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VITAMIN C LEVELS IN PREGNANT WOMEN AND EFFICACY OF VITAMIN C SUPPLEMENTS IN THE PREVENTION OF PRETERM BIRTH: A SYSTEMATIC REVIEW AND META-ANALYSIS

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ABSTRACT

We aimed to conduct a systematic review and meta-analysis to investigate if there is a significant difference in vitamin C blood levels in women who had preterm birth compared to control group who did not and evaluate the efficacy of vitamin C supplements in preventing it. This manuscript presents a part of a larger systematic review and meta-analysis which was registered in PROSPERO (CRD42022371644). PubMed/MEDLINE, Scopus and Web of Science were searched up to February 15, 2024. Forward and backward citation searching was also performed. Studies were selected according to prespecified inclusion and exclusion criteria. Data were analyzed using Meta-Essentials: Workbooks for meta-analysis (Version 1.5). A total of 10 studies (11 reports) met all eligibility criteria: 5 studies (5 reports) assessing vitamin C levels and 5 studies (6 reports) assessing efficacy. No significant difference was found in vitamin C levels between women who had preterm birth and controls who had term delivery (Hedges' g=0.33; 95% confidence interval [CI]: –0.22, 0.88; p=0.091; I^2=53.50%). Also, no differences were seen between women supplemented with vitamin C and controls taking placebo or having no vitamin C supplementation in the risk of preterm birth (risk ratio=0.94; 95% CI: 0.57, 1.55; p=0.730; I^2=50.13%). Significant level of heterogeneity was observed in both meta-analyses, but results were robust in all sensitivity analyses. Our results suggest that there are no significant differences in vitamin C levels between women with preterm and term birth and that vitamin C supplementation doesn't influence the risk of preterm birth.

Keywords: *Vitamin C, preterm birth, systematic review, meta-analysis.*

INTRODUCTION

Preterm birth is defined as birth after 20 and before 37 completed weeks of gestation (1). Preterm birth rates were estimated to range from 4 to 18% depending on the country, with a global rate of about 11%, but these figures seem to be rising in recent years (2). Approximately 50% of preterm births have an unknown cause, about 25% are associated with premature (prelabor) rupture of membranes (PROM), and the remaining 25% are attributed to medically indicated or elective preterm deliveries (3,4).

Vitamin C, or ascorbic acid, is an essential water-soluble vitamin which has a role in the synthesis of collagen and acts as an antioxidant (5,6). However, the role of antioxidants, including vitamin C, in preterm and term birth is not entirely clear (7). Some studies reported that pregnant women who had preterm birth had significantly higher vitamin C level in their blood compared to those who gave birth at term (7,8), while others found no significant difference (9–11). To the best of our knowledge, up to now there are no systematic review and meta-analysis evaluating vitamin C blood levels in women who had preterm birth compared to those who did not. However, there are some older systematic reviews and meta-analyses which evaluated the effects of vitamin C supplementation in the prevention of preterm birth, but with conflicting results. First, published in 2005, concluded that women supplemented with vitamin C were at increased risk of giving birth preterm compared to placebo (12). In 2015, the updated systematic review and meta-analysis reported no clear differences between women supplemented with vitamin C compared with placebo or no control for the risk of preterm birth (6). Also, a systematic review and meta-analysis conducted in 2017 and published as a congress abstract in 2018 reported that vitamin C was not associated with a reduction in risk of preterm birth (13).

Considering previous conflicting evidence, we aimed to conduct a systematic review and meta-analysis to investigate if there is a significant difference in vitamin C blood levels in women who had preterm birth compared to control group who did not and evaluate the efficacy of vitamin C supplements in preventing it.

METHODS

This systematic review and meta-analysis presents a part of a larger systematic review and meta-analysis registered in the International Prospective Register of Systematic Reviews PROSPERO (registration number: CRD42022371644). Current manuscript reports results regarding preterm birth, while results regarding PROM were reported elsewhere as well as the complete search strategy (14). Briefly, three electronic databases (PubMed/MEDLINE, Scopus, Web of Science) were searched independently by two authors from the beginning of indexing up to December 21, 2022, without any language or date restriction. The search was last updated on February 15, 2024. Both backward and forward citation searching on publications that met the eligibility criteria were conducted. Backward citation searching was conducted by inspecting the lists of references in these studies, while forward citation searching was conducted by using the Google Scholar citation index to identify citing studies on February 15, 2024. We followed the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Statement (15).

Eligibility criteria and selection process

For the part regarding vitamin C level, the inclusion criteria were an original clinical study of any type which reported maternal peripheral blood, serum or plasma vitamin C level measured at any point during pregnancy or at/after delivery with a study group consisting of pregnant women who experienced preterm birth and a control group of pregnant women whose pregnancy ended in term delivery.

For the part regarding vitamin C supplementation efficacy, the inclusion criteria were randomized controlled clinical studies evaluating the efficacy of vitamin C supplementation alone in preventing preterm birth in pregnant women in comparison with a control group who received no vitamin C supplementation or placebo.

We excluded studies with unavailable full text, conference abstracts, and studies for which we could not extract, calculate, or obtain information needed for calculation of combined effect sizes. Specific exclusion criteria for the part regarding vitamin C supplementation efficacy were non-randomized clinical studies and studies in which the efficacy of vitamin C in combination with other supplements was evaluated (study was not excluded if both groups received the same supplements).

Two authors independently evaluated the eligibility of retrieved publications based on their title and abstract. When these were insufficient for evaluation, we sought to retrieve and evaluate the full text. Also, we tried to contact the authors of a total of three reports (16–18) with a request for clarifications or providing information about relevant data that were not available in the retrieved full texts. Publications were included in the meta-analysis if all authors agreed that the eligibility criteria were met. Disagreements between individual judgements were resolved by the first author.

Data extraction

The data extraction sheet was created, and two authors independently extracted data from the included studies. We extracted the following for all studies: study ID, citation, country/region, characteristics of the sample (e.g., participant groups and their main characteristics, sample sizes, age), and relevant findings/conclusions of the study. Furthermore, we extracted the following for studies measuring vitamin C levels: mean and standard deviation (SD) of the vitamin C level, measurement method, blood sample type, time of taking the blood sample, gestational age, and percentage of smokers in each group. Also, we extracted the following for studies evaluating vitamin C supplementation efficacy: inclusion criteria

in relation to gestational age and previous history of preterm birth, information about vitamin C supplementation (e.g., timing of commencement/duration of supplementation, dosage), type of control (no vitamin C supplementation, placebo), information about blinding, frequency of preterm birth in each group, and reported information about observed side effects associated with vitamin C supplementation. The first author double-checked the accuracy of the extracted data and created the final data extraction table by collating the two tables. The final data extraction table is publicly available in the Figshare repository (19).

Methodological quality (risk of bias) assessment

The methodological index for non-randomized studies (MINORS) tool (20) was used to assess the methodological quality (risk of bias) of the included studies of vitamin C level measurements. The MINORS tool has a total of 12 methodological items (20). Each item is scored from 0 to 2: 0 (not reported), 1 (reported but inadequate) and 2 (reported and adequate) (20). The global ideal score for comparative studies is 24 (20). Quality assessment of individual studies according to total MINORS score was categorized as follows: very low (0–6), low (7–12), moderate (13–18), and high (19–24) quality (21).

Cochrane Risk of Bias 2 (RoB 2) tool (22) was used to assess risk of bias of included randomized studies assessing the efficacy of vitamin C supplementation and Robvis web app was used for visualizing assessment (23).

All authors individually assessed the risk of bias of each study, while differences in assessment were resolved by discussion until a consensus was reached.

Data analysis

Meta-Essentials: Workbooks for meta-analysis (Version 1.5) (24) was used to analyze the data.

For the part regarding vitamin C levels, we used the random effects model and estimated the combined effect sizes by using Hedges' g with its 95% confidence interval (CI), prediction interval (PI) and corresponding tests of significance. We used means and SDs of vitamin C levels and a corresponding number of participants in the analyses. Standard errors were converted to SDs using the number of patients when needed.

For the part regarding the efficacy of vitamin C supplementation, we used a random effects model with an inverse variance weighting method while the combined effect sizes were estimated using risk ratio (RR) with its 95% CI, PI and corresponding tests of significance. Numbers of participants with outcome of interest (preterm birth) and corresponding denominators (number randomized minus any participants whose outcomes were known to be missing) were used in the analyses. The random effects model was used in all analyses because of the clinical and methodological heterogeneity of included studies, while the combined effect sizes were considered significant if the associated 95% CI did not include zero (for Hedges' g) or one (for RR) and the associated two-tailed p-value was less than 0.05.

Cochran's Q test and I^2 statistic were used to evaluate statistical heterogeneity. Because Cochran's Q test can have low power when studies have a small sample size or are few in number, a p-value of <0.10 was considered to indicate the presence of statistically significant heterogeneity (25). An I^2 value >50% was considered significant. To explore sources of significant heterogeneity, additional subgroup analyses were performed in relation to available data (for levels in relation to measurement method, region [continent], blood sample type, and MINORS quality category; for efficacy in relation to overall risk of bias and region [continent]), along with moderator analysis with available continuous moderator variables (mean age for levels; total daily vitamin C dose for efficacy).

We carried out sensitivity analysis by removing one study at a time and recalculating the estimates of combined effect size for the remaining studies.

To assess the certainty of evidence for the efficacy outcome we used the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach for summarizing confidence in effects of interventions (26).

RESULTS

The PRISMA flow diagram describing the results of the search and selection process is provided in Figure 1. Only one author of one study published in two reports (16,17) provided the requested data and clarifications. A total of 10 studies (11 reports) met all eligibility criteria: 5 studies (5 reports) assessing vitamin C levels (7–11) and 5 studies (6 reports) assessing efficacy (16,17,27–30).

Main characteristics of included studies are summarized in Table 1 and Table 2. Some side effects associated with vitamin C supplementation were reported only in one study where one woman reported stomach pain after taking a vitamin C tablet (16,17). There were no side effects of vitamin C supplementation in two studies (27,28).

Quality (risk of bias) assessment for each study is shown in Table 1 (for studies assessing levels) and Figure 2 (for efficacy studies). The MINORS quality score ranged from 14 to 18 of 24, so all five studies assessing vitamin C levels had moderate quality. The overall risk of bias for efficacy studies according to the RoB 2 tool was judged as "high" and "some concerns" for two (40.0%) and three (60.0%) of five studies, respectively.

There was no significant difference in vitamin C levels between women who had preterm birth and controls (five studies, 297 participants; Hedges' g=0.33; 95% CI: –0.22, 0.88; PI: −0.69, 1.35; Z=1.69, p=0.091; forest plot shown in Figure 3), but between-study heterogeneity was significant (Q=8.60, p=0.072, I^2=53.50%). A subgroup of studies

conducted in Asia in which levels were measured using spectrophotometry (7,11) was without any heterogeneity (I^2=0.00%), while the combined effect size was significant (Hedges' g 0.51 with 95% CI from 0.41 to 0.61) and indicated that vitamin C levels were significantly higher in women who experienced preterm birth. Sensitivity analysis did not show significant changes with the exclusion of individual studies.

In addition, no differences were seen between women supplemented with vitamin C and controls in the risk of preterm birth (five studies, 1643 participants; RR=0.94; 95% CI: 0.57, 1.55; PI: 0.38, 2.35; Z=−0.34, p=0.730; forest plot shown in Figure 3) and these results were robust in sensitivity analysis, but there was a significant between-study heterogeneity (Q=8.02, p=0.091, I^2=50.13%). Subgroups of studies conducted in Africa (28–30) and continents other than Africa (16,17,27) had acceptable level of heterogeneity (I^2=43.86% and I^2=0.00%, respectively) and combined effect sizes were not significant (RR=1.10 with 95% CI from 0.56 to 2.18 and RR=0.52 with 95% CI from 0.17 to 1.59, respectively). Also, a subgroup of studies with high risk of bias (16,17,28) was without any heterogeneity (I^2=0.00%) and with a nonsignificant combined effect size (RR=0.77 with 95% CI from 0.03 to 22.90). Certainty of the evidence assessment using the GRADE approach for the efficacy outcome was rated as low indicating that further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate. We downgraded for two levels: one level for serious risk of bias (overall risk of bias in all included studies was rated as either "some concerns" or "high") and one level for serious imprecision (wide confidence intervals).

