $BrdU^+$ stained cells were manually counted for each image within the granule cell layer and subgranular zones. Total $BrdU^+$ cell count per hemisphere was used for analysis. For $NeuN^+/BrdU^+$ co-localisation, 50 randomly selected $BrdU^+$ cells per animal (25 per hemisphere) were imaged using 63× magnification Z-stacks (2 μM Z plane increments) within the DG granule cell layer and subgranular zone. Co-localisation was quantified as the number of co-expressing cells standardised to the total number of $BrdU^+$ cells imaged. $CD31^+$ DAPI images were obtained from within the SC and striatal regions at 40× magnification, within both contralateral and ipsilateral hemispheres. For each region, three images/hemisphere/slice were obtained across three slices per animal. Total $CD31^+$ staining per region per animal was quantified following background subtraction. All image analysis was performed using FIJI ImageJ (v1.54f; NIH, United States).

2.12. Nissl staining

To assess delayed infarct volume, whole cortex Nissl staining was performed using cresyl violet acetate (CV; C5042, Fisher, UK), using a regressive staining protocol. Briefly, mounted slides were submerged in 0.1% CV solution for 15 min. Staining was differentiated using acidified alcohol (70% industrial methylated spirit + 0.25% acetic acid), and slices were dehydrated using increasing concentrations of alcohol solution. Slides were sealed using distrene plasticiser xylene (HX86532379, Merck, UK), air-

dried and stored at room temperature until imaging. Slides were imaged using a light box with a digital single-lens reflex camera (Canon M50II, Canon, Japan).

Slices depicting an area of decreased/absent CV staining, indicative of ischaemic damage, were analysed. Three repeat measurements of the contralateral hemisphere, ipsilateral hemisphere and infarct (denoted by regions of decreased CV Nissl stain intensity in the region of the MCA territory, indicating neuronal loss) areas were delimitated manually by an experienced operator. To calculate volume, the area means were multiplied by slice thickness and series slicing distance (400 μM). Total infarct volume was adjusted for hemispheric swelling/shrinking per slice (infarct volume/[ipsilateral as a % of contralateral]). Image analysis was performed using FIJI ImageJ (v1.54f; NIH, United States).

2.13. Statistical analysis

Statistical analysis was performed using GraphPad PRISM (v10.3.1). All data were assessed for normality before statistical analysis, using the Shapiro–Wilk test. For two-group comparisons, an unpaired *t*-test with Welch's correction was performed. For comparisons of multiple groups, two-way repeated measures analysis of variance followed by a multiple comparison test with Šídák correction was used for *post hoc* analysis. Repeated measure matching levels were brain region, hemisphere, or time, depending on the experimental design. For survival analysis, the Mantel-Cox test was used. A significance level of $p<0.05$ was considered statistically significant. All data were expressed as mean ± standard deviation.

3. Results

3.1. Post-ischaemic NADPH oxidase inhibition does not attenuate brain injury or general wellbeing but improves survival probability

To evaluate post-ischaemic VAS2870-induced NADPH oxidase inhibition on brain injury, infarct volume was assessed at 48 h post-MCAO using a T2-weighted MRI, and at day 11 post-MCAO through Nissl quantification (Figure 2). Representative brain slice images from both modalities are shown in Figure 2A. VAS2870 treatment did not significantly alter infarct volume at 48 h (t [4.123] = 1.972, p=0.118), nor at day 11 (t [4.02] = 2.03, p=0.111) post-MCAO (Figure 2B). Survival analysis, which included animals removed from the study due to severity limits, showed that VAS2870 treatment significantly improved survival by 50% by day 2 post-MCAO (χ^2 [1 = 3.939, p=0.047; Figure 2C). No significant difference in weight loss, an indicator of general well-being, was observed between vehicle- and VAS2870-treated groups at any time post-MCAO (Figure 2D). A significant interaction between time and treatment was observed (F [11,99 = 1.98, p=0.038); however, treatment was not a significant source of variation (F [1,9 = 1.95, p=0.196). In contrast, time post-MCAO had a significant overall effect on body weight (F [2.43, 21.9 = 34.6, $p<0.001$; Figure 2D). *Post hoc* analysis revealed significant weight loss in the vehicle-treated group at day 3 post-MCAO compared to the baseline (p=0.07; Figure 2D), which was not observed in the VAS2870-treated group.

3.2. Post-ischaemic functional and cognitive impairments following NADPH oxidase inhibition

To assess post-stroke functional deficits, mNSS scores were measured. Results showed no significant difference was observed for the overall effect of treatment (vehicle vs. VAS2870) on mNSS scores (F [1,9 = 3.622, p=0.09), or between treatment and time (F [2,18 = 3.173, p=0.07). However, post-MCAO time had a significant effect on mNSS score (F [2,18 = 23.72, $p<0.001$; Figure 3A). *Post hoc* analysis revealed that both vehicle- and VAS2870-treated groups had significantly reduced mNSS scores at day 2 post-MCAO compared to the baseline (p=0.02 and $p<0.001$, respectively; Figure 3A). In addition, at day 2 post-MCAO, the VAS2870-treated group had significantly lower mNSS scores (p=0.02) compared to the vehicle-treated group. By day 10 post-MCAO, mNSS scores in both groups returned to baseline levels (Figure 3A). Cognitive deficits in spatial working memory, measured by percentage spontaneous alternations using the Y-maze, did not differ significantly between VAS2870- and vehicle-treated groups (t [9 = 0.183, p=0.859; Figure 3B). Similarly, there were no significant differences in total arm entries (t [5.91 = 0.708, p=0.51; Figure 3C) and total distance travelled (t [9 = 0.96, p=0.362; Figure 3D), suggesting that VAS2870 treatment did not affect cognitive function, motor activity, or exploratory behaviour.

3.3. Pro-inflammatory cytokine levels and circulating antioxidant capacity post- NADPH oxidase inhibition

The effect of post-ischaemic NADPH oxidase inhibition on circulating inflammatory markers and antioxidant capacity was evaluated at day 11 post-MCAO. Circulating total antioxidant capacity, expressed as the concentration of Trolox equivalents, did not significantly differ between VAS2870 and vehicle-treated groups (t [6.22 = 0.23, p=0.83; Figure 4A). Similarly, circulating concentrations of inflammatory cytokines IL-1β (t [6.28 = 0.301], p=0.77), TIMP1 (t [8.67] = 0.123, p=0.9), and VEGF (t [5.847] = 1.09, p=0.317) were not significantly different between the two groups at day 11 post-MCAO (Figure 4B-D).

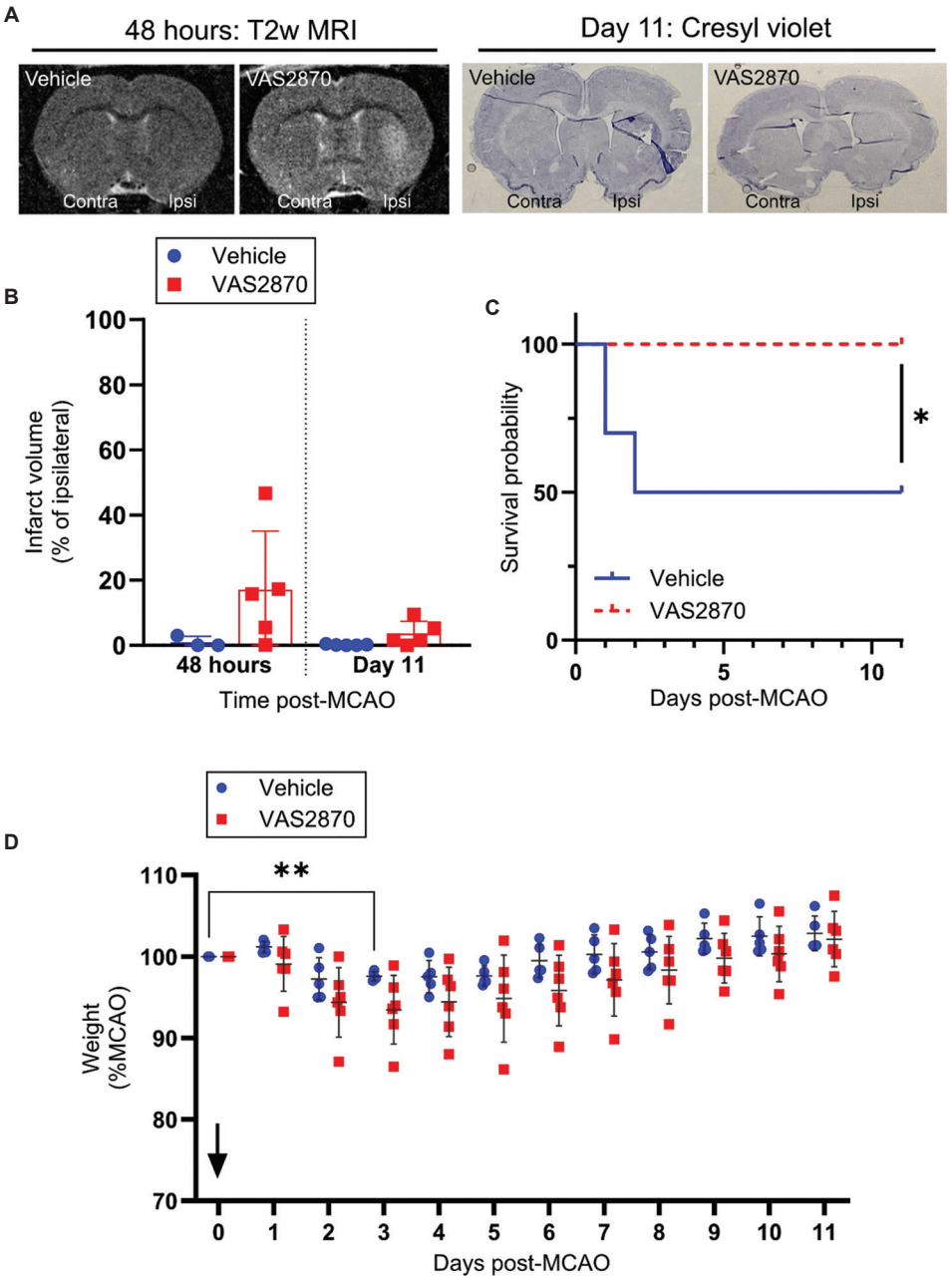

Figure 2. Effect of NADPH oxidase inhibition on post-MCAO infarct development, at acute (48 h) and chronic (11 days) post-MCAO time points, alongside post-MCAO survival probability. In addition, the effect of VAS2870 treatment following MCAO on well-being in rats was measured using daily weight assessment. (A) Representative images of T2-weighted (T2w) MRI scans (left panel) at 48 h post-MCAO and Nissl staining (right panel) at day 11 post-MCAO, in adult rats. T2w slice images depict infarct damage as a pale delineated area. Nissl-stained slices depict the infarcted area as an increased intensity of staining. Infarct volume, expressed as a percentage of the ipsilateral hemisphere, measured at 48 h post-MCAO using T2w MRI and at day 11 post-MCAO through Nissl staining. Analysis performed using unpaired *t*-test with Welch's corrections, within time point (*n*: vehicle = 5, VAS2870 = 5). All data are expressed as mean ± standard deviation. (C) Probability of survival analysis, including severity limit excluded animals, indicates NADPH oxidase inhibition significantly increased the probability of survival and reduced outcome severity (log rank test; *$p<0.05$; *n*: vehicle = 10, VAS2870 = 6). (D) Weight expressed as a percentage of weight at MCAO (baseline), measured daily for 11 days post-MCAO. The arrow indicates the MCAO procedure (two-way repeated measures analysis of variance with Sidak's multiple comparisons, assessing both between and within treatment group differences (**$p<0.01$; *n*: vehicle = 6, VAS2870 = 6). All data are expressed as mean ± standard deviation.
Abbreviations: Contra: Contralateral hemisphere; Ipsi: Ipsilateral hemisphere; MCAO: Middle cerebral artery occlusion; MRI: Magnetic resonance imaging; NADPH: Nicotinamide adenine dinucleotide phosphate.

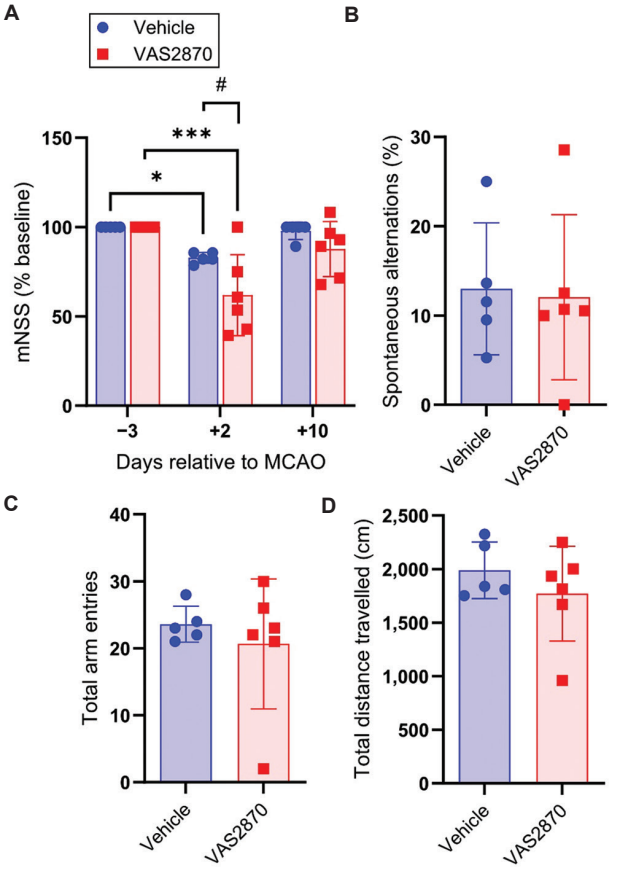

Figure 3. Effect of NADPH oxidase inhibition on functional sensorimotor, exploration, activity, and cognitive deficits in rats, following MCAO. (A) Sensorimotor neurological dysfunction assessed using modified neurological severity score (mNSS), expressed as percentage of baseline score (two-way repeated measures analysis of variance with Sidak's multiple comparison, across time (*) and between treatment groups (#) (#/*p<0.05, ***p<0.001). (B) Cognitive deficit assessment as measured using the Y-maze test, expressed as percentage spontaneous direction alternations (unpaired t-test with Welch's corrections). (C) Total spontaneous independent arm entries as measured during the Y-maze test (unpaired t-test with Welch's corrections). (D) Total distance travelled during Y-maze testing (unpaired t-test with Welch's corrections). All data are expressed as mean ± standard deviation. All graphs represent n: vehicle = 5 and VAS2870 = 6.
Abbreviations: MCAO: Middle cerebral artery occlusion; NADPH: Nicotinamide adenine dinucleotide phosphate.

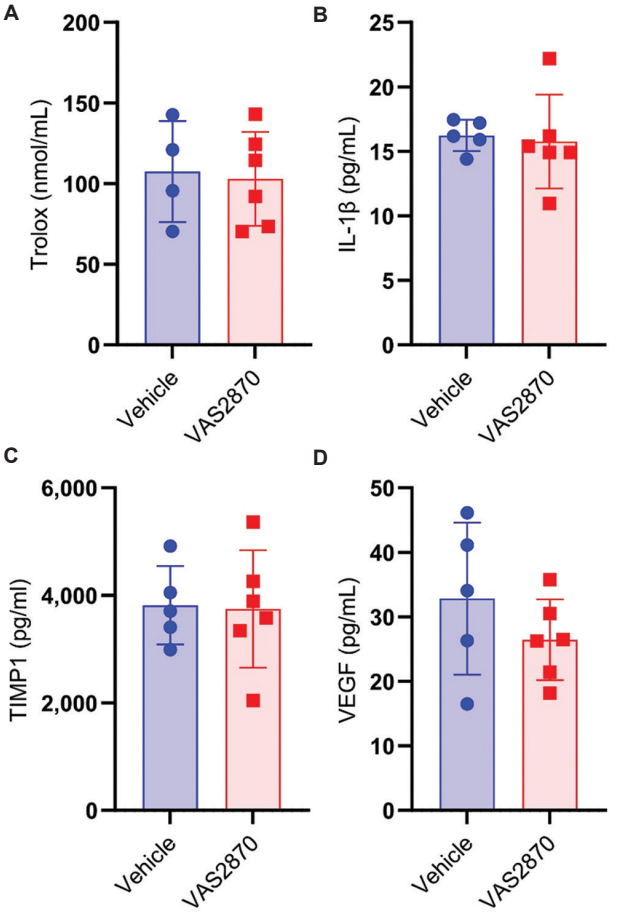

Figure 4. NADPH oxidase inhibition does not affect chronic circulatory inflammatory markers, measured at day 11 post-MCAO. (A) Plasma small molecule total antioxidant capacity, expressed as Trolox concentration (total antioxidant capacity; nmol/mL; n: vehicle = 4, VAS2870 = 6). (B) Proinflammatory cytokine interleukin-1 beta (IL-1β) circulating plasma concentration. (C) Tissue inhibitor of metalloproteinases-1 (TIMP1) circulating plasma concentration. (D) Vascular endothelial growth factor (VEGF) circulating plasma concentration. (B-D) n: vehicle = 5, VAS2870 = 6. All data were assessed using an unpaired t-test with Welch's corrections, expressed as mean ± standard deviation.
Abbreviations: MCAO: Middle cerebral artery occlusion; NADPH: Nicotinamide adenine dinucleotide phosphate.

3.4. Impact of post-MCAO NADPH oxidase inhibition on neurogenesis

Total neuronal cell number was assessed by NeuN staining at day 11 post-MCAO, and representative images depicting NeuN staining within contralateral and ipsilateral STR, SC, CA1, and DG regions for both vehicle- and VAS2870-treated groups are shown in Figure 5A. There was no significant overall effect of treatment (vehicle vs. VAS2870) on neuronal cell count normalised to the contralateral hemisphere (F [1,4] = 0.411, p=0.56), nor a significant interaction between treatment and brain region (F [3,12] = 0.201, p=0.89). *Post hoc* analysis of vehicle- versus VAS2870-treated groups revealed no significant differences in normalised cell count within DG, CA1, SC, and STR regions at day 11 post-MCAO (Figure 5B). Assessing raw total NeuN$^+$ cell count within hemispheres revealed no significant interaction between region and treatment (contralateral: F [3,12] = 0.062, p=0.98; ipsilateral: F [3,12] = 0.326, p=0.81), and in overall effect of treatment (contralateral: F [1,4] = 0.006, p=0.94; ipsilateral: F [1,4] = 0.029, p=0.87). Significant variation was seen across brain regions imaged within both

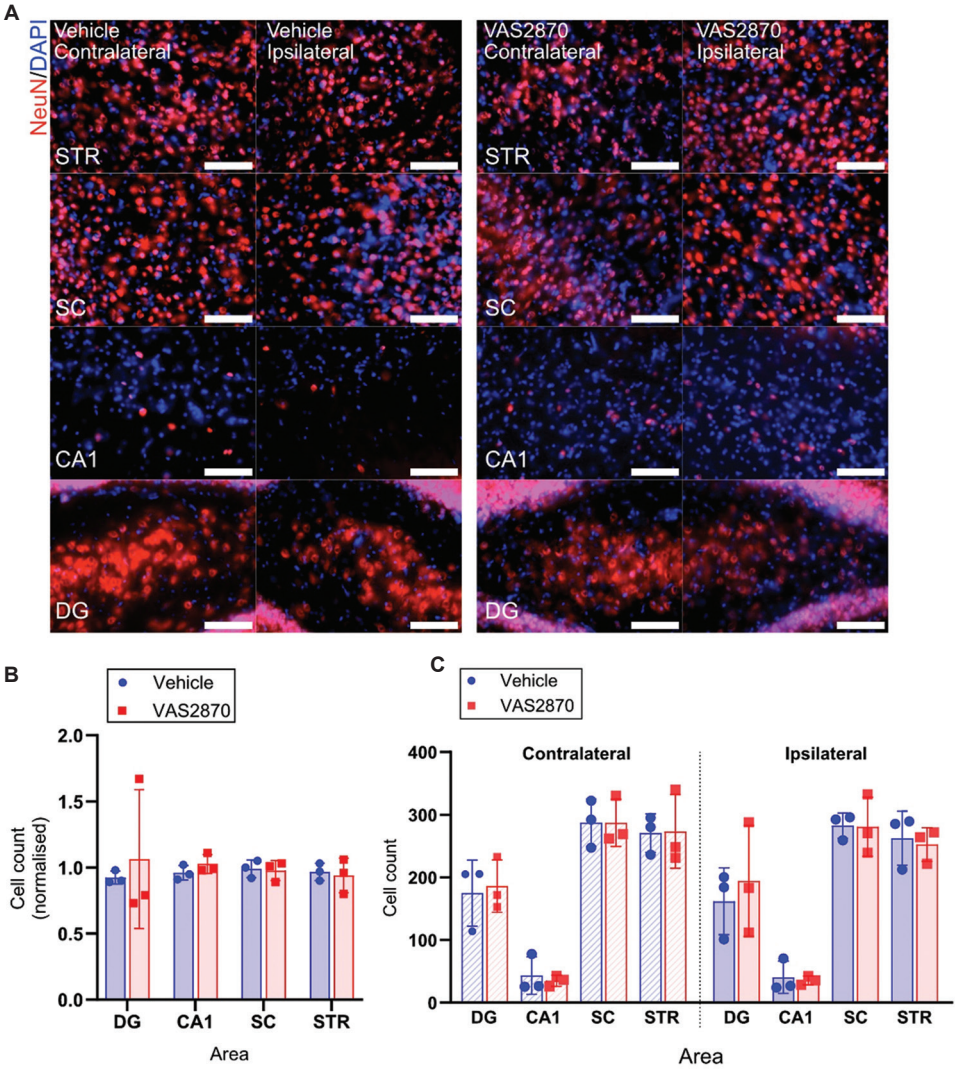

Figure 5. Region-specific total neuron density is not affected by NADPH oxidase inhibition at day 11 post-MCAO. (A) Representative immunofluorescent staining images, including neuron marker NeuN (red) and cell marker DAPI (blue). Panels show vehicle and treated brain region examples across both contralateral and ipsilateral hemispheres. Scale bar: 100 µM; magnification: 40×. (B) Ipsilateral hemisphere region-specific neuronal counts, expressed as a proportion of contralateral hemisphere (two-way repeated measures analysis of variance with Sidak's multiple comparisons test within regions between treatment groups). (C) Raw region-specific neuronal cell counts within both contralateral and ipsilateral hemispheres (Two-way two-factor repeated measures analysis of variance within hemisphere, with Sidak's multiple comparisons test across treatment groups within regions; solid bars=ipsilateral hemisphere, striped bars=contralateral hemisphere). All data expressed as mean ± standard deviation; *n*: vehicle = 3, VAS2870 = 3.
Abbreviations: CA1: Hippocampal CA1 region; DAPI: 4',6-diamidino-2-phenylindole nuclear DNA stain; DG: Dentate gyrus hilus; MCAO: Middle cerebral artery occlusion; NADPH: Nicotinamide adenine dinucleotide phosphate; NeuN: Neuronal nuclei; SC: Sensorimotor cortex; STR: Striatum.

contralateral (F [2.17,8.7] = 49.8, p<0.001) and ipsilateral hemispheres (F [1.58,6.31] = 42, p<0.001). However, *post hoc* analysis revealed no significant differences between groups within hemispheric regions (Figure 5C) and within treatment group and region between contralateral and ipsilateral hemispheres (Figure 5C).