Figure 1. Results of the search and selection process (PRISMA flow diagram)

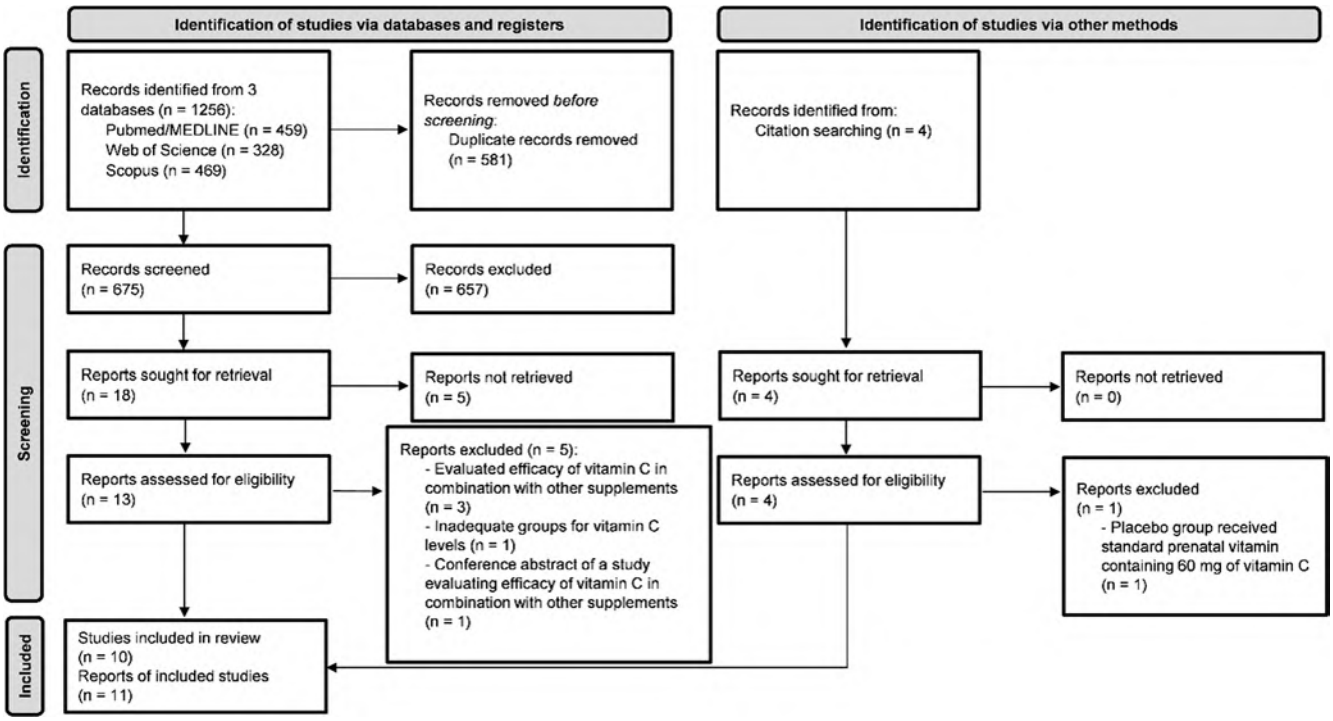

Figure 2. Risk of bias assessment of efficacy studies according to the RoB 2 tool

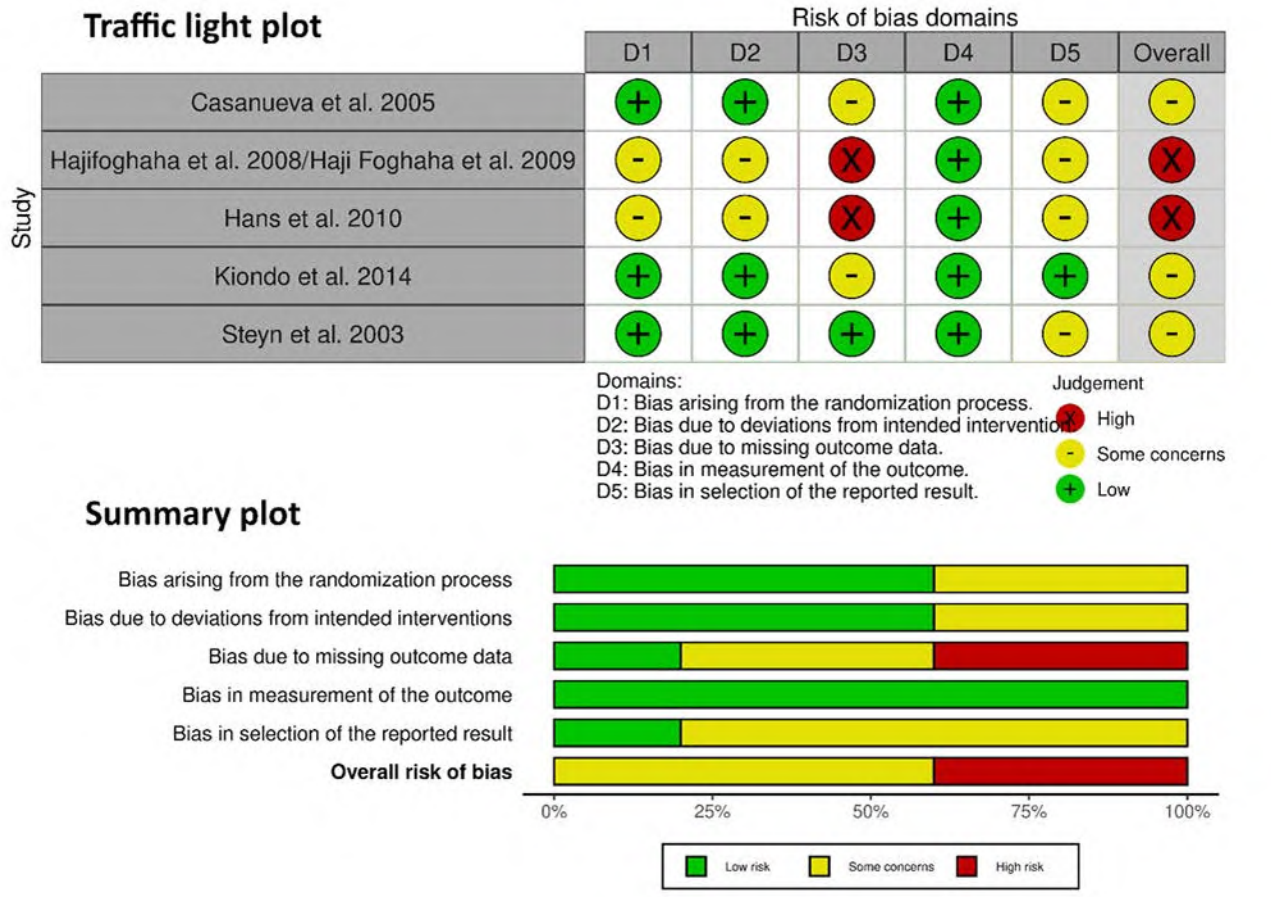

Figure 3. Forest plots showing effect sizes of differences in vitamin C levels between women with preterm birth and controls and effect sizes of efficacy of vitamin C supplementation in prevention of preterm birth

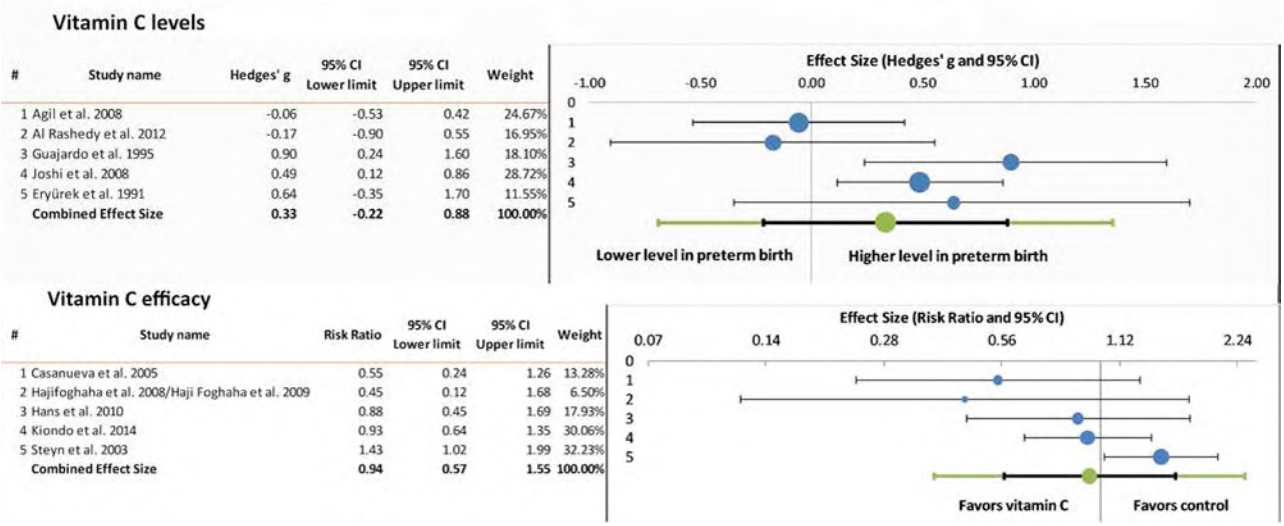

Table 1. Characteristics of included studies which evaluated vitamin C levels

	Study ID (first author, year)	Country	Blood sample type, measurement method, time of taking the blood sample	Number of participants		Mean age ± SD in years; Mean gestational age ± SD in weeks		Mean ± SD of vitamin C levels		Conclusion	MINORS quality score and category
				Preterm birth	Control	Preterm birth	Control	Preterm birth	Control		
1	Agil et al. 2008 (9)	Spain	Plasma, HPLC, at delivery (within 30 minutes)	40	30	NR±NR; NR±NR	NR±NR; NR±NR	59.33±16.32 µmol/L	60.12±9.04 µmol/L	Comparable levels in both groups.	17/24 Moderate
2	Al Rashedy et al. 2012 (10)	Egypt	Serum, HPLC, NS	15	15	24.6±3.2; 33.9±1.6 (of neonates)	25.5±3.4; 37.2±1.6 (of neonates)	23.5±11.2 µmol/mL	25.4±10.4 µmol/mL	No significant difference between groups.	15/24 Moderate
3	Guajardo et al. 1995 (8)	USA	Plasma, HPLC, at delivery	15	25	29.5±4.3; 31.4±3.4 (of neonates)	26.2±5.3; 39.6±0.94 (of neonates)	1.33±0.62 mg/dL	0.72±0.69 mg/dL	Level was significantly higher in the preterm group than in the term group ($p<0.05$).	16/24 Moderate
4	Joshi et al. 2008 (7)	India	Plasma, spectrophotometry, just after delivery	40	100	23.6±3.3; 34.9±1.8 (of neonates)	22.1±2.9; 38.9±1.0 (of neonates)	257.8±54.1 µmol/L	228.0±63.2 µmol/L	Level was significantly higher in the preterm group than in the term group ($p=0.01$).	18/24 Moderate
5	Eryürek et al. 1991 (11)	Turkey	Blood, spectrophotometry (ascorbate + dehydroascorbate), immediately after the delivery	7	10	NR±NR; NR±NR	NR±NR; NR±NR	6.08±1.75 µg/mL	4.91±1.72 µg/mL	No significant difference between groups.	14/24 Moderate

Abbreviations: HPLC – high-performance liquid chromatography; MINORS – methodological index for non-randomized studies; NR – not reported; NS – not specified; p – statistical significance; SD – standard deviation.

Table 2. An overview of included studies evaluating efficacy of vitamin C supplementation in prevention of preterm birth

	Study ID (first author, year)	Country	Inclusion criteria regarding gestational age and if previous history of preterm birth was required	Age of the participants in years (mean±SD, unless otherwise specified)	Vitamin C dosage, commencement/ duration of supplementation	Control, blinding	Frequency of preterm birth in vitamin C group (n/N)	Frequency of preterm birth in control group (n/N)	Study conclusions
1	Casanueva et al. 2005 (27)	Mexico	<20 weeks, no	Vitamin C: 27.5±7.4 Control: 27.4±7.7	100 mg daily, commenced after 20 weeks of gestation (duration not specified)	Placebo, double-blind	7/52	14/57	No significant difference (p=0.142).
2	Hajifoghaha et al. 2008 (17)/Haji Foghaha et al. 2009 (16)	Iran	20 weeks, no	Vitamin C: 23.88±4.62 Control: 24.00±4.56	100 mg daily, from 20 to 36 weeks of gestation	Placebo, single-blind	3/57	7/60	No significant difference (p=0.18).
3	Hans et al. 2010 (28)	Uganda	4 to 12 weeks, no	Median (range) Vitamin C: 24 (18-39) Control: 25 (18-37)	400 mg daily (two tablets of 100 mg two times a day), until delivery	No vitamin C, open-label	15/187	18/197	No significant difference (p=0.719).
4	Kiondo et al. 2014 (30)	Uganda	12-22 weeks, no	Age group (%) Vitamin C: ≤19 (19.5) 20-29 (53.0) 30-34 (17.0) ≥35 (10.5) Control: ≤19 (21.0) 20-29 (54.3) 30-34 (14.8) ≥35 (9.9)	1000 mg daily, until delivery	Placebo, triple-blind	47/415	51/418	No significant difference (p=0.7).
5	Steyn et al. 2003 (29)	South Africa	Before 26 weeks, history of previous preterm birth	Median (range) Vitamin C: 28 (18-44) Control: 28 (19-45)	500 mg daily (250 mg twice a day), until 34 weeks of gestation	Placebo, double-blind	50/100	35/100	Significantly more preterm birth in vitamin C group compared to control group (p=0.031).

Abbreviations: n – number of participants with preterm birth (outcome); N – number of randomized participants minus any participants whose outcomes were known to be missing (denominator); p – statistical significance; SD – standard deviation.

DISCUSSION

Our results confirm the conclusions of the previous meta-analyses that vitamin C is not effective in preventing preterm birth (6,13). This is also supported by our finding that vitamin C levels do not differ between women who had preterm birth and controls who had term delivery, as well as that both of our findings were robust in sensitivity analysis. On the other hand, part of the meta-analysis focusing only on PROM indicated that women with PROM, particularly those who develop it preterm, seem to have significantly lower levels of vitamin C, and that its supplementation seems to be effective in lowering the risk of PROM, particularly preterm PROM (14). Considering that PROM occurrence was associated with an increase in oxidative stress and abnormalities in the formation and structure of collagen, the potential ability of vitamin C to prevent PROM could be explained by its antioxidant effects and role in the synthesis of collagen (14,31,32). However, since only about 25% of preterm births is associated with PROM, while approximately 50% have unknown cause (3,4), it is likely that vitamin C doesn't play any role in these other causes which could explain its ineffectiveness in the prevention of preterm birth.

There is still no adequate standardization for reference ranges of blood vitamin C levels and units of measurement, which vary between laboratories and measurement methods (33). Reported mean vitamin C levels in the studies included in this systematic review and meta-analysis were generally above the level considered sufficient in both women who had preterm birth and controls who had term delivery (7–11). Two studies indicated that vitamin C levels were significantly higher in women who experienced preterm birth (7,8), with one study reporting that women who experienced preterm birth had mean vitamin C levels exceeding 250 μmol/L, which are usually regarded as high levels (7,33). It is well known that under normal circumstances, different tissue-recycling processes can absorb excess harmful radicals of vitamin C in blood (7,34). However, these radicals may accumulate inside the tissues and might not be as readily eliminated when antioxidant vitamin load surpasses the levels required to counteract endogenous oxidative stress which may cause a change from their beneficial antioxidant effect to a harmful prooxidant effect (7,35).