To assess the impact of post-ischaemic NADPH oxidase inhibition on acute phase neurogenesis, BrdU was administered at 48 h post-MCAO, and the DG, including the granule cell layer and subgranular zone, was analysed at day 11 post-MCAO. Representative NeuN$^+$/BrdU$^+$ immunofluorescence images across contralateral and ipsilateral DG areas are shown in Figure 6A. Total BrdU$^+$ cell counts (a measure of cell proliferation) revealed no significant interaction between hemisphere and treatment (F [1,8] = 1.14, p=0.32), nor any variation between treatment groups (F [1,8 = 0.214, p=0.66) or between hemispheres (F [1,8] = 1.16, p=0.31). *Post hoc* analysis confirmed no significant differences between groups

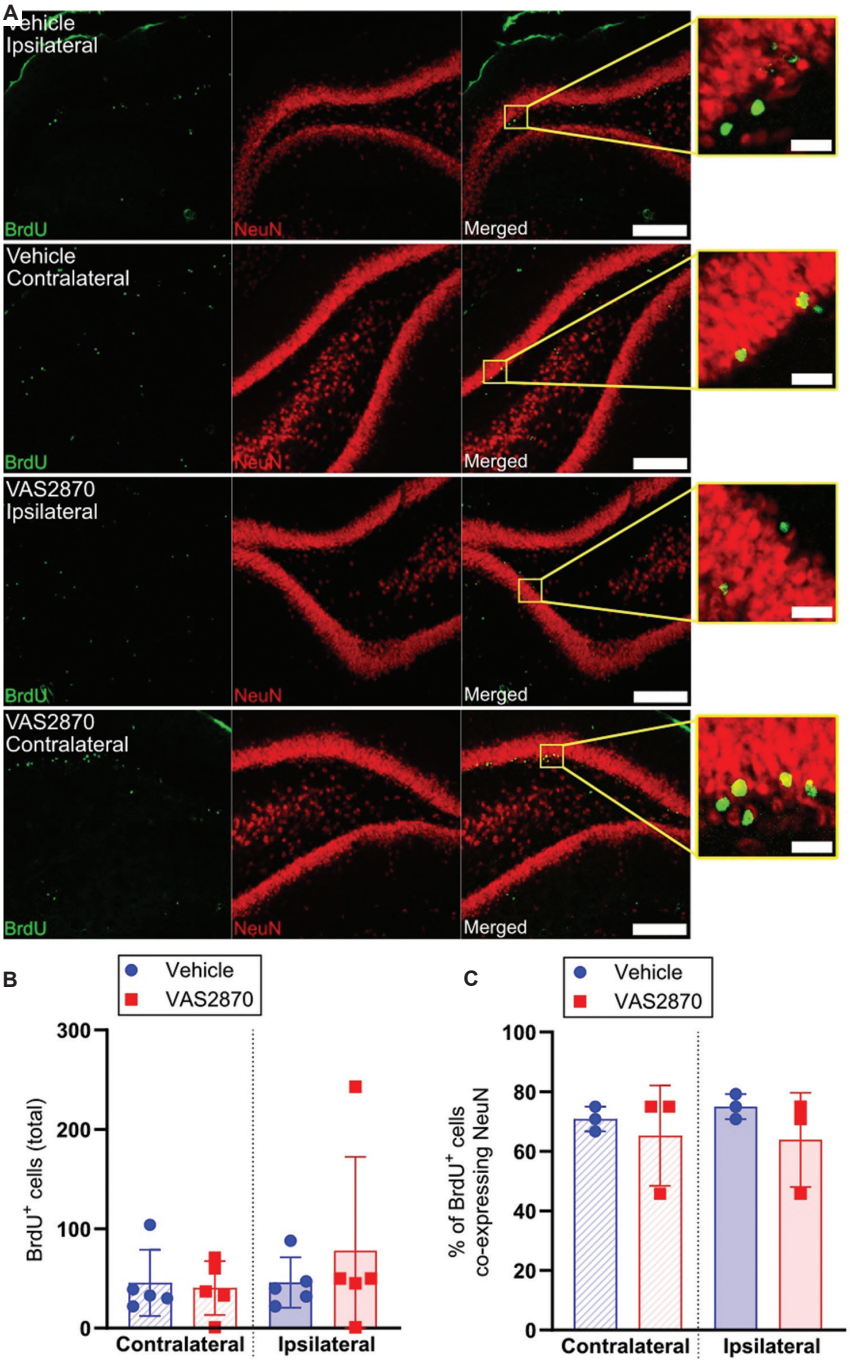

Figure 6. Immunofluorescent (IF) staining indicates that NADPH oxidase inhibition post-MCAO does not affect cell proliferation and neurogenesis within the dentate gyrus (DG), as marked at 48 h post-MCAO with the cell proliferation marker BrdU. (A) Representative IF images with cell proliferation marker BrdU (green) and neuron marker NeuN (red) staining. Panels show vehicle- and VAS2870-treated examples within both the ipsilateral and contralateral hemispheres. Zoom panels: yellow indicates co-expression of BrdU and NeuN. Scale bar: panel images: 200 μM, zoom images: 25 μM; magnification: 10×. (B) Total number of BrdU-expressing cells within the DG of both the ipsilateral and contralateral hemispheres. Two-way repeated measures analysis of variance with Sidak's multiple comparisons test, between treatment groups within hemisphere and within treatment group between hemispheres (*n*: vehicle = 5, VAS2870 = 5). Magnification: 10×. (C) Neurogenesis expressed as a percentage of cells co-expressing BrdU and NeuN, as a percentage of total BrdU-expressing cells (all proliferated cells), within the DG on both the ipsilateral and contralateral hemispheres. Magnification: 63×. Two-way repeated measures analysis of variance with Sidak's multiple comparisons test, between treatment group within hemisphere and within treatment group between hemispheres (*n*: vehicle = 3, VAS2870 = 3). All data are expressed as mean ± standard deviation.

Abbreviations: BrdU: 5-bromo-2'-deoxyuridine; MCAO: Middle cerebral artery occlusion; NADPH: Nicotinamide adenine dinucleotide phosphate; NeuN: Neuronal nuclei.

within the hemispheres nor between the hemispheres within treatment groups (Figure 6B). Similarly, the percentage of $NeuN^+/BrdU^+$ co-expressing cells (an assessment of acute phase neurogenesis) revealed no significant interaction between hemisphere and treatment (F [1,4] = 1.60, p=0.27), nor any variation between treatment groups (F [1,4] = 0.776, p=0.43) or between hemispheres (F [1,4] = 0.396, p=0.56). *Post hoc* analysis confirmed no significant differences between groups within the hemispheres nor between the hemispheres within treatment groups (Figure 6C).

3.5. Effects of post-MCAO NADPH oxidase inhibition on angiogenesis

Angiogenesis, measured as comparative post-MCAO sub-acute vascular density, was assessed using CD31 expression immunofluorescence staining at day 11 post-MCAO. Representative images showing CD31 staining within contralateral and ipsilateral CTX and STR regions for both vehicle- and VAS2870-treated groups are shown in Figure 7A. Ipsilateral CD31 immunofluorescence, when normalised to the contralateral hemisphere, showed no significant overall effect of treatment (vehicle vs. VAS2870; F [1,8] = 4, p=0.08), nor significant interaction between treatment and brain region (F [1,8] = 0.003, p=0.96; Figure 7B). *Post hoc* analysis of vehicle- versus VAS2870-treated groups revealed no significant difference in immunofluorescence values within CTX or STR at day 11 post-MCAO (Figure 7B). CD31 immunofluorescence within hemispheres revealed a significant overall interaction between region and treatment within the contralateral hemisphere (F [1,8] = 7.00, p=0.03), but not in the ipsilateral hemisphere (F [1,8] = 2.89, p=0.13; Figure 7C). No overall effect of treatment was observed within either hemisphere (contralateral: F [1,8] = 0.338, p=0.58; ipsilateral: F [1,8] = 0.506, p=0.506; Figure 7C). Significant differences between region variation were only observed within the contralateral hemisphere (contralateral: F [1,8] = 10.9, p=0.01; ipsilateral: F [1,8] = 0.593, p=0.46; Figure 7C). *Post hoc* analysis revealed a significant difference in CD31 expression between CTX and STR regions within the contralateral hemisphere of the vehicle-treated group (contralateral: p=0.006; ipsilateral: p=0.88). No differences were seen between regions in the VAS2870-treated group (contralateral: p=0.88; ipsilateral: p=0.78; Figure 7C). No within-region difference was observed when comparing vehicle- to VAS2870-treated groups within the contralateral (CTX: p=0.09; STR: p=0.98) or ipsilateral hemispheres (CTX: p=0.97; STR: p=0.47; Figure 7C). Additional *post hoc* analysis revealed no significant differences within treatment groups and regions between contralateral and ipsilateral hemispheres (Figure 7C).

4. Discussion

The present study investigated the sustained impact of acute pharmacological inhibition of NADPH oxidase using VAS2870 following experimentally induced stroke. Within the first 48 h following post-stroke VAS2870 administration, we demonstrated an improvement in survival probability. However, this survival benefit was paradoxically accompanied by a transient worsening of neurological function at day 2 post-stroke. Furthermore, despite improved survival, VAS2870 treatment did not yield significant improvements in other outcomes assessed at later time points. Specifically, we observed no significant reduction in infarct volume attributable to VAS2870 treatment at either 48 h or 11 days post-MCAO compared to the vehicle-treated group. Similarly, functional recovery and cognitive function showed no significant benefit of VAS2870 treatment at days 10 and 11. Furthermore, analyses of circulating biomarkers, neurogenesis, and vascular density at day 11 did not demonstrate any benefit of VAS2870 treatment.

The enhanced survival following VAS2870 treatment during the early post-stroke recovery phase has therapeutic benefit; however, the mechanisms underlying this improved survival are unclear. Considering the role of OS in early oedema and BBB breakdown,[6,15] it is plausible that VAS2870 attenuates these processes, particularly as cytotoxic oedema formation is a known contributor to early mortality.[15] Evidence suggests that intravenous administration of VAS2870, given before reperfusion at 5 h post-MCAO, resulted in reduced BBB permeability, NOX2/4 expression, AQP4 expression and oedema at 24 h following ischaemia,[49] supporting the potential that enhanced survival may be related to an attenuation of these processes. Alternatively, VAS2870 may help mitigate early secondary injury events, such as haemorrhagic transformation or acute systemic inflammatory responses. However, this survival benefit did not correspond to improved demonstrable neuroprotection or functional recovery with VAS2870 treatment. Functional outcome worsened during the acute recovery phase following VAS2870 treatment, although it did not cause weight loss, a measure of general well-being. It is possible that the VAS2870 dose or administration time point, while effective in improving acute survival, was insufficient to provide substantial protection against cell death or to promote functional repair pathways. The pathways influencing early survival may be distinct from those determining tissue salvage and long-term recovery in this model. It is possible that post-stroke VAS2870 administration, while sufficient to impact acute mortality, was too late to salvage significant penumbral tissue compared to interventions initiated