Also, it is worth noting that significant level of heterogeneity was observed in both meta-analyses which could be explained with regional differences (for both levels and efficacy), differences in measurement methods (for levels) and risk of bias (for efficacy). The influence of trial quality on heterogeneity was also observed in some of the previous meta-analyses (6). Clear regional disparities in vitamin C status and prevalence of deficiency were already observed between high-income and low- and middle-income countries, probably due to geographic, social, economic, and cultural factors (36,37). In addition, vitamin C can be measured in blood with diverse methods, many of which have limitations and are prone to interference (37). Also, vitamin C is sensitive to oxidation, so appropriate handling, processing and storage of samples prior to analysis is very important for valid and precise measurement (37).

Our meta-analysis had some limitations that should be mentioned. First, the total number of included studies was relatively small. Therefore, our results should be interpreted cautiously, as analyses could have been underpowered. Second, although we initially planned to evaluate publication bias, we did not perform this analysis because the assessment methods are unreliable when less than ten studies are included in the meta-analysis (38). Third, significant between-study heterogeneity was observed. Although we evaluated some of the factors that could be potential sources of heterogeneity, some of them were not reported in all included studies and we also weren't able to evaluate the influence of some important factors like dietary vitamin C intake and gestational age, because these data were either not provided at all or were provided but inconsistently and only in the minority of the studies. Finally, we were not able to retrieve the full text of some reports to assess if they fulfil our criteria for inclusion, and some of the contacted authors did not reply to our request for data and clarifications, which also could have affected our results.

CONCLUSIONS

In conclusion, our results suggest that there are no significant differences in vitamin C levels between women with preterm and term birth, as well as that vitamin C supplementation doesn't influence the risk of preterm birth.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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COMPARATIVE PERFORMANCE OF TWO IMMUNOASSAY PLATFORMS FOR PEDIATRIC HORMONE TESTING

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ABSTRACT

To compare the analytical performance of the Roche Cobas Pro and Siemens Atellica Solution platforms for measuring serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and testosterone in pediatric samples, with an emphasis on analytical bias, agreement, and clinical interpretability. A method comparison study was conducted using paired serum samples from children (LH: n = 132; FSH: n = 140; estradiol: n = 413; testosterone: n = 125). An additional 125 adult female samples were included to extend the estradiol measurement range. Hormone concentrations were measured on both platforms according to CLSI EP09-A3 guidelines. Analytical agreement and bias were evaluated using Passing–Bablok regression, Bland–Altman analysis, and Spearman correlation. Strong correlations were observed for all analytes (Spearman $r \geq 0.899$; $p < 0.0001$). However, clinically relevant biases were detected. Compared with Cobas Pro, Atellica underestimated LH (slope 0.856; intercept –0.424 IU/L) and overestimated FSH (slope 1.087). Estradiol demonstrated the greatest disagreement, with Atellica showing higher values in pediatric samples (mean bias +24.65 pmol/L) and adult female samples (mean bias +142.18 pmol/L). Among pediatric estradiol measurements, 232/413 samples were below the limit of quantification (LOQ) on one or both platforms; notably, 147 samples were undetectable by Cobas Pro but quantifiable on Atellica. Testosterone showed near-proportional agreement (slope 0.99), although non-linearity was observed at lower concentrations. Although both platforms demonstrate strong analytical correlation, measurement biases, particularly for LH and estradiol, are of sufficient magnitude to influence clinical interpretation in pediatric endocrinology, including puberty assessment and longitudinal hormone monitoring.

Keywords: *Pediatric endocrinology, Immunoassay comparison, Luteinizing hormone, Estradiol; Testosterone, Cobas Pro, Atellica Solution.*

INTRODUCTION

Accurate measurement of gonadotropins and sex steroids—specifically luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and testosterone—is essential for the diagnosis and management of pediatric endocrine disorders. These hormones play a central role in sexual development, growth, and reproductive health, and their levels vary substantially with age and sex. Interpreting hormone levels in children and adolescents therefore requires not only high analytical precision but also age- and sex-specific reference intervals to support accurate clinical decision-making (1, 2). In Vietnam, pediatric endocrine services have expanded significantly over the past decade, yet limited access to LC-MS/MS and lack of locally validated immunoassay performance data remain key challenges. Clinical laboratories continue to rely on high-throughput immunoassay platforms, but their accuracy for pediatric hormone quantification has not been systematically evaluated in the Vietnamese setting. This gap raises concerns about potential misclassification, especially in prepubertal children with low hormone levels near the detection limits of conventional assays.

Pediatric hormone testing presents unique analytical challenges due to the naturally low concentrations of sex steroids and gonadotropins in prepubertal and early pubertal children. For instance, serum estradiol levels in young girls and testosterone levels in young boys often fall below 20 pg/mL and 30 ng/dL, respectively—concentrations near or below the functional sensitivity of many commercial immunoassays (3-5). These low levels increase the risk of imprecision, cross-reactivity, and false elevation, leading to potential misdiagnosis or inappropriate treatment.

Among the most commonly used immunoassay platforms in clinical laboratories are the Roche Cobas Pro and Siemens Atellica Solution systems. Both are high-throughput, fully automated analyzers designed for the measurement of a wide range of hormones and clinical chemistry parameters. Previous studies have demonstrated that both systems provide reliable and reproducible measurements of LH and FSH, with well-established pediatric reference intervals that align with international standards (1, 2). However, questions remain about their performance in detecting low hormone concentrations, especially in prepubertal children.

Immunoassays, despite being widely available and cost-effective, are prone to limitations such as cross-reactivity, matrix effects, and reduced specificity at low concentrations. These challenges are especially pronounced when measuring sex steroids in children. Studies have shown that immunoassays may overestimate estradiol and testosterone concentrations when compared to more specific methods like liquid chromatography–tandem mass spectrometry (LC-MS/MS), which is considered the gold standard for hormone quantification (3-5). Such discrepancies may result in diagnostic inaccuracies and suboptimal clinical management.

While LC-MS/MS offers superior analytical specificity and sensitivity, its adoption in routine clinical practice remains limited due to high costs, technical complexity, and the need for specialized personnel. Consequently, immunoassays remain the mainstay of hormone testing in most pediatric settings. It is therefore critical to evaluate the analytical performance and limitations of widely used platforms, particularly in populations where hormone concentrations are near the assay detection limits (6, 7).

The aim of this study was to compare the performance of the Roche Cobas Pro and Siemens Atellica Solution platforms in measuring serum LH, FSH, estradiol, and testosterone in pediatric samples. By assessing analytical agreement and identifying potential biases between platforms, we seek to provide evidence to inform assay selection, guide appropriate clinical interpretation, and support the development of platform-specific pediatric reference intervals in Vietnam.

MATERIALS AND METHODS

Study design, sample size and sampling procedure

This method comparison study was conducted in accordance with the Clinical and Laboratory Standards Institute (CLSI) EP09-A3 guidelines. Paired serum samples were collected from pediatric patients at the Vietnam National Children's Hospital between June 2024 and January 2025. The study included 132 samples for LH, 140 for FSH, 413 for estradiol, and 125 for testosterone. To ensure adequate coverage of the analytical measurement range for estradiol, an additional 125 adult female samples were included. All samples were anonymized and obtained from routine testing; thus, informed consent was waived. Ethical approval was granted by the Institutional Review Board of Vietnam National Children's Hospital (IRB-VN01037/IRB00011976/FWA0002 8418). This study is part of a broader project aimed at establishing pediatric reference intervals for Vietnamese children.

Sample handling and hormone measurement

Serum samples were excluded if visibly hemolyzed, icteric, or lipemic. Following centrifugation, serum was aliquoted into Eppendorf tubes and stored at –80°C until analysis. All hormone assays were performed on the Roche Cobas Pro and Siemens Atellica Solution platforms within two hours of thawing. Calibration and internal quality control procedures followed the manufacturers' instructions. Daily quality control was performed using Bio-Rad control materials.

Analytical characteristics of each assay—such as measurement principle, traceability, and analytical range—are summarized in Table 1. Detailed calibration procedures and traceability chains have been presented in Supplementary Table S1 to improve clarity and focus. Both platforms use electrochemiluminescence or chemiluminescence immunoassay technologies with standardized calibration materials.

Table 1. Summary of analytical principles, traceability, and analytical ranges of Cobas Pro (Roche) and Atellica Solution (Siemens) platforms

Measurand	Platform	Method principle	Traceability	Analytical range
LH	Atellica	CLIA (sandwich)	NIBSC 80/552	0.07–200 IU/L
LH	Cobas Pro	ECLIA (sandwich)	NIBSC 80/552	0.3–200 IU/L
FSH	Atellica	CLIA	WHO IS 94/632	0.3–200 IU/L
FSH	Cobas Pro	ECLIA	WHO IRP 78/549	0.3–200 IU/L
Testosterone	Atellica	CLIA	ID-LC-MS/MS	0.24–52.05 nmol/L
Testosterone	Cobas Pro	ECLIA	ID-GC/MS	0.087–52.0 nmol/L
Estradiol	Atellica	CLIA	ID-GC/MS (manufacturer)	43.31–11010 pmol/L
Estradiol	Cobas Pro	ECLIA	CRM 6004a via ID-GC/MS	18.4–11010 pmol/L

Precision and trueness verification and monitoring

Precision was evaluated following CLSI EP15-A3 guidelines. Short-term precision was assessed by testing three concentration levels (low, normal, high) five times per day over five consecutive days. Long-term precision was monitored through daily quality control over the course of the study. Trueness was verified via interlaboratory comparison using Bio-Rad materials and peer-group mean comparisons. External quality assurance (EQA) was conducted monthly through participation in the Randox RIQAS Chemistry and Immunoassay program, with satisfactory performance across all assays.

Statistical analysis

Data were analyzed using MedCalc version 23.2.1 (MedCalc Software Ltd., Ostend, Belgium). Method comparison was assessed using Passing–Bablok regression to evaluate systematic and proportional bias. Non-linearity was formally assessed using the cumulative sum (Cusum) test provided within the Passing–Bablok regression framework. Agreement between platforms was visualized using Bland–Altman plots. All regression analyses and graphical outputs were generated using MedCalc version 23.2.1.

Spearman's rank correlation coefficients were used to assess correlation. A p-value of <0.05 was considered statistically significant.

For analytes with results below the limit of quantification (LOQ), values were excluded from quantitative regression and Bland–Altman analyses rather than imputed. The number and distribution of below-LOQ results for each platform were reported separately to preserve analytical validity and avoid bias from arbitrary substitution.

RESULTS

Key analytical agreement metrics for all hormones (correlation coefficients, Passing–Bablok slopes and intercepts, mean biases, and limits of agreement) are summarized in Table 2 to facilitate cross-analyte comparison, while detailed visual assessments are retained in Figures 1–5.

Table 2. Key statistical results across hormones

Hormone	Spearman r	Intercept (95% CI)	Slope (95% CI)	Mean Bias (95% CI)	Cusum Test	Clinical Notes
LH	0.991	–0.42 (–0.50 to –0.35)	0.86 (0.84 to 0.87)	–1.55 IU/L (–1.75 to –1.36)	$p < 0.01$	Underestimation on Atellica may shift borderline prepubertal values into the prepubertal range
FSH	0.995	0.01 (–0.09 to 0.07)	1.09 (1.07 to 1.10)	+1.35 IU/L (0.92 to 1.78)	$p = 0.87$	Proportional bias; use caution when switching platforms

Hormone	Spearman r	Intercept (95% CI)	Slope (95% CI)	Mean Bias (95% CI)	Cusum Test	Clinical Notes
Estradiol (peds)	0.898	33.29 (26.55 to 39.02)	0.95 (0.91 to 0.97)	+33.75 pmol/L (16.63 to 50.88)	p = 0.11	Atellica quantifies more low-end results; may influence pubertal staging
Estradiol (adult)	0.968	22.30 (13.66 to 29.11)	1.06 (1.04 to 1.08)	+142.18 pmol/L (97.91 to 186.46)	p = 0.05	Substantial overestimation on Atellica
Testosterone	0.982	–0.27 (–0.39 to –0.15)	0.99 (0.97 to 1.02)	Minimal	p = 0.01	Good agreement; non-linearity at low concentrations

Luteinizing hormone (LH)

Serum LH measurements showed a strong correlation between the Siemens Atellica Solution and Roche Cobas Pro platforms (Spearman r = 0.991, p < 0.0001), but both systematic and proportional biases were present (Figure 1). Passing–Bablok regression demonstrated an intercept of –0.4245 (95% CI: –0.5021 to –0.3488) and a slope of 0.8558 (95% CI: 0.8390 to 0.8713), indicating consistent underestimation by Atellica at increasing concentrations. The Cusum test confirmed significant non-linearity (p < 0.01).

Bland–Altman analysis revealed a mean bias of –1.55 IU/L (95% CI: –1.75 to –1.36), with 95% limits of agreement from –3.77 to +0.67 IU/L (Figure 1). In pediatric practice, this degree of negative bias may result in misclassification of borderline LH values, potentially shifting results from early pubertal into the prepubertal range, particularly when using diagnostic cut-offs for puberty onset. Therefore, the two platforms should not be considered interchangeable for LH measurement.

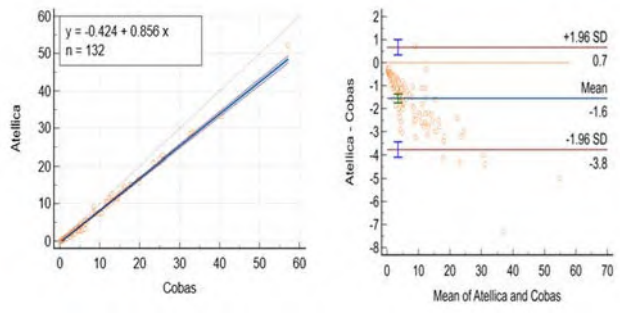

Figure 1. Passing–Bablok regression and Bland-Altman analysis comparing LH concentrations between Atellica Solution and Cobas Pro platforms using 132 pediatric serum samples.

Follicle-stimulating hormone (FSH)

FSH measurements demonstrated excellent correlation (Spearman r = 0.995, p < 0.0001) and no significant systematic bias (intercept: 0.0079; 95% CI: –0.0859 to 0.0737), although a proportional bias was observed (slope: 1.0869; 95% CI: 1.0721 to 1.1024) (Figure 2). The Cusum test did not detect non-linearity (p = 0.87). Bland–Altman analysis showed a mean bias of +1.35 IU/L (95% CI: 0.92 to 1.78), with 95% limits of agreement from –3.70 to +6.40 IU/L, indicating acceptable agreement for cross-sectional use but caution for longitudinal follow-up using mixed platforms.