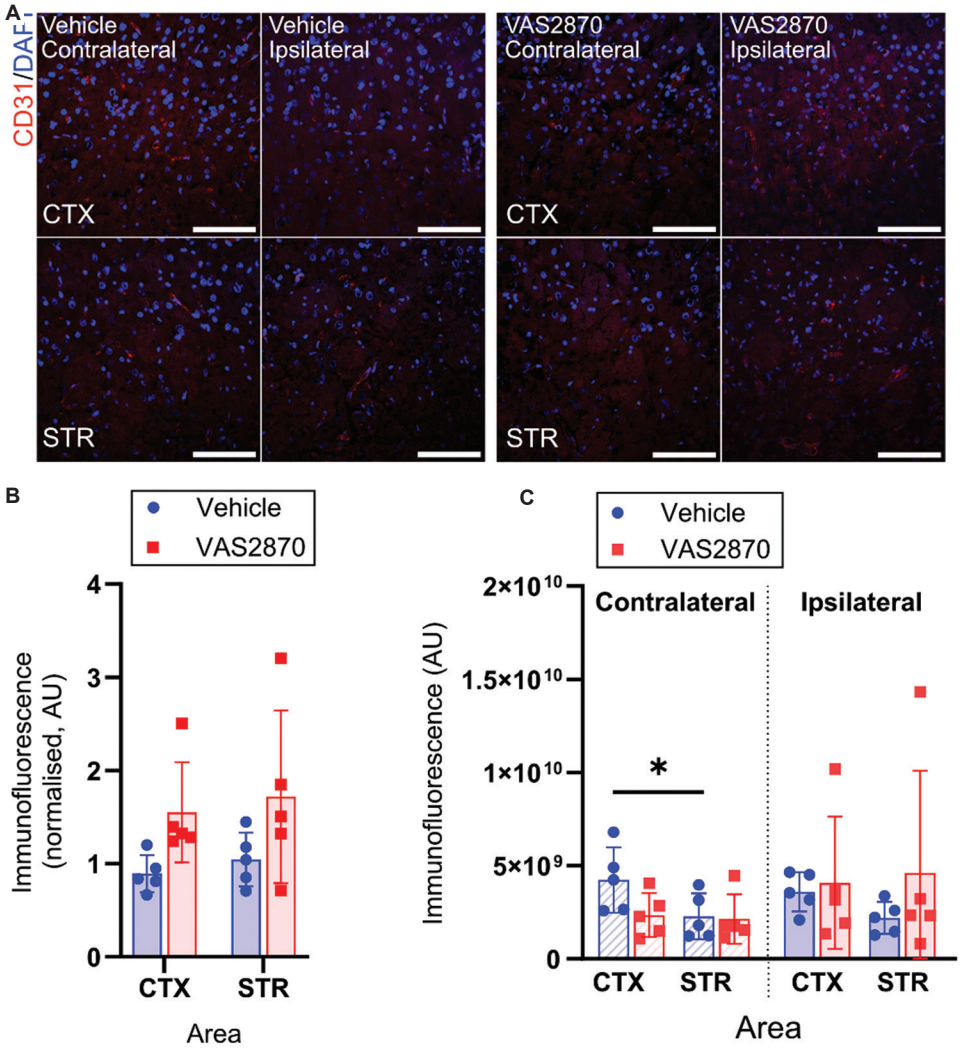

Figure 7. CD31 expression within the cortex (CTX) and striatum (STR), as measured by immunofluorescence (IF) reading, is not significantly affected by NADPH oxidase inhibition at day 11 post-MCAO. (A) Representative IF images depicting endothelial cell marker CD31 (red) and cell marker DAPI (blue). Images show vehicle- and VAS2870-treated examples across both contralateral and ipsilateral hemispheres. Scale bar: 100 μM; magnification: 40×. (B) Ipsilateral hemisphere CTX and STR CD31 expression, expressed as a proportion of the contralateral hemisphere (two-way repeated measures analysis of variance with Sidak's multiple comparisons, within regions between treatment groups). (C) CTX and STR region-specific CD31 expression levels across both contralateral and ipsilateral hemispheres (two-way two-factor repeated measures analysis of variance within hemisphere, with Sidak's multiple comparisons test across treatment groups within regions). All data expressed as mean ± standard deviation; *n*: vehicle = 5, VAS2870 = 5.
Abbreviations: AU: Arbitrary units; DAPI: 4',6-diamidino-2-phenylindole nuclear DNA stain; MCAO: Middle cerebral artery occlusion; NADPH: Nicotinamide adenine dinucleotide phosphate.

before reperfusion.[48] Further investigation incorporating BBB permeability analysis and oedema assessment during the acute phase is needed to explore the role of VAS2870 treatment during this acute post-reperfusion time point and its potential role in the improved survival reported here. Furthermore, the particularly higher level of mortality within the vehicle-treated group likely introduced a degree of survival bias, complicating direct comparisons between groups at sub-acute time points and skewing the surviving vehicle-treated group toward animals with milder initial strokes, potentially masking treatment effects.

Furthermore, neurological function assessment revealed complex results. While functional deficit scores did not differ significantly at a later time point (10 days post-MCAO), the VAS2870-treated group exhibited a significant worsening of neurological function at day 2. This counterintuitive finding, occurring alongside improved survival, warrants careful consideration. One possible explanation is that VAS2870 treatment enabled the acute survival of animals with more severe initial neurological deficits, typically a predictor of higher mortality.[61] Early mortality in the vehicle group, removing

the most severely affected animals, could explain the higher average mNSS score on day 2 in vehicle-treated survivors compared to VAS2870 survivors, where potentially more severely affected animals survived. Alternatively, this transient worsening could suggest complex acute pharmacological/off-target effects of NADPH oxidase inhibition immediately post-stroke, such as thiol alkylation at the ryanodine receptor-Ca^{2+} channel,[62] replication of some ROS effects on redox status, or inhibition of basal mitochondrial respiration and cytotoxicity.[63] In addition, the concentration-dependent role of ROS in excitatory pathway neuromodulation, including calcium modulation, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor transport, and long-term potentiation,[64-66] must be considered. Disentangling these possibilities requires further investigation. Systemic VAS2870 treatment side effects and impacts beyond NADPH oxidase inhibition, such as reduced platelet aggregation through NADPH-independent pathways blocking downstream protein kinase-C signalling,[67] and effects on cell proliferation and immune regulation,[68,69] necessitate safety evaluations and broader physiological impact assessment. Considering the complexity of NADPH oxidase roles,[15] including potential involvement in both detrimental and physiological signalling,[70,71] broad inhibition may counteract overall functional recovery.[62,63,72] Comparing with other neuroprotection studies[32,48] requires consideration of these mechanistic complexities and methodological differences in administration, timing, and how early functional scores relate to survival in those specific paradigms. The mNSS used here while assessing a range of sensorimotor functions, including postural abnormalities, forelimb strength, locomotor activity, hemi-neglect and sensory capabilities,[73] is a composite and subjective measure that does not provide behaviour-specific information, potentially masking deficits. Furthermore, spontaneous sensorimotor function recovery following unilateral brain injury[74-76] further impacts test accuracy. Neurological function post-MCAO positively correlates with infarct volume[77] and is affected by damage location. MCAO infarct volume can be highly variable, with tissue damage ranging from predominantly striatal to expansive infarcts encompassing much of the cortex,[78,79] leading to diverse outcomes in various behaviours, including locomotor activity, skilled motor control, sensorimotor processing, and motivation/reward-seeking behaviours.[80-83] This variability, combined with potential survival bias, incomplete 48-h MRI data, and reduced group sizes from differential mortality, limits definitive conclusions on the relationship between infarct volume/location and functional outcome, requiring more extensive assessment.

The present study also indicates that sub-acute (11 days post-MCAO) cognitive function, assessed using a Y-maze measure of working memory,[84] was not improved following VAS2870 treatment. Evidence suggests that NADPH oxidase-mediated ROS production in chronic and acute cerebral ischaemia/hypoperfusion results in cognitive impairment;[85-87] however, there is very little evidence exploring the effect of NADPH oxidase inhibition on cerebral ischaemic damage on induced cognitive function at sub-acute time points. Although one study demonstrated cognitive function recovery with NOX2 inhibition at a sub-acute time point using a Rac-1 guanosine triphosphatase inhibitor,[85] methodological differences exist, including pre-treatment versus our post-reperfusion administration and global versus focal ischaemia models, potentially explaining the differences in findings. Sub-acute cognitive effects of post-stroke NADPH oxidase inhibition remain sparsely investigated.

Furthermore, VAS2870 administration revealed no significant impact on neurogenesis and vascular density markers at day 11 post-stroke. This aligns with the proposed dual role of NADPH oxidase-derived ROS in both injury and repair processes,[32,44,50,51,88] with concentration-dependent effects on endothelial proliferation and migration,[88,89] as well as modulation of hippocampal plasticity and neurogenesis.[90,91] One potential explanation is that ROS concentrations achieved here were potentially below the threshold required to significantly alter these processes within the timeframe studied, or that the effects of inhibition were too subtle to detect using the methods utilised in this study. Similarly, the absence of significant changes in circulating cytokines or antioxidant capacity at day 11 suggests limited lasting impact from this acute intervention on these systemic antioxidant and inflammatory measures. This could indicate primarily central nervous system localised or transient effects of acute NADPH oxidase inhibition on these pathways.

Collectively, these results underscore the inherent challenge of achieving broad therapeutic success by targeting a single pathway within the complex pathophysiology of stroke. The limited efficacy of VAS2870 beyond acute survival likely reflects the multifactorial nature of ischaemic injury, driven by intricate interactions between OS, inflammation and neurodegeneration.[13,14,70,71] The documented interplay between inflammation and NADPH oxidase activity, where inflammation can drive NADPH oxidase activity and NADPH oxidase-derived ROS can perpetuate inflammation,[70,71] provides a strong rationale for why OS inhibition alone may prove insufficient. The therapeutic modulation of NADPH oxidase is further complicated by potential complex or even counter-regulatory effects.[50]

Moreover, when extrapolating these findings clinically, the prevalence of comorbidities, particularly diabetes and hyperglycaemia, in patients must be considered. Approximately 30% of ischaemic stroke patients have diabetes,[92] and an additional 40% of stroke patients develop stress hyperglycaemia during hospitalization.[93,94] These conditions profoundly affect vascular function and are known to modulate NADPH oxidase activity and OS burden.[95,96] This may likely impact responsiveness to NADPH oxidase-targeted therapies and highlight the importance of including comorbid groups in preclinical studies. Thus, a key limitation of the present study is our use of healthy, normoglycaemic animals, restricting direct translatability. The multifaceted nature of stroke and heterogeneity in patient populations, including comorbidities, is a recognised challenge in the development of effective, clinically translatable stroke therapeutics, highlighted by the Stroke Therapy Academic Industry Roundtable criteria.[97] Future preclinical studies incorporating comorbid models are crucial to better understand NADPH oxidase's role in clinically relevant contexts. Reinforcing the idea that combination therapies targeting multiple pathways, including anti-diabetic and anti-inflammatory treatments, may be essential for effective clinical translation.

Several limitations, beyond those highlighted above, should be acknowledged. This study utilised a single VAS2870 dose, administration route and time point, restricting dynamic understanding of VAS2870 action, necessitating explorations of a variety of doses, time points, and routes, alongside pharmacokinetics, for a full understanding of its effectiveness and applicability for ischaemic stroke. Considering the role vascular NADPH oxidase isoforms contribute to vascular tone through ROS production,[24,25] understanding VAS2870's effect on vascular tone through focal CBF analysis would be beneficial to understanding its mechanism of action at endothelial NADPH oxidase; however, only confirmatory filament placement CBF readings were taken. Consequently, a more comprehensive exploration of post-reperfusion cerebrovascular dynamics, through laser Doppler flowmetry or laser speckle contrast imaging, would provide valuable insights into VAS2870's impact on vascular reactivity and overall reperfusion. Furthermore, within this study, direct measurements of tissue-level OS, including ROS levels, BBB permeability and oedema measures, were not performed. Addressing these would provide valuable mechanistic insights. Confirmatory analysis of acute ROS levels and acute oxidative damage markers, such as lipid peroxidation, would validate VAS2870's action against NADPH oxidase. In addition, the lack of region-specific analysis for infarct damage and tissue-level OS markers are a technical limitation, restricting exploration of specific localised functional outcomes and cellular effects. Furthermore, using VAS2870 to modulate both NOX2 and NOX4 prevented assessment of isoform-specific contributions. In addition, despite *in vitro* evidence supporting NADPH oxidase inhibition,[45-47] *in vivo* pharmacokinetics remain unconfirmed. Specifically, cerebrovascular and brain tissue concentrations are not well-established, considering BBB impermeability and post-ischaemic BBB biphasic opening.[98-100] We assumed VAS2870 reached the cerebrovasculature, and therefore vascular NADPH oxidase, through intravenous administration due to high bioavailability; however, this was not verified, nor was brain tissue penetration tested. These unconfirmed aspects highlight a need for future research to quantify VAS2870's brain penetration and local concentration. Furthermore, MRI technical difficulties limited accurate 48-h infarct localisation across the groups, preventing direct correlation with delayed tissue analysis and hindering assessment of within-infarct post-ischaemia changes. Consequently, our analysis focused on established functional regions, the SC,[101,102] and areas highly susceptible to ischaemic injury after MCAO, including the STR and hippocampus CA1 region.[103,104] Neurogenesis was assessed solely in the DG due to its inherent lifelong neurogenic capacity.[105,106] This is a significant limitation, and future analysis within the damaged brain region is needed for a more precise assessment of therapeutic VAS2870 efficacy by determining neurogenic and inflammatory biomarkers.