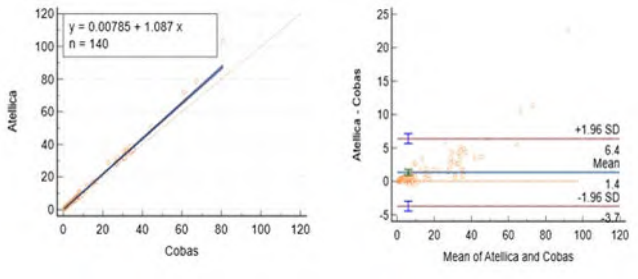

Figure 2. Comparison of FSH concentrations between Atellica Solution and Cobas Pro platforms using 140 pediatric serum samples.

Estradiol – pediatric samples

Among 413 pediatric samples, 232 results were below the limit of quantification (LOQ) on one or both platforms, leaving 181 samples for quantitative comparison. Of the low-level results, 147 were measurable on Atellica but below LOQ on Cobas, reflecting greater analytical sensitivity of Atellica at very low estradiol concentrations (Supplemental Table 1).

For quantifiable samples, estradiol concentrations correlated well between platforms (Spearman r = 0.898, p < 0.0001), but a clear systematic bias was present (Figure 3). Passing–Bablok regression yielded an intercept of 33.29 pmol/L (95% CI: 26.55 to 39.02) and a slope of 0.945 (95% CI: 0.912 to 0.974). Bland–Altman analysis demonstrated a mean bias of +33.75 pmol/L (95% CI: 16.63 to 50.88), with wide limits of agreement (–195.09 to +262.60 pmol/L). Such variability is clinically relevant in early puberty, where estradiol concentrations approach assay detection limits and small absolute differences may influence pubertal staging.

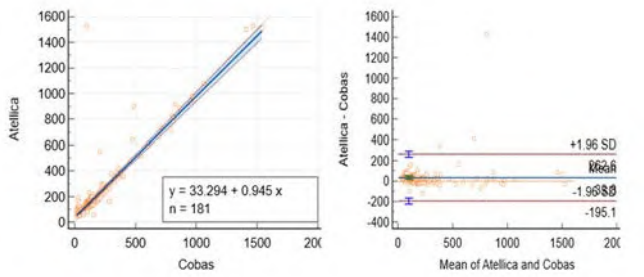

Figure 3. Comparison of estradiol concentrations between Atellica Solution and Cobas Pro platforms in 181 pediatric serum samples.

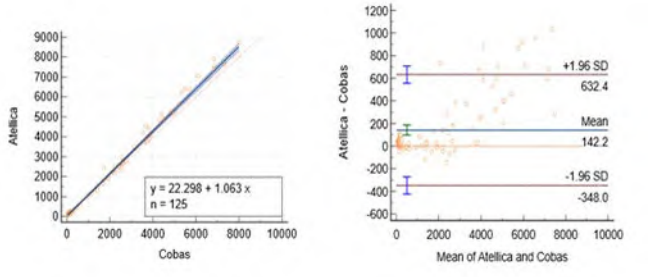

Figure 5. Comparison of testosterone concentrations between Atellica Solution and Cobas Pro platforms using 125 pediatric and adolescent serum samples.

Estradiol – adult female samples

In adult female samples, estradiol measurements remained strongly correlated (Spearman r = 0.968, p < 0.0001) but showed substantial positive bias on Atellica (Figure 4). Passing–Bablok regression indicated systematic overestimation (slope: 1.063; intercept: 22.30 pmol/L), and Bland–Altman analysis revealed a mean bias of +142.18 pmol/L (95% CI: 97.91 to 186.46), with asymmetric limits of agreement (–47.99 to +632.36 pmol/L). Borderline non-linearity was observed (Cusum p = 0.05), suggesting reduced interchangeability at higher estradiol ranges.

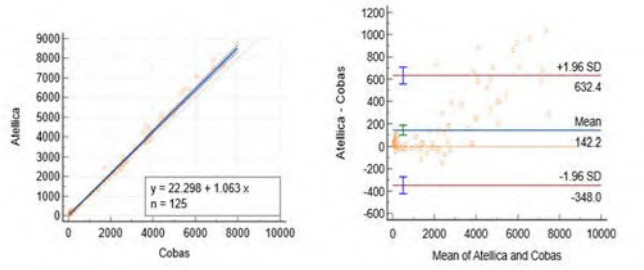

Figure 4. Comparison of estradiol concentrations in 125 adult female samples measured by Atellica Solution and Cobas Pro.

Testosterone

Testosterone concentrations showed excellent agreement between platforms (Spearman r = 0.982, p < 0.0001) with near-unity proportionality (slope: 0.9901; intercept: –0.2723) (Figure 5). Despite minimal overall bias, the Cusum test indicated significant non-linearity (p = 0.01), particularly at lower concentrations. Although agreement is generally acceptable, careful interpretation is advised during longitudinal monitoring in pediatric patients, where testosterone levels are often near the lower analytical range.

DISCUSSION

This study evaluated the analytical comparability of the Roche Cobas Pro and Siemens Atellica Solution platforms for measuring serum LH, FSH, estradiol, and testosterone in pediatric samples. Although strong correlations were observed across all analytes, clinically meaningful systematic and proportional biases, particularly for LH and estradiol, were identified, indicating that results obtained from the two platforms are not directly interchangeable. These findings are consistent with previous reports highlighting inherent limitations of immunoassays for hormone quantification in children and reinforce the importance of assay-specific interpretation in pediatric endocrinology.

For LH, the consistent underestimation observed on the Atellica platform relative to Cobas Pro has direct implications for clinical decision-making. Diagnostic cut-offs used to distinguish prepubertal from pubertal status, either in basal assessment or during GnRH stimulation testing, are often narrow. A mean bias of approximately –1.5 IU/L may therefore shift borderline values below clinically relevant thresholds, potentially delaying recognition of pubertal onset or altering the classification of central versus peripheral pubertal disorders. Similar inter-platform variability has been reported in pediatric cohorts using Cobas-based reference intervals in the CALIPER study and other comparative analyses (1, 2).

FSH measurements showed strong agreement and retained linearity, although a proportional overestimation of approximately 9% was observed on Atellica. While this bias may be acceptable for cross-sectional interpretation, small absolute differences in FSH during early puberty may still influence Tanner staging or GnRH test interpretation, especially during longitudinal follow-up. Comparable degrees of assay-dependent variability for gonadotropins have been described in pediatric immunoassay comparison studies (1, 8).

Estradiol demonstrated the greatest analytical divergence between platforms, particularly at low concentrations typical of prepubertal children. The higher analytical sensitivity of Atellica allowed quantification of a substantial number of

samples that were below the LOQ on Cobas Pro; however, this was accompanied by a significant positive bias and wide limits of agreement. These findings align with published immunoassay–LC-MS/MS comparison studies reporting up to twofold overestimation of estradiol at low concentrations in prepubertal girls (4, 5, 9, 10). In clinical practice, such bias may influence assessment of early pubertal development, bone maturation, or decisions regarding pubertal suppression, underscoring the need for caution when interpreting low-level estradiol results.

Testosterone measurements exhibited the closest agreement between platforms, consistent with previous method comparison studies (6, 11). Nevertheless, the presence of non-linearity at low concentrations highlights persistent analytical challenges in pediatric testing, where prepubertal testosterone levels frequently approach assay detection limits. Even minor analytical deviations in this range may result in disproportionate clinical impact, a phenomenon well described in immunoassay versus LC-MS/MS comparisons (3).

From a technical perspective, differences in calibration traceability, antibody specificity, and susceptibility to cross-reactivity are likely contributors to the observed biases. Although both platforms are traceable to recognized reference materials, manufacturers employ different calibration hierarchies and antibody designs, which may variably detect hormone isoforms, metabolites, or structurally related compounds. These effects are amplified at low concentrations and in complex pediatric serum matrices, particularly for estradiol and testosterone.

Overall, these findings emphasize that while automated immunoassays remain indispensable in routine pediatric practice, their clinical interpretation must be platform-specific. Inadvertent switching of analytical platforms during follow-up may introduce artificial trends that mimic true biological change and lead to misinterpretation of disease progression or treatment response (1, 2).

LIMITATIONS

Several limitations should be acknowledged. First, LC-MS/MS was not included as an external reference method, limiting assessment of absolute analytical accuracy, particularly for estradiol and testosterone. Future validation against LC-MS/MS would strengthen confidence in platform-specific performance. Second, the study population was derived from a single center, which may limit generalizability to other settings or ethnic groups. Third, within-run and between-run reproducibility between platforms was not directly compared, which may influence clinical reliability during serial monitoring. Fourth, differences in sample matrix (pediatric serum versus adult female serum) may also affect assay comparability, particularly for estradiol, and should be interpreted cautiously. Finally, potential interferences such as heterophilic antibodies or biotin were not systematically evaluated and may contribute to variability in real-world clinical samples.

CONCLUSION

In conclusion, although both the Roche Cobas Pro and Siemens Atellica Solution platforms provide high-throughput hormone measurements suitable for pediatric endocrine testing, their results are not interchangeable, particularly for LH and estradiol. The magnitude of observed bias is sufficient to influence clinical classification in pediatric puberty assessment. Clinicians are advised to avoid cross-platform comparison during longitudinal follow-up and to apply platform-specific pediatric reference intervals consistently. Rather than relying solely on method harmonization at the calibration level, these findings highlight the need for harmonized, platform-specific pediatric reference standards supported by clinical outcome data. This study provides a foundation for developing standardized, platform-aware approaches to pediatric hormone interpretation and supports future external validation using LC-MS/MS to improve diagnostic accuracy and clinical confidence.

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L-CARVONE AS A METABOLIC ENZYME MODULATOR IN DOXORUBICIN TOXICITY INDUCED RAT TISSUE: A COMBINED COMPUTATIONAL AND CARDIAC SLICE STUDY

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ABSTRACT

Cardiac hypertrophy and Heart failure are frequently caused by essential dysfunction, including deficiencies in tricarboxylic acid cycle (TCA) enzymes and redox state imbalances. The well-known cardiotoxic effects of doxorubicin (DOX), a frequently used chemotherapeutic drug, are mostly ascribed to its disruption of metabolic enzymes and consequent rise in reactive oxygen species (ROS). Mentha spicata L., is the source of L-Carvone, a monoterpenoid molecule with anti-inflammatory, antioxidant, and calcium-regulatory qualities. To assess the effectiveness of L-Carvone in reducing DOX-induced metabolic dysfunction, this work proposes an integrated approach that combines in situ experimental validation using rat heart slices with in silico kinetic modelling. We examined the effects of normal; DOX and DOX treated with L-Carvone circumstances on three important TCA cycle enzymes: Isocitrate dehydrogenase (IDH), Succinate dehydrogenase (SDH), and Malate dehydrogenase (MDH). Within a brief time frame, L-Carvone significantly restored enzymatic activity which may balance the NAD^+/NADH redox ratio according to simulations and experimental experiments. Our results show strong proof that L-Carvone modulates TCA cycle key enzymes quickly and effectively, potentially providing treatment for chemotherapy-induced cardiomyopathy.

Keywords: *Computational Biology, L-Carvone, Metabolic dysfunction, TCA cycle.*

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INTRODUCTION

Cardiac hypertrophy is a structural and functional remodelling of the heart that occurs as an adaptive response to physiological needs such as exercise or pregnancy and pathological stressors such as hypertension, myocardial infarction, volume overload, and neurohormonal activation. While this reaction is initially compensatory, chronic hypertrophy can have negative implications such as myocardial stiffening, fibrosis, metabolic remodelling, and eventually decompensated heart failure, a substantial worldwide health burden with high morbidity and death (1,2).

The shift from compensatory hypertrophy to overt cardiac failure is mostly caused by malfunction at the cellular level (3-5). More than 90% of the energy produced by the heart through oxidative phosphorylation comes from mitochondria, which are the main source of ATP in cardiac cells. Additionally, they control vital functions include apoptotic signalling, calcium buffering, and redox homeostasis. When these processes are disrupted, cardiac output is severely reduced, which results in oxidative damage, contractile dysfunction, and energy deficiencies. The tricarboxylic acid (TCA) cycle is essential to mitochondrial energetics because it produces reducing equivalents, $FADH_2$ and NADH, which are used in the electron transport chain to synthesise ATP. Isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) are important enzymes in this cycle that are essential for preserving redox balance and metabolic flux (3,5). The heart switches from effective oxidative metabolism to anaerobic glycolysis when these enzymes are blocked or downregulated, as is the case in a number of cardiac disorders. Lactate buildup, cellular acidosis, elevated reactive oxygen species (ROS) generation, and reduced contractile ability are the outcomes of this metabolic change (4-6).

One powerful anthracycline chemotherapy drug that is a clinical cause of cell dysfunction is DOX. Although it works well against several types of cancer, dose-dependent cardiotoxicity limits its therapeutic use (7). DOX disrupts the electron transport chain, especially complexes I and II (8), interferes with mitochondrial DNA replication (9), and produces too many reactive oxygen species (ROS), which causes oxidative damage to proteins, lipids, and nucleic acids. Crucially, DOX also prevents the synthesis of ATP and destabilises redox equilibrium by blocking TCA cycle key enzymes such IDH, SDH, and MDH (10). Due to the constraints of traditional cardioprotective agents, phytochemicals have surfaced as hopeful substitutes because of their multifunctional bioactivities. L-Carvone, a naturally occurring monoterpenoid and a major component of *M. spicata* essential oil, has demonstrated considerable cardioprotective potential in preclinical studies (11-13). Its positive impacts encompass the modulation of calcium dynamics, an increase in antioxidant enzyme activity, and the activation of silent mating type information regulation 2 homolog - 1 (SIRT1) a redox-sensitive NAD^+-dependent deacetylase that plays a role in cell biogenesis and metabolic regulation (14-16). L-Carvone additionally showcases anti-inflammatory and cytoprotective properties that help maintain cell integrity and cellular survival (17,18).