A further study limitation is that cognitive assessment was restricted to only spatial working memory at a sub-acute time point using the Y-maze. The Y-maze tests short-term spatial memory[107] and relies on the natural exploratory drive; animals can rapidly habituate, making it unsuitable for repeated longitudinal testing. It can also be influenced by environmental sensitivity, affecting the reliability and reproducibility of outcome measures. Given the Y-maze's reliance on varied intrinsic curiosity, with no use of positive or negative stimuli, training, or visual cues, and the limitations of the mNSS functional deficit score discussed earlier, future studies evaluating post-stroke VAS2870 treatment should more thoroughly assess its impact on cognitive and functional deficits. This necessitates incorporating a wider array of sensitive tests performed at varied and longer-term time points. For instance, the radial arm maze[108] has reported deficits up to 65 days post-MCAO.[109] The adhesive removal test[80,110] more sensitively assesses long-term sensorimotor deficits following mild injury,[111] and the foot fault test,[110] together, could provide a deeper understanding of cognitive, sensory, and motor impairments. As highlighted, the study was conducted in healthy young adult male rats, lacking clinically

relevant factors, such as advanced age, sex differences and comorbidities, affecting translatability. Considering the multifaceted nature of brain ischaemia, this study utilised a monotherapy approach; however, exploration of combined VAS2870 treatment with clinically relevant ischaemic stroke treatments (e.g., tissue plasminogen activator) would strengthen clinical relevance. In addition, high infarct volume variability within the MCAO model significantly reduces statistical power, resulting in large animal cohorts to detect meaningful differences between treatment groups.[112] Given the limited data on VAS2870's post-stroke safety and efficacy, in addition to animal use ethical considerations, the resource equation was used to calculate initial sample sizes. This approach allowed for the efficient use of resources, considering the uncertain nature of the success and potential adverse effects of this novel treatment paradigm. Consequently, the absence of a priori power analysis is a limitation. Furthermore, high vehicle-treated mortality led to reduced and uneven groups during later analyses. This differential mortality reduced sample sizes and statistical power, increasing the risk of type II error and limiting confidence for sub-acute analyses, such as cellular and circulatory marker levels, due to survivor bias. The reduced 48-h infarct analysis number, due to MRI technical difficulties and uneven sampling, may also indicate sampling bias and must be considered. It should also be noted that animals reaching humane endpoints, informed by the Ischaemia Models: procedural refinements of *in vivo* Experiments guidelines,[113] were included as events within survival analyses. These animals, due to severe stroke impact, even with extensive supportive care guided by the IMPROVE guidelines,[113] had a low probability of survival and, therefore, based on this survival assumption, were included in the survival analysis. While this captures overall severity, it may influence the interpretation of outcomes. Finally, expanding outcome assessments to include additional acute time points, a broader cytokine panel, direct region-specific tissue analyses (including hemispheric differences) and a wider variety of functional assessments would strengthen future investigations.

In summary, this study demonstrates that acute pharmacological NADPH oxidase inhibition utilising NOX2/4 inhibitor VAS2870 significantly enhances early survival following experimental stroke in rats. However, this survival benefit was not accompanied by reductions in cerebral damage or improvements in cognitive or functional recovery within the time points assessed. These findings suggest that while targeting NADPH oxidase-derived OS with this specific approach impacts survival pathways, it may not be highly effective as a monotherapy. These findings highlight the challenge of translating single-pathway interventions, such as OS inhibition, into broad neuroprotective efficacy, likely due to the inherently multifactorial pathophysiology of stroke associated with inflammation, neurodegeneration, alongside OS and the modulating influence of factors, such as clinical comorbidities. This study highlights these challenges and underscores the ongoing effort to elucidate and understand the complex roles of NADPH oxidase enzymes in stroke, revealing that while interventions targeting them can impact critical survival pathways, achieving comprehensive therapeutic success may require more nuanced or combined therapies.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

This study was conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986 (Project Licence No. PP9645035) and following ethical approval by the University of Nottingham Animal Welfare Ethical Review Body, reported in line with Animal Research Reporting of *in vivo* Experiments (ARRIVE) guidelines.

Consent for publication

Not applicable.

Availability of data

Data will be made available upon request to the corresponding author.

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Appendix

Table A1. Glossary of antibodies used in immunofluorescence

Immunohistochemistry staining combination	Endogenous block antibody (2-h room temperature incubation)	Primary antibody (4°C overnight incubation)	Secondary antibody (2-h room temperature incubation)	Fluorescent probe
NeuN and DAPI	-	Rabbit anti-NeuN (1:500; AB177487, Abcam, UK)	Goat anti-rabbit IgG AlexaFluor 546 (1:500; A1101, Invitrogen, UK)	DAPI 10 mg/mL (1:1000; D3571, Invitrogen, UK)
NeuN and BrdU	Goat anti-rabbit IgG (heavy and light chains) (1:300; 15450834, Fisher, UK)	Rat anti-BrdU (BU1/75 [ICR[1]) (1:200; AB6326, Abcam, UK); Rabbit anti-NeuN (1:500; AB177487, Abcam, UK)	Goat anti-rabbit IgG AlexaFluor 546 (1:500; A1101, Invitrogen, UK); Goat anti-rat IgG AlexaFluor 488 (1:400; AB150157, Abcam, UK)	-
CD31	-	Rabbit anti-CD31 (1:200: AB222783; Abcam, UK)	Goat anti-rabbit IgG AlexaFluor 546 (1:800; A1101, Invitrogen, UK)	DAPI 10 mg/mL (1:1000; D3571, Invitrogen, UK)

Abbreviations: BrdU: 5-bromo-2'-deoxyuridine; DAPI: 4',6-diamidino-2-phenylindole nuclear DNA stain; IgG: Immunoglobulin G; NeuN: Neuronal nuclei; UK: United Kingdom.

28-point rat neurological scoring						
	0	1	2	3	4	5
Paw placement (max 4)	0 paw placements	1 paw placement	2 paw placements	3 paw placements	4 paw placements	N/A
Response to vibrissae touch	no response on either side	no response on affected side	weak response on affected side	symmetrical response	N/A	N/A
Horizontal bar	if animal falls	in animal hangs from the bar	if one hindlimb raised onto bar	if both hindlimbs raised onto bar	N/A	N/A
Visual forepaw reaching (max 2)	0 paw placements	1 paw placement	2 paw placements	N/A	N/A	N/A
Circling	spinning	small circles (>15 cm radius)	medium circles (15–50 cm radius)	large circles (>50 cm radius)	tends to one side	no circling
Grip strength	no grip	weak strength	good but weakened strength	normal strength	N/A	N/A
Mobility	no movement	unsteady	lively (moves around but no walls)	very active (up to 3 walls)	normal mobility (4 walls)	N/A
General condition	poor (weight loss & secretions)	fair (weight loss)	good (maintaining weight)	very good (gaining weight)	normal (back to pre-stroke weight)	N/A

Figure A1. A 28-point modified neurological severity score, assessing motor and sensory deficits. Components include paw placements (number of paw retractions from edge; motor coordination), vibrissae touch (stimulated investigative turning; sensory response), horizontal bar (forepaw pull-up; forelimb strength), visual paw reaching (forepaw extension induced by whisker stimulation; sensorimotor integration), circling (open field observation; motor asymmetry), grip strength (45-degree wire grid hang and removal; forelimb strength), and general mobility (cage exploration and climbing ability; motor activity).

ACCSCIENCE PUBLISHING

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SPECIAL ISSUE ARTICLE

COVID-19 impact on school-based dental sealant programs: De-implementation, re-implementation, challenges, and adaptations

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Abstract

Background: The COVID-19 crisis significantly affected school-based dental sealant programs (SBDSPs). Nationwide mitigation efforts, including school closures, led to the de-implementation of SBDSPs by default. **Aims:** We examined how COVID-19 crisis management planning by SBDSPs, or lack thereof, influenced: (i) de-implementation-related adaptations, (ii) re-implementation processes, (iii) workforce capacity required for re-implementation, (iv) the role of organizational resources in early re-implementation, and (v) overall school reach. **Methods:** We conducted an embedded multiple case study using a stratified random sample of organizations delivering SBDSPs in Oregon. Semi-structured interviews were conducted with program personnel (n = 10) from the six organizations. We performed quantitative (e.g., counts and percentages) and qualitative (i.e., directed content analysis, within, and across case study analysis) analyses to identify crisis management efforts, de-implementation adaptations, re-implementation timelines, and related challenges. **Results:** A universal absence of proactive crisis management during SBDSP de-implementation was observed and resulted in challenges for re-implementation. SBDSPs initiated different adaptations (e.g., mobile dental vans) to reach their targeted population. Re-implementation timeline varied (i.e., partial, intermediate, and full) and followed different rates (i.e., full rapid, full gradual, and intermediate slow). Challenges with workforce capacity, organizational resources, program policies, schools' response, and inter-organizational communication influenced re-implementation. Re-implementation occurred more rapidly for SBDSPs that: (i) formed "crisis management teams" and quickly rebuilt their workforce and (ii) operated within well-sourced organizations that retained staff during de-implementation. However, school responses and COVID-19-related policies often created complex approval systems that limited re-implementation and overall school reach. Results suggested that COVID-19 crisis management planning largely overlooked dental public health programs (i.e., SBDSPs). **Conclusion:** Public health and school-related organizations must develop proactive crisis management plans that support the continuity of dental public health programs during the crises. **Relevance for patients:** School reach is foundational to SBDSPs' implementation processes. Addressing barriers to re-implementation during crises is essential to ensure continued dental care access for the target population.

Keywords: School-based dental sealant programs; COVID-19 crisis; De-implementation; Re-implementation; Dental public health; Implementation research

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1. Introduction

Dental caries, especially in permanent teeth, is the most common preventable chronic condition among 6–19-year-old children and adolescents worldwide.[1,2] The rates of untreated pediatric dental caries (PDC) in the United States (>50%) and Oregon (49%) are relatively high. The prevalence of PDC among children aged 6–9 years in certain Oregon counties has reached as high as 68%.[3-5] Dental sealants, protective material applied to the occlusal surfaces of permanent molars, are clinically proven to prevent dental caries.[6,7] Community-based programs such as school-based dental programs and community water fluoridation are evidence-based interventions effective in reducing PDC.[5,8] However, only 26.3% of individuals in Oregon receive fluoridated water from a public utility, and overall, Oregon ranks 49th out of 50 states in providing fluoridated water.[9]

To address this gap, the Oregon Health Authority's (OHA) Oral Health Unit began disseminating and implementing statewide school-based dental sealant programs (SBDSPs) in 2006.[10] By 2020, 20 organizations, including OHA, dental care organizations, federally qualified health centers (FHQCs), non-profit organizations, and privately owned for-profit organizations, adopted and implemented SBDSPs in 778 schools in all 36 counties.[10] These programs are evidence-based, cost-effective, and effective in reducing dental caries among elementary and middle school children, especially in high-risk schools (schools with 30 – 50% free or reduced lunch student enrollment).[4,7,8,11-14] However, multiple factors such as dental sealant retention, program design, funding, policies, and implementation factors (e.g., school reach, workforce capacity, and inter/intra-organizational communications) may influence SBDSP's effectiveness.[15,16]

Prior to the COVID-19 pandemic, we examined that how SBDSPs were implemented and identified factors influencing school reach.[15] We outlined a multi-stage implementation process by which the programs reached eligible participants: (i) school recruitment, (ii) client recruitment, and (iii) client attendance. School recruitment, also known as school reach, is the first and most critical step; without access to schools, SBDSPs face a significant challenge in implementing their services.[15]

The COVID-19 pandemic, a global-scale public health crisis that significantly impacted health and mortality and overwhelmed health services and resources,[17] severely disrupted this process. From March 2020 to March 2021, federal and state mitigation strategies led to school closures, including in Oregon,[18] which halted all SBDSP services or modified them to offer limited, contact-free services in compliance with COVID-19 mitigation guidelines. Even after schools reopened, concerns about COVID-19 infection and decentralized decision-making regarding the resumption of school-based programs created further challenges for re-implementation.[19,20]

Although we use the term *de-implementation* in this study, many of the interruptions to SBDSP services more accurately reflect program disruption, *abandonment, or interruption* the unplanned cessation of services due to external factors such as workforce issues, funding, or, in this case, the COVID-19 pandemic.[21-23] In contrast, *de-implementation* refers to the intentional, evidence-informed reduction or discontinuation of an intervention.[24,25] While these concepts are theoretically distinct, they often overlap in practice, particularly when the timeline for re-implementing services is unclear or indefinite. Importantly, the current literature does not define a specific time threshold separating program abandonment or interruption from de-implementation. Rather, the distinction lies in the nature and intent of the discontinuation. Moreover, several studies use these terms interchangeably, reflecting the lack of consensus in the field.[24,26] Given the absence of consensus, we adopt a pragmatic definition tailored to our study's context: De-implementation is defined as a total cessation of all program services due to external factors, followed by re-implementation efforts with unknown timelines. *Re-implementation* refers to reintroducing programs in the same settings after disruption or cessation, with adaptations to either the programs themselves or their implementation processes.[23]

Under normal circumstances, the implementation of evidence-based interventions is influenced by a wide range of factors (e.g., organizational, programmatic, personnel, and available resources).[27-31] The current study examined crisis-related organizational factors that fall under the general conceptual frameworks of crisis management and implementation science. In addition, we examined the workforce capacity and available resources to support the re-implementation of programs at the school level following crisis-related closures.