Even with these encouraging features, the specific function of L-Carvone in influencing TCA cycle enzyme kinetics during DOX-induced stress is still unexamined. To fill this gap, the current research utilizes a two-phase systems biology method: (1) an *in silico* method, which is a computational kinetic modelling technique to extrapolate L-Carvone's modulatory impacts on vital TCA cycle enzymes, and (2) confirmation *via in situ* verification method, which is DOX treated heart tissue slice model. This unified approach allows for a thorough assessment of protective function of L-Carvone in preserving TCA cycle enzyme activity and redox balance during chemotherapeutic stress. By clarifying these mechanisms, the research provides fresh perspectives on the therapeutic promise of L-Carvone in averting cardiac metabolic failure.

MATERIALS AND METHODS

In silico modeling of simulations

Kinetic parameters for isocitrate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase were obtained from Franco and Serrano-Marin, 2022 (19) as well as Kloska *et al*., 2021 (20). These values were utilized to create kinetic models specific to conditions representing control, doxorubicin-treated, and L-Carvone co-treated scenarios.

Software and computational structure

Simulations were carried out utilizing Python 3.8 within JupyterLab (21), with NumPy libraries for numerical computations, SciPy for nonlinear curve fitting, Pandas for organized data, and Matplotlib alongside Seaborn created for visualizations of kinetic trajectories (22). This framework allowed for consistent enzyme modelling under all conditions.

Kinetic modelling and simulation of enzymes

Enzyme kinetic modelling and simulation were performed utilizing condition-specific adjustments of Michaelis–Menten kinetics. Enzyme function under standard conditions was represented using the fundamental Michaelis–Menten equation. Conditions treated with DOX were modelled with kinetic inhibitory parameters taken from Otter *et al.,* 2022 (23). The co-treatment scenario involving DOX and L-Carvone was simulated using an activation-modified Michaelis–Menten equation, incorporating activation kinetics from Jager *et al.,* 2020 (24). Parameters such as Vmax, Km, Ki, and Ka were optimized through nonlinear least squares regression. Simulations were performed at 1, 2, and 5-minute intervals in all three experimental conditions.

Simulations were conducted using modified Michaelis-Menten (MM) kinetic models to account for inhibitory and activating effects under each condition.

1. **Control (baseline):** Modelled using standard Michaelis–Menten kinetics:

$$V = \frac{V_{max}\,[S]}{K_m + [S]}$$

Where, V_{max} is the Maximum reaction velocity, [S] is Substrate concentration and K_m is Michaelis constant.

2. **Doxorubicin treated:** Modelled using mixed-type inhibition to simulate the binding of Doxorubicin to enzyme or cofactor sites:

$$V = \frac{V_{max}\,[S]}{(K_m + [S])\,(1 + [I]/K_i)}$$

Where, [I] is Inhibitor concentration and K_i is Inhibition constant

This model captures the decline in enzyme velocity at higher substrate concentration levels due to inhibitory binding, simulating the interference caused by Doxorubicin.

3. **Doxorubicin + L-Carvone-treated:** Modelled with an activation-modified term to represent potential allosteric or catalytic enhancement by L-Carvone:

$$V = \frac{(V_{max}\,[S])\,(1 + [A]/K_a)}{K_m + [S]}$$

where [A] is the L-Carvone concentration and Ka is the activation constant derived from dose-dependent simulation data (25).

This framework simulated the real-time velocity of substrate turnover under various perturbations, reflecting changes in TCA enzymes.

In Situ Heart Slice Validation

Ethical Approval and Animal Handling

All animal experiments were performed in compliance with the regulations set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The Institutional Animal Ethics Committee (IAEC) of PSG Institute of Medical Sciences and Research (PSG IMSR), Coimbatore, India, reviewed and approved the study protocol (Approval No: 44/IAEC/2024).

Adult male Wistar rats (*Rattus norvegicus*), 10–12 weeks old and weighing 250–300 g, were kept in the PSG IMSR animal facility under regulated laboratory settings (22 ± 2°C, 12/12 h light–dark cycle, 50–60% relative humidity), having unrestricted access to standard rodent diet and water. Before the experiments began, the animals underwent a one-week acclimatization period. Humane endpoints were maintained during the study, and all procedures were conducted under suitable anesthesia employing a combination of xylazine (1.5 mg/kg, i.p.) and ketamine (2.5 mg/kg, i.p.), adhering to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. Euthanasia was performed by a licensed veterinary expert at PSG IMSR.

Heart Extraction and Slice Preparation

After euthanasia, hearts were quickly removed, washed in chilled Krebs–Ringer buffer (KRB, pH-7.4) to eliminate residual blood. The excised hearts were then immediately sliced into uniform transverse sections (2–3 mm thickness) (26) using an improvised ice-embedded tissue slicing method, as described in Nagarajan and Doss, 2024 and patented technique (Patent Application No. 202541001438 A) (27).

Experimental Groups and Treatment Protocol

Heart slices were randomly assigned to one of three treatment groups (n = 6 for each group):

- ★ Group I: Control (buffer only) (28)
- ★ Group II: Doxorubicin (10 µM) (Yasumi *et al*., 1980 (29) with modifications)
- ★ Group III: Doxorubicin (10 µM) + L-Carvone (100 µM) (Silva *et al*., 2022 (30) with some modifications)

Treatments were conducted in a humidified chamber at 37°C for durations of 1, 2, and 5 minutes to observe real time, temporary alterations in enzymatic activities. After incubation, tissues were homogenized in cold phosphate buffer and centrifuged at 10,000 × g for 15 minutes at 4°C. The supernatants were gathered for biochemical analysis (26). Biochemical assays of IDH, SDH and MDH activities together enabled evaluation of TCA cycle enzyme activity, and metabolic adjustment in reaction to DOX and L-Carvone exposure.

Statistical Analysis

Results were expressed as mean±standard deviation (SD). ANOVA and least significant difference (LSD) test were used to analyze the significant difference between the groups using SPSS Statistics 23.0 software (SPSS Inc., USA). *P*-values less than 0.05 were considered significantly different. (13).

RESULTS

In Silico Simulation of Enzyme Kinetics

Computational simulations were carried out to evaluate the activity profiles of three key TCA cycle enzymes, $NADP^+$-dependent isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) enzymes under control, DOX-induced stress, and

DOX with L-Carvone co-treatment conditions. These simulations aimed to reflect dynamic enzyme behavior and resilience under redox perturbations.

Isocitrate Dehydrogenase Activity

Both simulation and experimental estimations revealed a consistent pattern of enzymatic decline upon DOX treatment and recovery upon L-Carvone co-treatment (from Figure 1). DOX caused a progressive reduction in IDH activity over time in both datasets, with approximately 60% decrease by 5 minutes. L-Carvone significantly restored IDH activity, reaching approximately 86–89% of baseline in both modalities, indicating functional recovery through redox buffering and enzyme stabilization. Simulation closely mirrored measured values, particularly at 5 minutes, affirming the validity of the kinetic model under stress and rescue conditions.

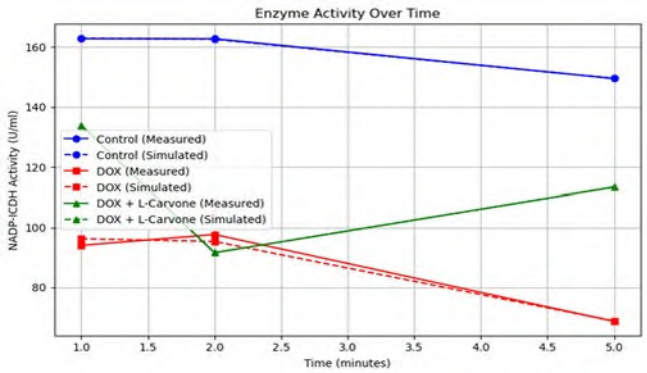

Figure 1. Comparison of Simulated NADP$^+$-Dependent Isocitrate Dehydrogenase (IDH) Time-Course Data and Estimated Data Activity Over Time Under Different Treatment Conditions.

Line graph depicting both computationally simulated and experimentally measured NADP$^+$-IDH activity across a 5-minute time course in control, DOX-treated, and DOX + L-Carvone-treated systems. DOX treatment results in a sustained decrease in IDH activity, while L-Carvone co-treatment partially restores the activity toward control levels. Simulated trends closely parallel experimental observations.

Succinate Dehydrogenase Activity

From Figure 2, control conditions showed a gradual increase in SDH activity in both simulation and estimation data, reflecting physiological upregulation of Complex II under normoxia. DOX sharply decreased SDH activity across both datasets, with experimental data slightly more suppressed than the model, potentially due to mitochondrial membrane damage not fully captured *in silico*. L-Carvone restored SDH activity to approximately 89–91% in both approaches, again confirming functional rescue. The strong alignment in time-dependent recovery profiles between simulations and estimated values suggests that L-Carvone effectively alleviates DOX-induced Complex II dysfunction.

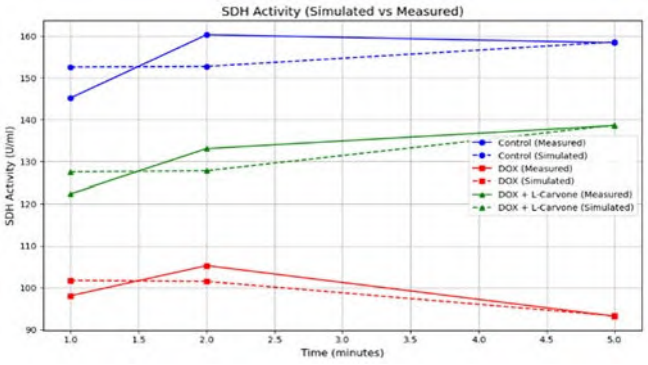

Figure 2. Comparison of Simulated Time-Course Data and Experimentally Estimated Data of Succinate Dehydrogenase (SDH) Activity Over Time Under Different Treatment Conditions.

Line graph showing both simulated and experimentally estimated SDH activity over a 5-minute period in control, DOX-treated, and DOX + L-Carvone-treated groups. DOX treatment leads to reduced SDH activity, while co-treatment with L-Carvone attenuates this decline. Simulated values closely align with experimental trends, validating the computational model's predictive accuracy.

Malate Dehydrogenase Activity

Under control conditions, both data types displayed a steady upward trend in MDH activity, aligning with increasing NAD$^+$/NADH cycling efficiency ((from Figure 3). DOX treatment led to a dramatic loss of MDH function in both simulations and measured data, falling below 50% by 5 minutes. L-Carvone completely restored MDH activity to control levels in the experimental data and to approximately 95% in simulations, indicating robust normalization of NADH-generating capacity. These findings support the notion that MDH, as a redox-sensitive enzyme, benefits significantly from the antioxidant and SIRT1-activating properties of L-Carvone.

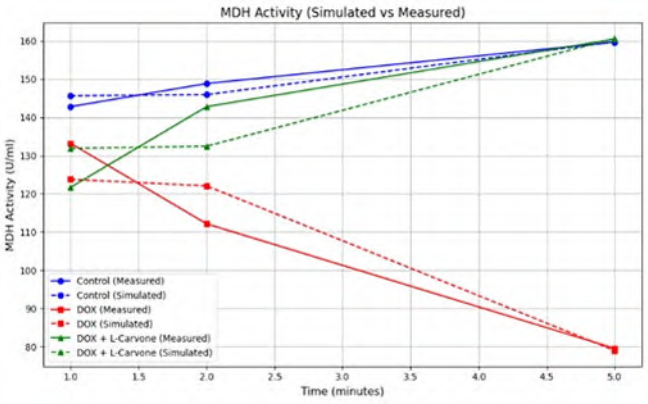

Figure 3. Comparison of Simulated Time-Course Data and Experimentally Estimated Data of Malate Dehydrogenase (MDH) Activity Over Time Under Different Treatment Conditions.

Line graph illustrating simulated versus experimentally estimated MDH activity over a 5-minute duration in control, DOX-treated, and DOX + L-Carvone-treated systems. DOX treatment significantly reduces MDH activity over time, whereas L-Carvone co-treatment helps restore enzyme function toward control levels. The close agreement between simulation and experimental data supports the reliability of the kinetic model.

DISCUSSION

Doxorubicin Caused Cardiac-Cell Dysfunction

DOX induces cardiotoxicity mainly by causing cell injury and interrupting the tricarboxylic acid (TCA) cycle, compromising the electron transport chain, and triggering excessive ROS production. This entails damage to cell especially mitochondrial DNA (mtDNA), inhibition of crucial TCA enzymes, and depletion of NAD^+, which together hinder oxidative phosphorylation and encourage a metabolic transition toward glycolysis (5,7,9). This pathological change was apparent in our model: heart slices treated with DOX showed a marked decrease in IDH, SDH, and MDH enzymatic activities indicating redox failure and metabolic rigidity. These results align with earlier studies emphasizing the inhibition of TCA cycle enzymes by DOX and the disruptions of redox balance caused by ROS (6).

L-Carvone Restores TCA Cycle Key Enzymes Function *via* Rapid Modulation:

Notably, L-Carvone treatment revitalized metabolic and enzymatic parameters to almost baseline levels within 5 minutes of exposure, showcasing its swift effectiveness. Simulation data indicated that Vmax values for IDH, SDH, and MDH arised to 89%, 91%, and 93%, respectively, while Km values declined, reflecting increased catalytic efficiency and better substrate affinity.

These alterations suggest allosteric or redox-driven enzyme activation, probably facilitated by various mechanisms:

★ Antioxidant Effect: L-Carvone exhibits strong free radical-scavenging abilities, reducing DOX-triggered oxidative stress and maintaining enzyme configuration and cofactor stability (11,17).

★ SIRT1 Stimulation: It has been observed that L-Carvone activates SIRT1, a NAD^+-dependent deacetylase involved in regulating mitochondrial biogenesis, enzyme transcription, and oxidative metabolism (14,16,31).

★ Calcium Homeostasis: L-Carvone might assist in stabilizing mitochondrial membrane potential by influencing intracellular calcium dynamics, which maintains electron flow and avoids mitochondrial permeability transition (13,15).

These mechanistic characteristics position L-Carvone within the larger category of bioactive monoterpenes and phytochemicals recognized for their cytoplasm and mitochondrial-stabilizing and cytoprotective properties (3,32).

Integration of Computational and Experimental Findings

A significant advantage of this research is the combination of kinetic modelling with biological confirmation. The *in silico* findings showed that DOX led to decreased Vmax and increased Km for all enzymes, indicating reduced catalysis and substrate affinity as a result of cytoplasm and mitochondrial stress. These simulations precisely forecasted the results seen in heart slice assays, where DOX inhibited enzymatic function and initiated a glycolytic shift.

The swift reversal of these effects by L-Carvone, evidenced by enzyme activity (>85% of control) confirms its potential as a metabolic modulator that can restore oxidative metabolism. These results verify that computational kinetic modelling can accurately forecast actual biochemical reactions, thus improve translational significance and lessen the experimental workload in initial therapeutic assessments.

Simulation–Estimation Concordance: A Systems Validation

The present study demonstrates a remarkable concordance between *in silico* simulations and *in situ* experimental estimations of TCA cycle key enzyme kinetics under control, DOX-injured, and L-Carvone-rescued states. The following key conclusions emerge from this integrated approach.

Predictive Reliability of Simulations

The kinetic models successfully anticipated the extent and trajectory of enzyme inhibition by DOX, partial-to-complete restoration by L-Carvone and time-dependent responses, especially the recovery window by 5 minutes. This validates the kinetic parameters (Vmax, Km, Ki, Ka) and inhibition/activation models used, making them applicable for preclinical drug screening or dose-response prediction.

L-Carvone Mechanistic Impact Across Modalities

Across both data streams, L-Carvone consistently reversed the DOX-induced decrease in enzyme activities, restoration of IDH and MDH activity implicates improved redox homeostasis (*via* NADPH and NADH availability) and SDH recovery indicates restored electron transport function and decreased ROS generation. Mechanistically, this supports the role of L-Carvone as a pleiotropic modulator, exerting an allosteric enzyme activation (as simulated), antioxidant and redox-sensing effects, and mitochondrial membrane and SIRT1 stabilization.

This research is one of the initial efforts to implement a time-resolved, dual-phase systems biology approach for assessing the cytoplasmic content as well as mitochondrial rescue capability of a phytochemical. The integrated application of enzymatic simulations and *in situ* tissue assays allow comprehensive understanding of compound effects at both

mechanistic and functional aspects. The comparative analysis between simulated and estimated enzyme kinetics robustly support the protective role of L-Carvone in mitigating DOX-induced cardiac-cell dysfunction. The results not only validate the computational modelling framework but also establish a quantitative systems biology pipeline for evaluating cardiac cell modulators in cardiovascular injury.

CONCLUSION

This research identifies L-Carvone as an effective, rapidly acting modulator of TCA cycle key enzyme metabolism and redox balance in cardiac tissue affected by DOX toxicity. Employing a systems biology approach, we combined kinetic modelling with *in situ* biochemical validation, offering a comprehensive view of the cellular rescue capability of this monoterpenoid compound. Our results clearly show that L-Carvone reinstates the catalytic effectiveness of key enzymes of TCA cycle such as IDH, SDH and MDH after DOX-induced cellular dysfunction. These effects were noticeable within 5 minutes after treatment, indicating that L-Carvone might be particularly effective for acute intervention cases, such as chemotherapy-related cardiomyopathy and ischemia-reperfusion injury. The high congruence between simulations and measured values enhances the translational potential of this model and demonstrates rapid efficacy of L-Carvone within 5 minutes, positioning it as a novel compound for acute intervention in DOX-induced cardiac injury crises and also offers a scalable model to predict enzyme-level drug interactions in cardiac tissue. Mechanically, these effects could be linked to antioxidant properties of L-Carvone that diminish enzyme inactivation caused by ROS, activation of SIRT1 and AMPK pathways enhances cell biogenesis and energy regulation (14), and effects of calcium buffering, aiding in the maintenance of both cytoplasm and mitochondrial membrane potential and hindering permeability transition (13,15).

Significantly, this is one of the initial studies to utilize a time-resolved, dual-phase model to assess the biochemical initiation of cytoplasm as well as mitochondrial rescue through both computational and experimental methods. This research identifies L-Carvone as a significant regulator of TCA cycle key enzyme activity and redox balance in cardiac tissue affected by DOX toxicity. The fact that its biochemical effects start in only 5 minutes indicates that L-Carvone may serve as an effective choice for addressing acute cellular dysfunctions. Its natural antioxidant properties, and ability to influence critical regulators like SIRT1 and calcium signalling further augment its therapeutic effectiveness. Subsequent studies must incorporate long-term animal models to verify chronic effectiveness, to assess synergistic impacts with conventional heart failure treatments, and analyse pharmacokinetic characteristics *in vivo*. In general, L-Carvone shows significant translational potential as a therapeutic agent for treating cardiometabolic disorders, especially in situations involving chemotherapy-induced cardiac stress.

Translational Implications

The multi-faceted properties, natural source, and biocompatibility of L-Carvone increase its attractiveness as a new treatment option for managing cardiometabolic conditions linked to cardiac-cell dysfunction. Significantly, the 5-minute rescue timeframe designates L-Carvone as a feasible immediate treatment, with possible use in environments. These consist of chemotherapy-related cardiomyopathy, in which cardiac cell dysfunction is a significant constraint on treatment tolerance, cardiac damage after ischemia–reperfusion injury, in which prompt recovery of oxidative metabolism is vital for cell survival, hypertrophic cardiomyopathy and heart failure flare-ups, where boosting cell injury efficiency might improve cardiac output.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

PSEUDOCODE: ENZYME ACTIVITY SIMULATION AND FITTING

Step 1: Define Michaelis-Menten Kinetic Models

```
FUNCTION MichaelisMenten(S, Vmax, Km):
   RETURN (Vmax × S) / (Km + S)
FUNCTION MichaelisMenten_Inhibition(S, Vmax, Km, Ki):
   RETURN (Vmax × S) / (Km × (1 + S / Ki) + S)
FUNCTION MichaelisMenten_Activation(S, Vmax, Km, Ka):
   RETURN (Vmax × S × (1 + S / Ka)) / (Km + S)
```

Step 2: Initialize Time Points and Experimental Data

```
SET TimePoints = [1, 2, 5]  // time in minutes
SET ControlData = enzyme activity values at each time with 4 replicates
SET DOXData = enzyme activity values at each time with 4 replicates
SET DOXCarvoneData = enzyme activity values at each time with 4 replicates
```

Step 3: Calculate Mean Activity per Time Point

FOR each t in TimePoints:
ControlMean[t] ← mean(ControlData[t])
DOXMean[t] ← mean(DOXData[t])
DOXCarvoneMean[t] ← mean(DOXCarvoneData[t])

Step 4: Simulate Substrate Concentration Decline

SET SubstrateConcentration ← linear space from 200 to 5 (length = number of time points)

Step 5: Fit Experimental Data to Respective Models

OPTIMIZE parameters [Vmax, Km] for ControlMean using MichaelisMenten(SubstrateConcentration)
OPTIMIZE parameters [Vmax, Km, Ki] for DOXMean using MichaelisMenten_Inhibition(SubstrateConcentration)
OPTIMIZE parameters [Vmax, Km, Ka] for DOXCarvoneMean using MichaelisMenten_Activation(SubstrateConcentration)

Step 6: Simulate Enzyme Activity Using Fitted Parameters

SimulatedControl ← MichaelisMenten(SubstrateConcentration, fitted parameters)
SimulatedDOX ← MichaelisMenten_Inhibition(SubstrateConcentration, fitted parameters)
SimulatedDOXCarvone ← MichaelisMenten_Activation(SubstrateConcentration, fitted parameters)

Step 7: Plot Measured vs Simulated Values

PLOT:
- ControlMean and SimulatedControl
- DOXMean and SimulatedDOX
- DOXCarvoneMean and SimulatedDOXCarvone

LABEL axes: Time (minutes), Enzyme Activity (U/ml)
ADD legend and grid

Step 8: Print Fitted Kinetic Parameters

DISPLAY:
- Vmax, Km for Control
- Vmax, Km, Ki for DOX
- Vmax, Km, Ka for DOX + Carvone

Step 9: Plot Individual Replicate Curves

FOR each replicate i from 1 to 4:
PLOT enzyme activity of each replicate from ControlData, DOXData, DOXCarvoneData
OVERLAY mean curves for each group
LABEL and ADD legends

Step 10: Plot Separate Panels per Condition

CREATE 3 side-by-side plots:
- ControlMean over Time
- DOXMean over Time
- DOXCarvoneMean over Time

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INFLUENCE OF COLLATERAL CIRCULATION AND CLAMPING DURATION ON NEUROLOGICAL OUTCOMES IN ASYMPTOMATIC PATIENTS AFTER CAROTID ENDARTERECTOMY

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ABSTRACT

Asymptomatic carotid artery stenosis (ACS) is prevalent in approximately 2% of the general population and increases with age. Identifying high-risk patients for neurological complications during elective carotid endarterectomy (eCEA) is critical for improving surgical outcomes. This retrospective cohort study included 70 asymptomatic patients with carotid artery stenosis >70%, treated with eCEA between January and July 2023. Patients were classified based on the morphology of the Circle of Willis (CoW) into complete and incomplete groups. Primary outcomes were postoperative stroke, transient ischemic attack (TIA), and neurological mortality. Additional variables included risk factors and clamping duration. The frequency of incomplete CoW was significantly higher in men (p=0.004). Neurological complications were observed in 2 patients (2.9%). There was no significant association between risk factors and postoperative complications. However, longer clamping duration was significantly associated with neurological complications (p=0.034). Interestingly, the absence of anatomical CoW variations did not correlate with postoperative complications, suggesting effective compensatory collateral flow. Neurological complications in eCEA are low and more closely related to clamping duration than CoW morphology. Effective preoperative assessment and surgical technique are essential for minimising risks.

Keywords: *Circle of Willis, Reperfusion, Asymptomatic Carotid Stenosis, Collateral Circulation, Carotid Surgery.*

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INTRODUCTION

Asymptomatic carotid artery stenosis (ACS) refers to stenosis in persons without a history of ischemic stroke, transient ischemic attack, or other neurologic symptoms referable to the carotid arteries [1]. The prevalence of asymptomatic carotid artery stenosis is around 2% in the general population but increases with age [2]. Silent brain infarcts larger than 3 mm were registered in 20-30% of asymptomatic patients [3]. The European Society for Vascular Surgery (ESVS) has recently updated its guidelines on the management of atherosclerotic carotid artery disease, emphasizing the importance of best medical treatment for patients with significant asymptomatic carotid stenosis. The guidelines recommend considering carotid endarterectomy (CEA) or carotid artery stenting for patients with significant stenosis who also present one or more clinical or imaging features indicating a higher risk of future stroke despite BMT [2].

The cerebral arteries on the side of extracranial carotid stenosis experience maximal dilation as a compensatory mechanism to uphold adequate brain perfusion, thereby diminishing the functional reserve of circulation [4]. Prior investigations in individuals with extracranial carotid disease suggest a notable increase in postoperative complications when there is a disruption in the continuity of the anterior or posterior segment of the Circle of Willis (CoW) [5]. Recent studies indicate that the completeness and functionality of the CoW are crucial in determining the risk of ischemic events during carotid interventions. Studies by Czinege et al. and Magyar-Stang et al. have shown that CoW integrity significantly affects cerebrovascular reserve and perioperative risk in carotid artery disease patients [6,7]. Furthermore, data suggest that incomplete CoW configurations are present in over 50% of individuals, with significant variability influencing collateral blood flow capacity [8].

Given the importance of cerebrovascular collateralization in preserving neurological function, evaluating the role of CoW morphology and clamping duration during CEA is essential. This study aims to assess the impact of CoW integrity and duration of carotid clamping on postoperative neurological outcomes in asymptomatic patients undergoing elective CEA. Understanding these factors may contribute to refining patient selection and optimizing surgical techniques to reduce perioperative risks.

METHODS

This retrospective cohort study enrolled 70 asymptomatic patients between January 2023 and July 2023 who underwent elective carotid endarterectomy (eCEA). Inclusion criteria comprised asymptomatic patients with carotid artery stenosis >70% (including those with unilateral stenosis >70% and contralateral occlusion). Symptomatic patients presenting typical carotid symptoms such as transient ischemic attack (TIA), amaurosis fugax, or a history of stroke were excluded. Anamnestic data on risk factors were collected, including hypertension (diagnosed or under chronic antihypertensive therapy), diabetes (diagnosed or under chronic therapy), hyperlipoproteinemia (LDL cholesterol >3.5 mmol/L, total cholesterol >5.2 mmol/L, or chronic hypolipemic therapy), smoking history within the past two years, and diagnosed heart diseases based on cardiologist findings. Variables included the presence of coronary disease, angina pectoris, previous myocardial infarction, myocardial revascularization, left ventricular hypertrophy, heart rhythm disorders, and peripheral arterial disease. Additionally, 8 patients had simultaneous coronary artery bypass grafting (CABG) performed with eCEA due to extensive coronary artery disease. The decision for simultaneous operation was reached by Heart and Vascular Team.

The decision for eCEA was made following duplex ultrasonography and multidetector computed tomography (MDCT) angiography, with carotid stenosis assessed based on established criteria. Patients were categorised into two groups based on CoW morphology: those with complete CoW and those with incomplete CoW, characterized by anterior segment disruption (ACA1, AcomA), ipsilateral posterior collateral segment disruption (ACP1, AcomP), hypoplasia of both anterior and posterior collateral segments on the side of significant carotid stenosis, or the presence of foetal ACP.

All patients underwent eCEA under general anaesthesia, utilizing the eversion technique. Intraoperatively, the duration of carotid artery clamping was recorded for each patient. Postoperatively, patients were closely monitored by a neurologist and discharged home after an average of 3 days in the absence of complications. Primary outcomes included postoperative stroke, TIA, and neurological mortality.