Crisis management planning provides a structured approach to navigating and organizing crisis response while incorporating feedback mechanisms (e.g., formative evaluation) to address the implementation challenges.[27,32-37] Program planning is essential for implementation under the normal conditions.[35,38-44] However, the focus shifts to immediate threat mitigating during a crisis, often overlooking adjacent public health programs. By adjacent programs, we mean programs, such as SBDSPs and other non-critical health services that might be temporarily closed or reduced in scope to prioritize crisis management

goals such as disease transmission reduction (Dr. Ashley Schuyler, oral communication, January 5, 2023). Because neither the field of crisis management nor implementation science explicitly incorporates adjacent programs in their planning processes, we expected programs such as SBDSPs to lack formal crisis management plans and instead respond reactively to unfolding events, which is an area of investigation that we examined.

Based on expert opinions and crisis management models, effective crisis management planning should include five key components: Key informants committees, planning processes, decision-making and actions, written guidance, and expert consultations.[27,33,36,37,39-41] For instance, involving school personnel, parents, and SBDSP staff in the planning process could help identify challenges and necessary adaptations before re-implementation.[39,44] In addition, strong program-to-school communication, critical under normal conditions,[15] remains essential for successful re-implementation, emphasizing the need for formal crisis protocols within and between SBDSPs and the school system.

Workforce capacity, including adequate staffing and training, is fundamental to the successful implementation of evidence-based interventions under normal conditions.[27,45-49] Conversely, insufficient staffing or high turnover negatively impact both organizational and intervention-level outcomes.[15,50,51] For example, high staff turnover rates have been associated with reduced program reach (e.g., school and client recruitment), effectiveness, and overall implementation quality.[15,48] These workforce capacity issues may be exacerbated during crises, and inadequate staffing can both hinder crisis response and result from poor crisis management planning. Therefore, our study emphasizes staff size, turnover, and workforce rebuilding as key factors in understanding the de-implementation and re-implementation of SBDSPs.

This research falls under the general rubric of implementation science and crisis management, both of which aim to support successful recovery from crisis and deliver evidence-based programs in the real-world settings, an area of study that has received limited attention in existing research. Specifically, the current study aimed to examine: (i) how SBDSPs were impacted by de-implementation and how they adapted, if at all, to school closures, (ii) how de-implementation affected SBDSPs' ability to maintain their pre-COVID workforce during school closures, (iii) how crisis management planning and organizational resources influenced SBDSPs' ability to maintain their pre-COVID workforce, and (iv) school re-engagement, re-implementation timelines, and factors that influenced the re-implementation processes of SBDSPs from the perspective of SBDSP administrators and providers.

2. Methods

2.1. Sample and study design

A list of all SBDSP organizations delivering SBDSPs in Oregon during the 2019–2020 school year was obtained (n = 20) from the OHA. OHA's SBDSP (n = 1) and participants in our pre-COVID-19 pandemic study (n = 5) were excluded from the sample frame. A stratified two-stage random list sampling technique was used to select the current study sample.[52] The sample frame of counties (n = 11) and the remaining organizations (n = 14) operating SBDSPs were stratified into small (n = 6) and large (n = 5) counties based on each county's population size. The sample was stratified by county to represent organizational differences (i.e., size and type). It is important to note that multiple organizations may provide services to different schools in the same county; thus, the number of organizations is not equal to the number of counties in the sample list. A first-stage random sample selection was obtained using a random assignment generator, after assigning a number to each county.[53] A second-stage random selection was conducted when more than one organization was operating in one randomly selected county. Eight organizations delivering SBDSPs were randomly selected and invited to participate in the study. Two organizations refused to participate, resulting in a final sample of n = 6 (three/stratum). This number of cases meets the general criteria (i.e., a minimum of five cases) for analytical generalizability within a multiple-case study design.[54,55]

We conducted a cross-sectional, embedded multiple-case study design with organizations delivering SBDSPs as the unit of analysis and personnel within each organization as embedded subunits.[54] This design is crucial for addressing the intricate complexities present in scenarios with more variables of interest than units available for the analysis.[56] It also allows us to collect the data from multiple sources (i.e., SBDSP personnel) and provides an in-depth examination of each participant's experience and perspective. However, it is essential to note that the data collected from SBDSP personnel were evaluated and interpreted to make conclusions about the organizations and not about the individuals within the organization.[54]

2.2. Procedures

Study procedures were approved by the Oregon State University's Institutional Review Board. Participating programs' directors, managers, and dental hygienists were contacted individually through E-mail and invited to

participate in the study. Initial contacts were made through the SDBSP directors within each organization to obtain basic information about the organization and program (e.g., organization type, SBDSP service area, and number of SBDSP personnel), identify program supervisor/manager (if different than director) and dental hygienists, and request permission to contact program personnel. SBDSP personnel were then contacted by telephone or email to explain the study, answer prospective participants' questions, and schedule interviews for those who agreed to participate. The participants were not reimbursed for their participation. We conducted semi-structured telephone interviews with each participant. All interviews were conducted in English, audio-recorded with participants' permission, lasted 1 – 3 h, and transcribed by the first author. All interviews were de-identified during the transcription process.

2.3. Interview measures

Interview protocols were developed by the authors and were informed by our pre-COVID-19 study, which was conducted to understand the implementation processes, challenges, and adaptations of SBDSPs under the normal conditions.[15] The interview protocols were developed to elicit responses about each organization's experience with SBDSPs during the COVID-19 crisis and were tailored to the participants' roles in the program. The interview obtained background data (e.g., demographics, training histories, and employment histories), as well as the type of program de-implementation (total disruption or suspension of all program services), related adaptations, and crisis management planning (i.e., establishing a key informant committee, planning processes, planning decisions and actions, written guidance, and expert consultants). It also covered re-implementation timelines (the time between school closure and re-implementation and the de-implementation phase) and processes, workforce capacity (i.e., staff size, turnover, and rebuilding), and organizational resources during the COVID-19 crisis and how these factors differ compared to before the onset of the crisis. Table 1 provides the examples of the content area assessed and corresponding interview questions.

Quantitative questions about the re-implementation processes, timelines, and number of schools and students served were also assessed. The following questions were asked to evaluate the program's experience during the COVID-19 crisis: "When did you stop delivering the program?" "After your program was shut down, when did you start contacting schools again?," "When did you reopen the program in the schools?," and "Since the schools reopened, how many schools have you served?." Specific questions about the number of schools served before the pandemic, and the number of schools each SBDSP was able to re-implement its services at were asked to measure each SBDSP's school reach. The full interview protocol is available from the first author.

2.4. Data management and analysis

All interviews were audio-recorded, transcribed, and compared to the original audio recordings for accuracy. The transcripts were reviewed for content, annotated, and de-identified. Data were organized by organization and personnel within an organization. Data analysis was conducted using directed (i.e., deductive) content analysis informed by our prior research.[15,54] Quantitative data analysis involved counts, sums, averages, percentages of workforce capacity, and de-implementation timelines in months. Qualitative data analysis involved initial case coding (i.e., structural, descriptive, categorical, and causal coding) to categorize and identify the causal inferences of major content areas (i.e., workforce capacity, crisis management planning, adaptations to de-implementation, re-implementation timelines, and challenges and adaptations related to school re-engagement for each case).[57,58] A codebook with operational and conceptual code definitions was developed and updated throughout the coding process. All the codes were reviewed for reliability, and consensus was achieved on discrepancies through group discussion by two independent coders.

Case study templates were then developed to guide case report construction, which facilitated within- and between-case analysis, followed by inter-case comparative analysis. The study design and analytical approach utilized facilitated across-case analysis, enabling the identification of patterns of variables both within and between cases, which may support replication logic.[56,59] Using replication logic and across-case analysis, we assessed consistencies and inconsistencies across cases, thereby enhancing the findings' analytical generalizability. Furthermore, the number of case replications is associated with the level of confidence in the study findings. Therefore, the current study maintained a minimum of five cases as recommended.[54]

3. Results

3.1. Organizational and participant characteristics

Six organizations delivering SBDSPs in Oregon participated in the study. Table 2 summarizes each organization's characteristics. SBDSPs in Oregon were more frequently provided by FHQCs ($n = 4$) and infrequently by non-profit ($n = 1$) and for-profit/privately owned ($n = 1$) organizations. We collected the data from 10 participants (≥18 years of age) from the six organizations under the study. This includes

Table 1. Workforce capacity, crisis management, and re-implementation processes, challenges, and adaptation measures assessed for school-based dental sealant programs

Variables assessed	Measures	Interview question examples
COVID-19 impact	We assessed the impact of COVID-19 on each organization, SBDSP, and workforce capacity.	(i) How did the closure of your program because of COVID-19 impact your job? (ii) How did the COVID shutdown impact the SBDSP staff? What happened to them? Did they file for unemployment or transfer to other programs?
Workforce capacity Staff size Staff turnover Staff rebuilding	We assessed each organization's SBDSP workforce capacity regarding staff size, training, turnover, and scale-up concerning school recruitment before and during the pandemic.	(i) How many employees did you have working with the SBDSP before the pandemic? What happened to them when the program was shut down? (ii) How many employees do you have working with the SBDSP now? Are they the same as before the pandemic?
De-implementation-crisis management planning: Key informant Planning process Planning decisions Guidance and consultants De-implementation adaptations	We assessed each organization's SBDSP's pre-implementation planning to resume program services during the pandemic, specific to the key informants' committee and associated planning processes (e.g., length of the planning processes, who was involved, planning meetings frequency, length, modality, topics discussed, and participatory decision-making processes). We also assessed each organization's planning considerations specific to the school recruitment/engagement, communication, and workforce capacity, including staff size, turnover, and rebuilding.	(i) How did the agency plan to implement the program during the pandemic? • Was there a planning committee? • Who was involved in the committee? • What were the topics/issues addressed by the committee? (ii) Did you pilot-test the SBDSP before implementing it during the pandemic? • Can you describe what was done in the pilot test? What was learned from that? (iii) Does the agency have an operation manual specific to its SBDSP? (Yes, No) • (If yes) Does the manual include processes specific to implementing the program during the pandemic? (yes, no)
Re-implementation process, challenges, and adaptations	We assessed the full re-implementation involved in delivering the SBDSPs. We also assessed the re-implementation timelines, the challenges experienced, and adaptations utilized during that process.	(i) Can you walk me through your program delivery routine during the pandemic? (ii) Did you face any challenges recruiting schools into the program during the pandemic? What type of challenges? How did you solve them? Were you involved in the problem-solving process?

Abbreviation: SBDSP: School-based dental sealant program.

Table 2. Summary of organizational characteristics

Characteristics	Case					
	1	2	3	4	5	6
Organization type	Nonprofit	For-profit	FQHC	FQHC	FQHC	FQHC
Service area[a]	Rural	Rural	Urban	Urban	Rural	Rural
Number of employees	4	4	4	4	3	4
Number of schools served[b]	28	22	22	17	10	15
Number of personnel interviewed[c]	2	1	2	2	1	2

Notes: [a]Refers to the service area: the single county where the program is providing services; [b]Refers to the number of schools served during the 2021 – 2022 school year; [c]Refers to the number of personnel interviewed: Program personnel who participated in the current study. Participating personnel from organizations 2 and 5 held multiple roles within the organization, including administrative (e.g., director, manager, and supervisor) and clinical (i.e., expanded practice dental hygienists). Main interviews were collected approximately 2 years post the COVID crisis.
Abbreviation: FQHC: Federally qualified health center.

program managers and dental hygienists (n = 6) within three FQHCs (Cases 3, 4, and 6). Case 5 participant (n = 1) occupied multiple roles (manager and dental hygienist). Case 1 participants (n = 2) included the program director and a dental hygienist. Case 2 participant (n = 1) was the SBDSP director and dental hygienist.

3.2. De-implementation and crisis management development

As seen in Table 3, the de-implementation phase ranged from 3 months (Case 4) to 21 months (Case 6). In terms of the total observation window (3 years), Case 6 faced complete de-implementation approximately 58% of the time compared to only 8% for Case 4. In subsequent sections, we will examine the reasons for the variations in the de-implementation phase.

During de-implementation, most SBDSPs developed informal crisis management teams (Cases 1, 3, 4, 5, and 6), while Case 2 relied on the program director to make crisis management decisions. Among the five SBDSPs with informal crisis management teams, four of them were FQHC SBDSPs/organizations (i.e., 3, 4, 5, and 6), and the remaining case (Case 1) was a non-profit organization. Unlike the non-profit organizations, the FQHCs were more directly involved in the crisis management of the SBDSPs and offered more human capital (i.e., parent organizations' dental directors [n = 4]) to address crisis management development and decision-making. Case 2, a smaller organization with fewer employees, was unable to develop a crisis management team.