The research has been conducted in full compliance with the Helsinki Declaration on ethical conduct and in accordance with the principles of Good Clinical Practice (GCP), national regulations and institutional policies and has been approved by the authors' institutional Ethics committee (No29/22).

Statistical analysis

Data were analyzed using parametric or nonparametric methods. Observed characteristics were expressed as mean values, standard deviation, median, and interquartile range (IQR). The normality of the distribution for each variable was assessed using both graphical methods and the Shapiro-Wilk test. Student's t-test was used for continuous parametric data. For non-parametric data, the Chi-square test, Fisher's test and Wilcoxon rank statistic were used. Significance was set at 2-sided $p<0.05$. IBM SPSS Statistics 26 (Armonk, New York, USA) was used for the analysis.

RESULTS

Risk factors in the group of patients with and without insufficiency of the ring of Willis are given in Table 1. There was no statistically significant difference in age between patients with complete and incomplete CoW ($p>0.05$). There is

a significant difference in the frequency of CoW insufficiency in men compared to women (p=0.004). Twenty patients (28.6%) reported vertigo on admission. There is a statistically significant difference in the frequency of vertebrobasilar basin insufficiency in these patients compared to those without vertigo symptoms (p<0.05).

Postoperative neurological complications were recorded in 2 patients (2.9%). Both had ischemic stroke. None of the examined risk factors was associated with the occurrence of postoperative neurological complications in asymptomatic patients (Table 2). All patients with postoperative neurological complications had a preserved polygon of Willis.

The average duration of the clamp was 20 minutes (range 9-30 minutes). There is a statistically significant difference in the duration of the clamp in patients with postoperative neurological complications compared to patients without complications (p=0.034). In univariate logistic regression analysis, the length of clamping proved to be a significant predictor of neurological complications (OR: 1.191; 95% CI: 0.999-1.419; p=0.049).

In 8 (11.4%) patients, CABG was performed simultaneously with the operative treatment of carotid disease. No postoperative neurological complication was recorded in this group of patients.

In-hospital mortality was recorded in one patient (1.4%) who died as a result of stroke. In this patient, CoW inefficiency was not described, and the clamp lasted 30 minutes.

The average length of hospitalization was 5 days (range 4-92 days). Length of hospitalization was not significantly different in patients with and without neurological complications. Hospitalization was significantly longer in patients who underwent myocardial revascularization in addition to carotid surgery (22 vs 4.5 days; p<0.001).

Table 1. Risk factors in the group of patients with and without Circle of Willis insufficiency

Risk factors	CoW-C n=34	CoW-IC n=36	p
Male sex (%)	76.5	41.7	0.004
Age (median, min-max)	73 (40-80)	71 (54-84)	0.701
DM (%)	47.8	25	0.135
HTA (%)	100	87.5	0.234
Dyslipidaemia (%)	87	87.5	0.955
Smoking (%)	63.6	71.4	0.547
Previous MI (%)	34.8	16.7	0.193
Previous PCI/CABG (%)	34.7	29.2	0.688
CHF (%)	17.4	0	0.050
AF (%)	13	12.5	0.955
PAD (%)	8.7	16.7	0.666
COPD (%)	8.7	16.7	0.666
CKD (%)	0	4.2	1

CoW-C - complete circle of Willis, CoW-IC - incomplete circle of Willis, DM - diabetes mellitus, HTA - hypertension, MI - myocardial infarction, PCI - percutanes coronary intervention, CABG - coronary artery bypass grafting, CHF - congestive heart failure, AF - atrial fibrillation, PAD - peripheral arterial disease, COPD - chronic obstructive pulmonary disease, CKD - chronic kidney disease

Table 2. Predictors of adverse neurological events

Factor	OR	95% CI	p
Male sex	0.150	0.016-1.422	0.098
Duration of clamping	1.191	0.999-1.419	0.049
Age	0.997	0.877-1.133	0.958
DM	0.833	0.125-5.556	0.851
Smoking	6.667	0.641-69.344	0.112
Previous MI	1.419	0.143-14.111	0.765
COPD	0.541	0.050-5.852	0.613

DM – diabetes mellitus, MI – myocardial infarction, COPD – chronic obstructive pulmonary disease

DISCUSSION

While prior research has emphasized anatomical variations in the CoW, our findings suggest that functional insufficiency-the inability of existing collaterals to compensate for hypoperfusion-is a more critical determinant of perioperative risk. Patients with an incomplete CoW do not necessarily experience neurological deficits if functional collateral flow is adequate.

The CoW serves as the primary source of contralateral blood supply in patients who undergo carotid artery cross-clamping (CC) for CEA. Its anatomical variations influence outcomes, with fully developed collaterals present in 35-50% of the population. [9,10]. In these individuals, hemodynamic disturbances are minimal, reducing the risk of neurological deficits [11]. However, collateral remodelling in response to carotid stenosis suggests that many CEA candidates have adapted over time, preventing preoperative neurological deficits.

Incomplete CoW has been linked to watershed infarcts and ischemic lesions, with anterior collateral disruption considered an independent risk factor for cerebral ischemia [12-14]. According to some authors, the interruption of continuity in the anterior collateralization represents an isolated risk factor for the occurrence of cerebral ischemia even without the presence of extracranial carotid disease [12,15,16]. These results led to the hypothesis that asymptomatic patients with incomplete CoW have an increased risk of developing a neurological deficit, and that such patients would benefit the most from surgical treatment. Surgical treatment performed in asymptomatic patients represents primary prevention before the onset of neurological deficit [2,17]. On the other hand, in the same patients due to incomplete CoW, there is an assumption that there will be a higher risk of perioperative neurological complications. Prior studies report an 87.3% frequency of incomplete CoW in symptomatic patients, with Göksu et al. noting a 72.6% prevalence in those with atherosclerotic carotid disease [18]. The study by Myrcha et al. examined the role of cerebral collateral circulation, particularly the CoW, in carotid artery cross-clamping tolerance during CEA [9]. Their findings showed that contralateral carotid stenosis >70% or occlusion significantly increased the risk of intraoperative neurological deficits ($p<0.001$), while an incomplete CoW alone was not a predictor. However, data were insufficient to assess its impact on early postoperative outcomes. Similarly, Banga et al. observed a tenfold increase in neurological risk with incomplete anterior or posterior collateralization during CEA without shunting, reinforcing the importance of routine shunt placement in high-risk patients [19]. Conversely, evidence suggests that CEA significantly improves functional reserve and normalizes cerebrovascular reactivity in both asymptomatic and symptomatic patients.

In high-volume institutions, performing more than 150 carotid surgeries annually, perioperative complications in asymptomatic patients are rare, occurring in less than 2% of cases [20]. Our sample did not record any instances of reversible cranial nerve damage, indicating an adequate surgical approach.

Hyperperfusion syndrome was observed in one patient who fully recovered and was discharged for home treatment. The reported incidence of cerebral hyperperfusion after CEA is about 20% [21]. Previous analyses have shown that cerebral hyperperfusion is dependent on the completeness of collateralization within the CoW, poorly controlled arterial tension, and the duration of the clamp. There are currently a few papers discussing the use of ischemic postconditioning (IPCT) in the prevention of hyperperfusion syndrome [22,23].

In patients with disruptions in both anterior and posterior collaterals, a selective shunt was required in 80% of operated patients, compared to only 7% in those with at least one collateral pathway [21]. Hendrikse et al. found that collateral diameter decreases post-CEA, indicating a dynamic CoW capable of flow adaptation [24]. While operative treatment enhances circulatory reserve, particularly in patients with incomplete CoW, the extent to which this influences preoperative and postoperative stroke risk remains unclear. This underscores the need to differentiate between anatomical insufficiency and functional collateral compensation, as functional adaptation may mitigate ischemic risk despite an incomplete CoW.

Patients with a complete CoW have greater tolerance to clamping duration and fewer perioperative neurological complications. Our research showed that the prevalence of CoW variation in asymptomatic patients is 51.4%. There is a significant difference in the frequency of CoW insufficiency between men and women, and a significant association between preoperative dizziness and incomplete CoW in the posterior circulation, likely due to VB insufficiency [25,26].

The absence of anatomical variations of the CoW in asymptomatic patients with postoperative neurological complications may be explained by the development of functional collateral flow over time, which compensates for chronic hypoperfusion and keeps these patients asymptomatic despite significant stenosis [27]. This compensatory mechanism may protect them from neurological complications during surgery. In our study, neurological complications were more dependent on clamping duration, supporting recent research that suggests perioperative risk assessment should consider both the anatomy and functionality of the CoW. Other factors such as inadequately regulated hypertension, anaesthesia type, cardiac instability, and comorbidities also influence outcomes.

In our study, neurological complications correlated more with CC duration than CoW anatomy, emphasizing the need for functional assessments beyond anatomical variations. Our low postoperative stroke rate (2.9%) highlights the protective role of CEA, optimized by surgical expertise and

techniques such as IPCT. Notably, patients with functional collateral insufficiency preoperatively exhibited improved hemodynamic stability postoperatively, suggesting that CEA enhances cerebrovascular reserve over time.

Limitations

This study is limited by its small sample size (70 patients, 2 index events) and retrospective design, which introduces selection bias. Additionally, we focused on anatomical variations without directly assessing functional collateral circulation using advanced imaging like perfusion MRI. Larger prospective studies are needed to validate these findings and further explore CoW functionality in surgical risk stratification.

CONCLUSION

Half of the patients with asymptomatic extracranial carotid disease exhibit CoW discontinuity, yet operative treatment effectively restores perfusion with a low complication rate. CoW remodelling and functional collateral adaptation likely compensate for hypoperfusion, explaining the lack of association between CoW insufficiency and neurologic complications. Other compensatory mechanisms, including collateral recruitment and autoregulation, further support cerebral circulation. However, extended clamping duration remains a key risk factor, highlighting the need for careful intraoperative management.

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TRENDS IN PANCREATIC CANCER MORTALITY IN SERBIA: A JOINPOINT REGRESSION ANALYSIS

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ABSTRACT

Pancreatic cancer is a significant public health problem worldwide. It is currently ranked as the 12th most common malignancy and the 6th leading cause of cancer mortality. This study aimed to investigate sex-specific and age-specific pancreatic cancer mortality trends and disparities in the Serbia from 2000 to 2021. A descriptive epidemiological population-based study analyzing pancreatic cancer mortality was conducted. Age-standardized mortality rates (ASMRs) were calculated using the Segi's world standard population. Temporal trends for pancreatic cancer mortality were assessed using the joinpoint regression analysis. Age-standardized rate of pancreatic cancer in Serbia was 6.4/100,000 (7.9/100,000 in men, 5.1/100,000 in women). The rates were found to be 1.6 times higher in men than women. Pancreatic cancer mortality trend significantly increased from 2000 to 2010 in men (APC=+2.4%, 95%CI: 0.3 – 1.4), after 2010 rates nonsignificantly decline (APC=-0.4%; (95%CI: -2.5 – 0.5). In women, mortality trend significantly increased by +1.8% at an annual level (95%CI: 0.7–2.7). The mortality of pancreatic cancer increased by age. The joinpoint analysis showed a significant rising trend of mortality rates in all age groups 60+, with the highest average annual percent change being reported in the age groups 85+ (AAPC=+3.1 (95%CI: 1.6–4.5)). The provision of updated statistics on the occurrence and outcomes of pancreatic cancer, time trends among various population groups, along with a better understanding of its etiology and identification of causal changeable risk factors – are essential when it comes to the primary prevention of this particular disease.

Keywords: *Pancreatic cancer, age-standardized rates, mortality trend, joinpoint analysis.*

INTRODUCTION

Pancreatic cancer is a significant public health problem worldwide (1, 2). Despite advances in cancer therapy, pancreatic cancer is one of the most aggressive and most lethal malignancies, with the five-year survival rate less than 10% even in developing countries (3).

Globally, the number of new cases of pancreatic cancer has significantly increased for the past two decades (4), and it is currently ranked as the 12th most common malignancy (2.6% of all carcinomas) and the 6th leading cause of cancer mortality (4.8% of all carcinomas). In the year of 2022, pancreatic cancer caused 467,409 deaths globally, including 247,589 (52.9%) deaths among males and 219,820 (47.1%) deaths among females. Most of the deaths were recorded in Asia (212,243; 45.4% of the total), followed by Europe (138,644; 29.7%); whereas the least number of death cases was recorded in Africa (17,770; 3.8%) and Oceania (4,389; 0.9%). China (106,295), the US (49,491) and Japan (43,265) had the highest number of deaths from pancreatic cancer in 2022 among both sexes. Globally, the ASR of pancreatic cancer mortality was 4.2 per 100,000 (the ASR was 5.0/100,000 for men and 3.5/100,000 for women) with considerable variations in mortality rates by country and region (5). The age-standardized incidence and mortality rates are four to five times higher in countries with a high/very high Human Development Index (HDI) when compared to low/middle HDI countries.

Based on the estimates from the GLOBOCAN 2022, pancreatic cancer was the sixth most common malignant tumor in the Republic of Serbia, accounting for 3.4% of the total number of all malignant tumors (1,433 cases) and the standardized incidence rate of 7.6/100,000. In the year of 2022, 1,343 people died of pancreatic cancer in the Republic of Serbia (5.6% out of the total number of deaths caused by malignant tumors), whereas the standardized mortality rate was 6.9/100,000, which is why it is considered the fourth most common malignant tumor as regards the structure of mortality from malignant tumors (5).

The etiology of pancreatic cancer is complex and it is still insufficiently elaborated. Apart from unchangeable genetic factors, age and gender, there are a few significant modifiable pancreatic cancer risk factors such as the following: tobacco smoking, obesity, type 2 diabetes, alcohol consumption, physical inactivity, nutrition factors, chronic pancreatitis and exposure to certain chemicals, hypertension and high cholesterol level (2).

This study aimed to investigate sex-specific and age-specific pancreatic cancer mortality trends and disparities in the Serbia from 2000 to 2021.