Among cases who reported having developed crisis management teams, the teams typically consisted of 2–3 members (e.g., SBDSPs managers, dental directors, and/or dental providers; Cases 1, 3, 4, 5, and 6) and met remotely, weekly to bi-monthly. The content of the crisis management meetings focused on COVID-19 infection control protocols, staff size and training, school re-engagement timelines, consent form updates to include COVID-19 mitigations, and modifications needed to the program's delivery processes. Most importantly, all crisis management teams reported discussing plans for re-implementation.

None of the cases had proactively developed crisis management teams or plans. It is important to notice that SBDSPs are adjacent to the central crisis, and it is atypical for such programs to have crisis management plans in advance of a crisis. In addition, none of the cases formed key informant committees, used specific crisis management guidance (i.e., a formal planning model), or obtained expert consultation on crisis management. However, two cases (Cases 3 and 4) received additional input from an external organization that provided these programs' management and quality assurance oversight.

De-implementation also had a significant effect on workforce disruption. Four FQHCs SBDSPs/organizations maintained their workforce during de-implementation by transferring workers to different departments or COVID-19-related programs in the parent organizations (e.g., COVID-19 testing and vaccination clinics). The remaining two SBDSPs reported reducing (Case 1) or completely losing (Case 2) their workforce during the de-implementation phase.

3.3. Re-implementation patterns and timelines

Our analysis revealed that SBDSPs initiated and adapted various strategies to re-implement program services at different points in time. Case 4 spent 33 months engaged in re-implementation activities, while Case 6 spent 15 months engaged in re-implementation over the 3-year observation window of the study. Some re-implementation strategies were implemented outside of school venues to meet the pandemic guidelines.

In addition, our study found that programs were re-implemented at varying capacities compared to before the COVID-19 crisis. A review of Table 4 and Figure 1 illustrates our findings regarding the different patterns in the re-implementation process, including partial, intermediate, and full re-implementation. The implementation patterns are not sequential; programs may initiate full re-implementation without partial or intermediate re-implementation (e.g., Cases 3 and 5).

Table 3. De-implementation and re-implementation pattern timelines by months

Case	De-implementation	Re-implementation							Total re-implementation	
		Partial		Intermediate			Full			
	Months	Percentage	Months	Percentage	Months	Percentage	Months	Percentage	Months	Percentage
3	15	42	0	0	0	0	21	58	21	58
5	18	50	0	0	0	0	18	50	18	50
4	3	8	18	50	3	8	12	33	33	92
2	15	42	15	42	0	0	6	17	21	58
1	6	17	9	25	21	58	0	0	30	83
6	21	58	0	0	15	42	0	0	15	52

Note: Cases are ordered based on the timing of achieving full re-implementation (earliest to latest).

Table 4. Re-implementation patterns of school-based dental sealant programs

Re-implementation pattern	Definition
Partial re-implementation	Indicates a reduction in the delivery of the SBDSP core component[8] and whether delivery of these partial components was provided to fewer schools than before the pandemic. Sealant application was the most common core component to be removed due to a high risk of aerosols and spatter velocity, leading to an increased risk of COVID-19 transmission. However, other core components were provided (e.g., education, dental screenings, diagnostic dental exams, or dental referrals). These partial services may or may not have been provided on the school premises and, at times, provided more limited reach than school-based programming.
Intermediate re-implementation	Indicates that SBDSPs provided all program core components to fewer schools than before the pandemic. Programs are defined as intermediate if they did not provide services to at least one school they served pre-COVID, and all program services were provided within the school premises in the served schools.
Full re-implementation	Indicates that all SBDSP core components were provided to the same number of (or more) schools compared to before de-implementation, and all program services were provided on the school premises.

Note: The re-implementation patterns were key findings of the study and were categorized using two pieces of information: The program core components provided and the number of schools being served relative to the number of schools served before the COVID-19 crisis. SBDSP core components include oral health education, dental screenings, diagnostic dental exams, clinical dental caries prevention treatments (e.g., dental sealants, fluoride varnish, silver diamine fluoride, and dental cleaning), personal oral/dental hygiene instructions, and dental referrals.[8]
Abbreviation: SBDSP: School-based dental sealant program.

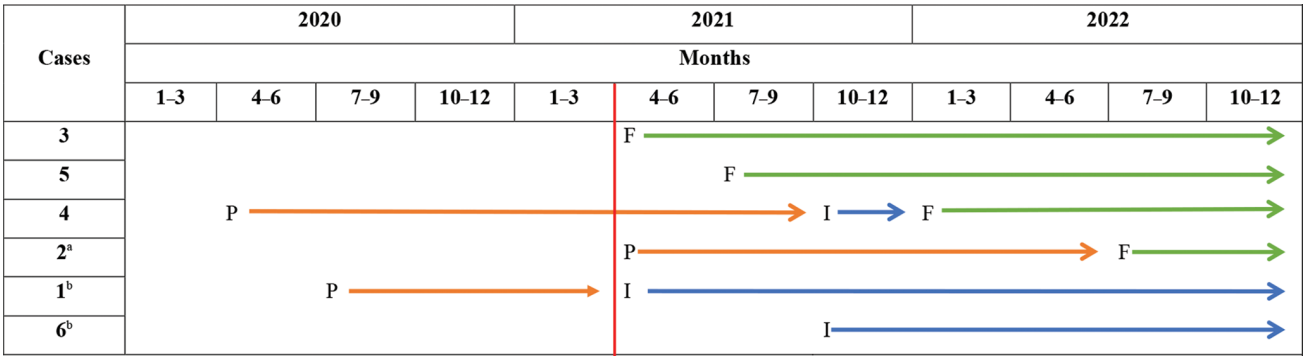

Figure 1. Timeline pattern analysis for school-based dental sealant program (SBDSP) re-implementation. March 16, 2020 (i.e., schools' shutdown due to COVID-19) is our reference timeline, against which all re-implementation timelines are compared. P is coded in orange (refers to a reduction in SBDSP core components, but some other SBDSP components were provided). I is coded in blue (all SBDSP core components were provided, but fewer number of schools were served compared to before the pandemic). F is coded in green (all SBDSP core components were provided to the same or a greater number of schools served before the pandemic, and the services were provided on the school premises). The red vertical line indicates when all elementary schools reopened for a hybrid or full in-person sessions on March 29, 2021, while 6 – 12 grades reopened on April 19, 2021. *The third quarter of each year (7 – 9) generally reflects summer vacation for public schools in Oregon, and some SBDSPs reduce services to some schools during this time. However, if they were providing services to all their schools prior to summer vacation, we interpolated for the summer months and included that period in the full re-implementation category. Initial start dates for re-implementation may have occurred within a given quarter, but we have standardized all start dates to reflect the quarter in which they were initiated ± 2 weeks. [a]Case 2 reported being able to re-engage with all of the schools served before COVID during the follow-up interview. [b]Cases 1 and 6 did not reach full re-implementation; these two cases prioritized serving elementary schools and served fewer schools.
Abbreviations: F: Full re-implementation; I: Intermediate re-implementation; P: Partial re-implementation.

3.3.1. Partial re-implementation

Partial re-implementation of SBDSPs occurred at different times across the cases. While three cases (3, 5, and 6) did not initiate any partial re-implementation strategies, the other three engaged in re-implementation efforts over periods ranging from 9 to 18 months. Cases 1, 2, and 4 adapted their services to align with crisis mitigation strategies. In cases 1 and 4, partial re-implementation was conducted before schools were fully reopened. SBDSPs in Cases 1 and 4 adapted their delivery modality to accommodate the lack of school and student access. The utilization of mobile dental units by Case 1, the development and dissemination of an online oral health education program, and the utilization of the parent organization's dental clinic by Case 4 allowed for partial re-implementation. These adaptations were feasible and successful due to a positive school response (the schools' general reaction and attitude toward SBDSPs' re-implementation efforts) and solid collaborations with the schools in the service areas.

Additionally, partial re-implementation also occurred when programs began providing services at the schools (i.e., not all core components were provided) (i.e., Case 2). Furthermore, the ability to swiftly rebuild the workforce (Cases 2 and 4) allowed for rapid re-implementation. Although Case 1 was not able to maintain its workforce during de-implementation, a complete workforce was not necessary for partial re-implementation since the program was not operating at full capacity.

Organizational size and resources (e.g., staff size, affiliation to dental clinics, and funding) did not seem to be contributing factors to successful partial re-implementation. However, one of the larger FQHC organizations (Case 4) had a dental clinic that provided additional workforce opportunities when the SBDSP's program was de-implemented. Smaller organizations were successful in partially re-implementing their SBDSPs. Even though Case 1 is a small nonprofit organization, it was able to make crisis management adaptations feasible and successful through fundraising.

3.3.2. Re-implementation rates

Using a Gantt chart and pattern analysis, we further identified and subcategorized each case into one of three distinct subcategories. This subcategorization considers the rate at which SBDSPs move through the re-implementation patterns. The re-implementation timelines are displayed in terms of the standard academic quarters broken out by winter, spring, summer, and fall (i.e., we used this division despite some schools being on semesters rather than quarters) (Figure 1). We stratified SBDSPs into three subcategories reflecting the rate at which they were re-implemented (rapid, gradual, and slow):

(i) Full rapid re-implementation: Refers to the cases observed to be the first to re-implement their SBDSPs fully (all SBDSP core components were delivered to all the schools served before the COVID crisis) (i.e., Cases 3 and 5).

(ii) Full gradual re-implementation: Refers to the cases observed to gradually reach full re-implementation by re-implementing SBDSPs partially or intermediately before reaching the full re-implementation (i.e., Cases 2 and 4). Although these cases reached full re-implementation, their timeline was longer than those in the full rapid re-implementation category.

(iii) Intermediate slow re-implementation: Refers to the cases observed to never reach the full re-implementation, as defined in this study (Table 4) during the 3-year study period, and only reached intermediate re-implementation compared to other cases in the study (i.e., Cases 1 and 6).

3.4. Full rapid re-implementation

Cases 3 and 5 fully and rapidly re-implemented their SBDSPs in 2021 (Figure 1). They provided all SBDSP core components to the same number of schools they served before the pandemic. Among all the cases, they initiated full re-implementation earlier and, consequently, had the longest span of full implementation services during our 3-year observation period (21 – 18 months, respectively) (Table 3). Cases 3 and 5 were the only cases to fully re-implement their SBDSPs without first going through partial or intermediate re-implementation. Both SBDSPs' crisis management teams decided not to conduct partial re-implementation to marshal their resources for full implementation when the schools were ready to host the program. Contributing factors to full rapid re-implementation included:

(i) SBDSPs resources (organizational and workforce capacity). Cases 3 and 5 maintained their workforce during de-implementation (transferred SBDSP workforce to other departments within the parent organization) and rapidly rebuilt it for re-implementation. Both cases were FQHCs and utilized similar resources with dental clinic affiliations and valuable organizational resources (i.e., financial and human).

(ii) School response (COVID-19 policies and inter-organizational communication). Cases 3 and 5 had efficacious communication with the schools and experienced positive school responses to re-implementation. Neither case reported COVID-19 school-related policy challenges during re-implementation.

3.5. Full gradual re-implementation

Cases 2 and 4 initiated partial re-implementations prior to transitioning to full re-implementation (Figure 1). Both Cases 2 and 4 made a deliberate decision to implement partial re-implementation adaptations because they believed that this would facilitate the return to full implementation down the road. Unfortunately, this goal was not realized. In particular, Cases 2 and 4 were confronted with schools that were reluctant to allow full re-implementation at an earlier date. By our definitions, these cases were categorized as full gradual re-implementation. Case 4 was the first SBDSP to initiate partial re-implementation 3 months after de-implementation (Table 3). Case 4 also spent 3 months in intermediate re-implementation, wherein they provided full services to some, but not all, of their pre-pandemic schools. Overall, Case 4 had the longest span of total re-implementation (i.e., 33 months) during our 3-year observation period. Case 2, on the other hand, progressed from partial to full without going through

intermediate re-implementation (i.e., they initiated full re-implementation in their entire pre-pandemic school network).

3.5.1. SBDSPs resources (organizational and workforce capacity)

Cases 2 and 4 were of different organizational sizes and types, and thus had different resources that influenced their advancement to full re-implementation. Case 2 was a small for-profit organization with limited financial and human resources compared to Case 4, a large FQHC with abundant resources. The depth of resources allowed Case 4 to maintain its workforce despite voluntary staff turnover, which had no impact on its re-implementation because additional resources (i.e., dental clinic) provided staff to fill those vacancies. Case 2 also rapidly rebuilt its workforce after the catastrophic loss of workers during de-implementation.

3.5.2. School response (COVID-19 policies and inter-organizational communication)

The gradual transition from partial to intermediate to full re-implementation was influenced by program-to-school communication challenges and unevenness in the school systems' decisions and policies throughout the re-implementation process. Despite having open communication channels and being included in the schools' re-opening blueprints, delays in re-implementation were experienced by Case 4 due to inconsistent messages from the schools and COVID-19 policies (i.e., schools in the services area limited the number of non-essential personnel (SBDSP staff) from entering the schools). Case 2's program-to-school communication changed significantly (i.e., school district-level nurses acted as school gatekeepers); in turn, the program no longer had direct contact with the schools compared to before the pandemic. As a result of the disruption in communication, Case 4 had prolonged partial and unanticipated intermediate re-implementation, and Case 2 temporarily lost access to 6 schools. In summary, challenges in program-to-school communication and misaligned crisis management practices within the school systems delayed full re-implementation. Once these challenges were resolved, SBDSPs were able to reach full re-implementation

3.6. Intermediate slow re-implementation

Cases 1 and 6 were the only two cases that did not reach full re-implementation during our 3-year study period. Case 1 spent 21 months, and Case 6 spent 15 months in intermediate re-implementation, where they provided full services to some, but not all, pre-pandemic schools. Cases 1 and 6 followed different pathways to a prolonged period of intermediate re-implementation. The prolonged intermediate re-implementation, paired with failure to reach full re-implementation, stemmed from programmatic decisions, particularly the reduction in the number of schools served. The programs' decision to limit their services to elementary schools only caused a reduction in school reach and the inability to achieve full re-implementation.

3.6.1. SBDSPs resources (organizational and workforce capacity)

Cases 1 and 6 were of different organizational sizes and types (Case 1: Small nonprofit with limited resources; Case 6: Large, FQHC with abundant resources), and thus may have had different resource capacities to devote to re-implementation. Case 1's SBDSPs were short-staffed, and the program was not able to maintain or rebuild its dental providers. Case 1 differed from other cases in the study in that it had a mixture of direct and subcontracted workforce. During de-implementation, Case 1 was only able to maintain its direct staff. As a result, the number of subcontracted dental providers declined from 13 to 3, resulting in significant workforce challenges. Case 6 had a change in management, which might have prolonged de-implementation and slowed decision-making processes.

3.6.2. School response (COVID-19 policies and inter-organizational communication)

Case 1 maintained open communication with the schools in its service area, while Case 6 did not. As expected, Case 6 faced more challenges with the school's response and policies towards re-implementation than Case 1. One school district in Case 6's service area required all SBDSPs providing services at their schools to be vaccinated, while the other district did not. The conflicting vaccination policies enacted by the schools in conjunction with poor SBDSP-to-school communication had a negative effect on Case 6's re-implementation. School vaccination policies required Case 6 to add additional workers drawn from their dental clinic, but these new workers needed training and time to learn field skills. Consequently, vaccine policies had an indirect impact on delaying full re-implementation.

4. Discussion

4.1. Re-implementation patterns

This study examined the de-implementation and re-implementation of SBDSPs in the context of a large-scale crisis, identifying three distinct re-implementation patterns: Full rapid, full gradual, and intermediate slow re-implementation. These patterns were influenced by crisis management planning or lack thereof, SBDSPs'

resources (i.e., organizational and workforce capacity), and school policies/response to SBDSPs' re-implementation (e.g., inter-organizational communication). These observed re-implementation patterns broaden the existing literature by describing re-implementation efforts, adaptations, and re-implementation factors influencing program variability in reaching full re-implementation following crisis-related disruptions.[23]

4.2. Adaptation's role in re-implementation

None of the SBDSPs had a proactive crisis management plan ahead of the COVID-19 crisis. However, some programs developed crisis-induced transformational (i.e., fundamental) adaptations, such as virtual oral health education programs or alternative care delivery settings.[23,60] These adaptations enabled partial re-implementation of SBDSPs. In the context of full and gradual re-implementation patterns, such adaptations acted as interim mechanisms to preserve elements of program delivery and maintain organizational engagement with school systems. However, their effectiveness was contingent upon alignment with school policies, staff readiness, and resource availability.

Importantly, the school's response to these adaptations and the re-implementation of SBDSPs varied considerably across the cases. In full re-implementation contexts, the school system (i.e., schools and school districts) offered consistent support and clear communication channels. Conversely, in cases of gradual and slow re-implementation, program staff experienced uncertainty due to unclear directives and inconsistent school district policies. Furthermore, variations between and within the school districts' COVID-19 mitigation strategies, policies, and adaptations led to a chaotic SBDSP re-implementation process that delayed full re-implementation. These findings are consistent with the literature in other fields, indicating that conflicting, ambiguous, or overly complex policies contribute to implementation delays, even under non-crisis conditions.[61-63] Therefore, programs that introduced adaptations without concurrent organizational support or key informants' buy-in (e.g., schools, school district nurses) experienced limited traction, underscoring the importance of co-developed adaptation strategies during crisis contexts. These findings highlight the critical need for organizational readiness and multi-level systems coordination, ensuring adaptations during a crisis are not only implemented but also effectively aligned, integrated, and accepted.[23,27,64,65]

Several questions arise from our findings on partial re-implementation. First, it was not feasible for SBDSPs to conduct evaluations of the adapted programs within the crisis context. That is, neither the field of implementation science nor crisis management addresses how to effectively develop and evaluate adaptations under the intense time pressures and resource constraints posed by a health or environmental crisis.[66,67] Secondly, there was no evaluation of the negative impact on oral health of not having developed these partial re-implementation strategies. Post-COVID research has found that prolonging the de-implementation of school-based dental services was associated with: (i) delayed access to dental care, (ii) poor oral hygiene, (iii) increased prevalence of dental caries, and (iv) a decline in dental prevention services utilization.[68-72] This reinforces findings from broader public health literature that delays in re-implementation following service disruption are associated with worsened health outcomes, particularly in underserved populations.[73,74]

4.3. Factors influencing re-implementation patterns

This study highlights the impact of SBDSPs' resource (i.e., organizational and workforce capacity) availability during de-implementation and re-implementation. The prolonged de-implementation of SBDSPs resulted in the loss of program personnel, and rebuilding the workforce became a critical step for re-implementation. Programs that were re-implemented more rapidly tended to have larger organizational infrastructure and access to internal clinical services, which collectively supported workforce maintenance and quicker re-implementation of SBDSPs. In contrast, programs that were re-implemented gradually were smaller organizations with fewer resources and faced greater challenges, highlighting the critical role of organizational resources in managing workforce losses during de-implementation and rebuilding them for re-implementation.

These results are consistent with prior research showing that, under non-crisis conditions, insufficient staff size affects all aspects of the implementation process, including program planning, implementation, fidelity, sustainability, and reach.[15,48,75-77] Our findings reinforce this literature by demonstrating how these challenges become more pronounced during a crisis. The differential ability of larger versus smaller organizations to retain or redeploy staff suggests that baseline organizational infrastructure, resources, and support are key determinants of re-implementation capacity in crisis contexts.[23]

At a fundamental level, program recovery was hindered by the loss of personnel who may have been valuable to the crisis management process. Inexperience and the absence of crisis management training may have exacerbated delays in crisis management and hindered crisis management teams' ability to cope with rapid workforce disruption. Prior work under crisis conditions suggests that a lack of preparedness negatively impacts workforce capacity.[78] Consistent with this, our data findings suggest

that preparedness gaps, particularly those related to workforce planning, were not uniformly distributed across organizations, disproportionately affecting smaller, community-based providers.

Workforce disruption, in turn, negatively affected school re-engagement and ultimately reduced school reach by delaying the return to full re-implementation. High turnover and insufficient staff size were associated with poor school re-engagement, delays in full re-implementation, and limitations in school reach (i.e., reducing the number of schools served and, consequently, reducing the number of students reached). We have found a relationship between the quality of school engagement and school reach under non-crisis conditions,[15] indicating the importance of workforce capacity to a wide range of implementation and re-implementation outcomes. These findings reinforce the interdependency between workforce maintenance and external engagement, particularly in community-based programs, such as SBDSPs, that rely on repeated, trust-based interactions between school and program personnel.

The workforce impact of the COVID-19 pandemic was not specific to the six programs in the current study. Data have been reported showing a pervasive disruption of the dental hygiene workforce due to various COVID-19 crisis-related factors.[79] These types of crisis-related outcomes serve as an important reminder of the need to implement crisis management in advance of any potential crisis. Beyond COVID-19, these insights are increasingly relevant given the growing frequency of large-scale crises, such as extreme weather events or regional public health emergencies, that can similarly destabilize local health and social service systems. Programs need crisis management plans to address workforce capacity disruptions during crises.

Furthermore, clear and consistent inter-organizational communication between the school systems and SBDSP is critical for successful implementation.[15] Our findings illustrate how inter-organizational communication disruption can significantly hinder timely re-implementation during a crisis. The variability in school key informants and policy interpretations contributed to inconsistencies in access to schools, even when SBDSPs were otherwise ready to resume services. Proactive crisis management planning would be expected to help the SBDSPs and the schools cope with this disruption. However, we found multiple cases where this type of crisis management did not occur. We suspect that the absence of communication between the crisis management components of the SBDSPs and the schools contributed to inter-organizational communication challenges and delayed re-implementation. This gap points to a broader issue in crisis preparedness: The lack of integrated crisis communication protocols that include adjacent programs such as SBDSPs. Because most crisis planning focuses inward on the direct functions of the school system, it often neglects the complex web of services embedded within the school infrastructure.[80] Further research is needed on strategies for developing crisis management programming for adjacent programs, particularly those requiring inter-organizational cooperation to fully implement the program.

4.4. Program-related recommendations

The present study provides data that suggest a number of practical solutions to what appeared to be relatively common challenges for the SBDSPschool system during the COVID-19 crisis. First, establish crisis management teams that develop crisis management plans for various potential crises. Second, a crisis management plan should be developed that considers strategies to deal with various challenges (e.g., workforce capacity, internal mitigation strategies, and mitigation strategies imposed by external organizations). Third, conduct an after-action review[81,82] to review and evaluate SBDSP's response to the crisis (e.g., challenges, successes, and changes needed) and make corrections for future efforts. Lastly, a larger systemic-level recommendation is to include adjacent programs in the Department of Public Health (DPH) crisis management. Adjacent programs, such as SBDSPs, need to begin interacting with the DPH to make them aware of how the imposed mitigation strategies impacted their functioning and jeopardized oral health services. The DPH should be able to provide guidance and potentially expert consultants to adjacent programs to aid in developing their crisis management teams and plans.

5. Limitations

The current study has some methodological limitations. First, our findings are based on a sample of SBDSPs in the state of Oregon, and consequently, have limited generalizability beyond SBDSPs in Oregon. In addition, the study is primarily qualitative; thus, we cannot estimate the relative impact of those variables identified. Finally, the data are based on the retrospective self-reports of unknown validity and are subject to potential recall bias.

6. Conclusion

Although all SBDSPs in the study experienced de-implementation due to the COVID-19 crisis, there were distinct variations in their re-implementation patterns and rates. Partial re-implementation, for instance, was delivered by three SBDSPs out of six and involved crisis-related adaptations of unknown fidelity. Crisis-related adaptations and re-implementation efforts were influenced by organizational resources, workforce capacity, school response, COVID-19 policies, inter-organizational

communication, and, in some cases, programmatic decisions.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Eiman E. AlEissa
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Writing – review & editing: Eiman E. AlEissa

Ethics approval and consent to participate

The study was approved by Oregon State University's Institutional Review Board (approval ID: IRB-2021-1171). Written consent was not required, and verbal consent from the participants was obtained at the time of data collection.

Consent for publication

Not applicable.

Availability of data

Data are not openly available as to protect the participants confidential. Additional analyses are still underway, and releasing the data at this stage could compromise the integrity of future findings.

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