MATERIALS AND METHODS

Data sources

A descriptive epidemiological population-based study analyzing pancreatic cancer mortality was conducted. The data on pancreatic cancer mortality in Serbia (excluding the territory of Autonomous Province of Kosovo and Metohija) for the period 2000 – 2021, were obtained from the Statistical Office of the Republic of Serbia. The primary data source refers to the death certificates completed by a physician who determines the time and cause of death – based on which special statistical reports on death are completed (DEM-2) by health institutions. The annual number of deaths was collected in accordance with the codes listed in International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10) (code C25) and it is stratified according to age and 5-year age groups. The data related to the exact number and structure of the population according to age and gender, used as the denominator in calculations of death indicators, were derived from the Statistical Office of the Republic of Serbia.

Statistical analysis

In this particular paper, we calculated the crude rates, age-specific rates and age-standardized rates (ASRs) of pancreatic cancer per 100,000 inhabitants. Gender-specific age-standardized mortality rates (ASMRs) were calculated using the Segi's world standard population.

In order to estimate mortality trends according to age groups and gender for the period 2000 – 2001, we used the linear regression and joinpoint regression analyses (Joinpoint Regression Program, Version 5.3.0.0, Statistical Research and Applications Branch, National Cancer Institute). The joinpoint regression analysis was employed to determine the Annual Percent Change (APC), the Average Annual Percent Change (AARC), complete with the points leading to significant changes in trends. All the results with the p-value less than 5% ($p < 0.05$) are considered statistically significant.

RESULTS

From 2000 to 2001, the total number of 22,488 pancreatic cancer deaths were reported in Serbia, which is why pancreatic cancer is considered the fourth most frequently occurring malignant tumor in the structure of malignant tumor mortality. Among the above-mentioned cases, 12,053 (53.6%) were males and 10,435 (46.4% %) were females. The average number of deaths was 1,022 (548 men and 474 women), whereas the average ASR of pancreatic cancer was 6.4/100,000 (7.9/100,000 in men, 5.1/100,000 in women). The rates were found to be 1.6 times higher in men than the ones detected among women.

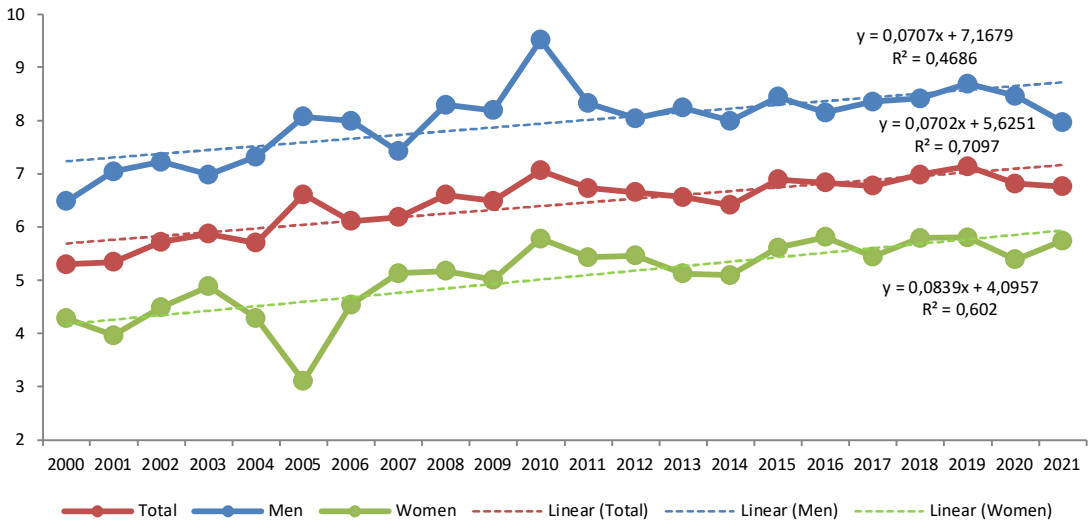
Estimates for death cases due to pancreatic cancer, crude rates and ASRs per 100,000 people from 2000 to 2021 are presented in Table 1.

Table 1. Number of death cases, crude rates and ASRs (per 100,000) for pancreatic cancer in Serbia, 2000 – 2021.

Year	Total			Men			Women		
	N	Crude rate	ASR[a]	N	Crude rate	ASR[a]	N	Crude rate	ASR[a]
2000	747	9.94	5.30	401	10.97	6.48	346	8.96	4.28
2001	772	10.29	5.34	445	12.20	7.04	327	8.48	3.96
2002	835	11.13	5.72	466	12.78	7.22	369	9.58	4.49
2003	855	11.43	5.87	451	12.40	6.98	404	10.51	4.88
2004	852	11.42	5.70	476	13.12	7.32	376	9.81	4.29
2005	988	13.32	6.61	518	14.32	8.07	470	6.25	3.11
2006	910	12.28	6.11	520	14.43	7.99	390	10.24	4.54
2007	966	13.09	6.18	491	13.68	7.42	475	12.52	5.13
2008	1034	14.07	6.60	561	15.70	8.29	473	12.53	5.17
2009	1020	13.03	6.49	546	15.34	8.19	474	12.60	5.01
2010	1088	14.92	7.06	566	15.96	9.51	522	13.94	5.78
2011	1055	14.58	6.73	570	16.18	8.32	485	13.06	5.43
2012	1099	15.26	6.65	573	16.34	8.04	526	14.24	5.46
2013	1070	14.93	6.56	585	16.76	8.24	485	13.19	5.12
2014	1056	14.81	6.41	573	16.50	7.99	483	13.20	5.09
2015	1134	15.98	6.89	618	17.89	8.44	516	14.18	5.61
2016	1151	16.31	6.83	599	17.42	8.15	552	15.25	5.81
2017	1156	16.47	6.77	619	18.10	8.35	537	14.91	5.44
2018	1168	16.73	6.98	615	18.08	8.41	553	15.44	5.79
2019	1206	17.36	7.14	640	18.91	8.69	566	15.89	5.80
2020	1169	16.94	6.81	625	18.60	8.46	544	15.37	5.39
2021	1157	16.93	6.76	595	17.88	7.97	562	16.02	5.74
Overall	22488	14.15	6.43	12053	15.62	8.24	10435	12.55	5.06

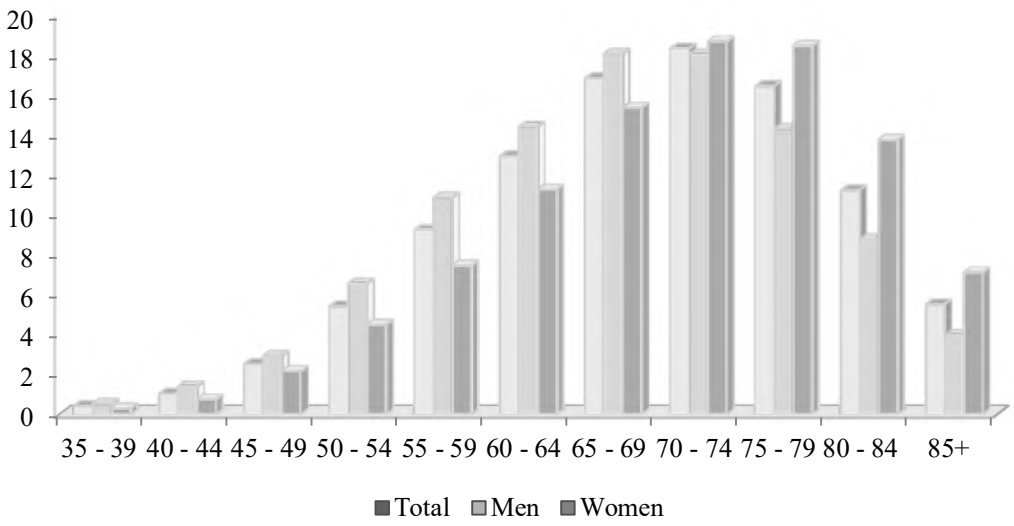
A significant rising trend in the mortality of pancreatic cancer was reported to be present in the population (y=0.0072x + 5.6251, p<0.001), during the observed period in both men (y=0.00707x + 7.1679, p=0.001) and women (y=0.0839x + 4.0957, p<0.001) (Figure 1).

Figure 1. Trends of age-standardized pancreatic cancer mortality rates in Serbia by sex, in the period 2000-2021.



Age groups that are most affected by the burden of pancreatic cancer mortality were the following: 70-74 (18.3%), 65-69 (16.8%) and 75-79 (16.4%). It is significant to emphasize that the number of death cases in men constantly surpasses the number of deaths in women until the age-group of 70-74 years, whereas after the age of 70 – women are more likely to die when compared to men. The majority of death cases were registered in the age group of 65-69 years, with the total number of 2,174 death cases (18.0%) in men, whereas 37,609 death cases (18.7%) were reported in women in the age group of 70-74 years (Figure 2).

Figure 2. Distribution of pancreatic cancer mortality by age groups in Serbia, 2000 - 2021.

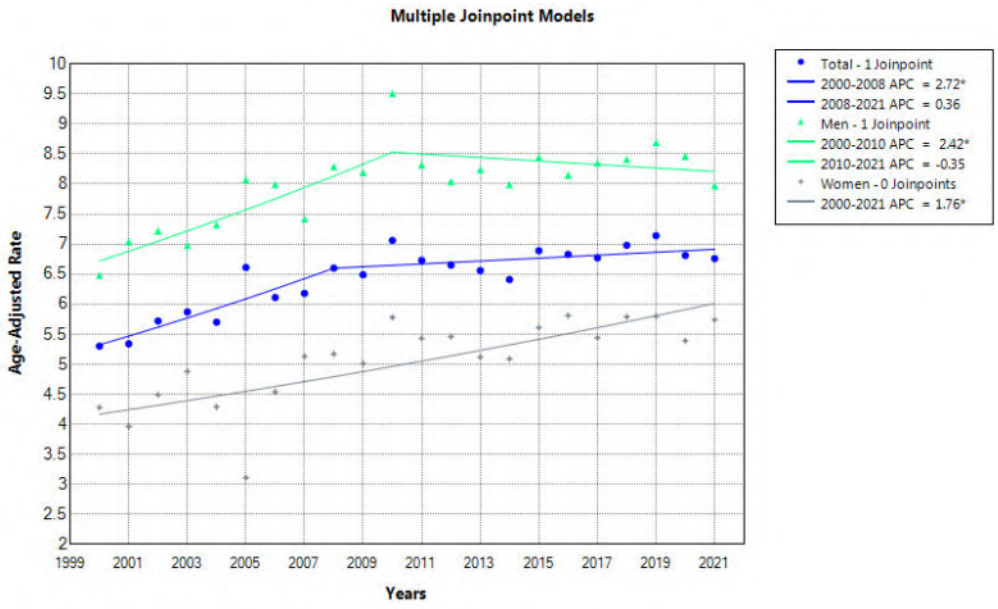


The joinpoint regression revealed a statistically significant increase in the mortality rates of pancreatic cancer for the total level, with an AAPC of 1.3% (95% CI: 0.9–1.6); p<0.001). More specifically, the joinpoint regression analysis identified one time point in which the trend significantly changed. An abrupt rise in the mortality rates was detected in the period from 2000 to 2008, by approximately 2.7% yearly (95% CI (1.7–6.2), p<0.001), followed by a slight, but not statistically significant decline in the mortality rates until the end of the observed period, with an average change of -0.4% per one year (95% CI: -0.8–0.8, p=0.331).

A similar trend in the mortality rates was reported in men as well (AAPC=0.95 (95% CI: 0.5–1.5; p=0.008). The mortality rates of pancreatic cancer in men were constantly increasing from 2000 to 2010, with an APC of 2.4% (95% CI: 1.4 – 5.5), which was considered to be statistically significant (p<0.001). After the year of 2010, there has been a decline in the above-mentioned mortality rates, with the APC of -0.4% (95% CI: -2.5 – 0.5). However, this change was not statistically significant (p=0.392).

Unlike men, what was observed in women was a continuously rising trend in mortality of pancreatic cancer by 1.8% at an annual level (95%CI: 0.7–2.7. p=<0.001). The joinpoint regression analysis did not identify a single time point in which the trend in mortality rates of women significantly changed (Figure 3).

Figure 3. Trend in mortality of pancreatic cancer in Serbia, 2000 – 2021.



The mortality of pancreatic cancer increased by age both in men and in women. The highest ASR among men was in the age group of 80-84 years (77.9±18.1) and among women in the same age group (65.2±12.2).

The joinpoint analysis showed a significant rising trend of mortality rates in all age groups 60+ during the whole study period, with the highest AAPC being reported in the age groups 85+ (AAPC=+3.1 (95% CI: 1.6–4.5)). The age specific mortality rates in males increased significantly by +1.1% (95% CI: 0.1–2.2) yearly in the age group 60–64, +1.75% (95% CI 1.00-2.5) yearly in the age group 65–69, +1.59% (95% CI: 0.6–2.6) yearly in the age group 70–74 and +2.10% (95% CI: 0.6–3.6) yearly in the age group 75-79. A decreasing trend in the mortality of pancreatic cancer was recorded only in males aged 40 – 44, 45 – 49, 50 – 54 and 55 – 59 years, but these changes were not considered to be statistically significant.

The rate for women increased significantly in the age groups of 55–59, 65–69, 70–74, 74–79, 80–85, and 85+ years. The corresponding AAPC values are 1.9% (95% CI: 0.6–3.2), 2.1% (95% CI: 0.6-3.7), 0.9% (95% CI: 0.01–1.9), 2.9% (95% CI: 0.3–3.6), 2.1% (95% CI: 0.7–3.42), and 4.4% (95% CI: 2.1–6.4) (Table 2).

Table 2. Joinpoint regression analysis* of pancreatic cancer mortality by age in Serbia, 2000 - 2021.

	Total		Men		Women	
	ASR±SD	AAPC[a] (95% CI[b])	ASR±SD	AAPC[a] (95% CI[b])	ASR±SD	AAPC[a] (95% CI[b])
25 - 29	0.2±0.2		0.1±0.2		0.2±0.3	
30 - 34	0.3±0.3		0.3±0.4		0.3±0.5	
35 - 39	0.4±0.4	-0.9 (-4.5-2.9)	1.2±0.7		0.3±0.5	
40 - 44	2.1±0.6	-1.3 (-3.6-1.2)	3.1±1.5	-1.5 (-4.3-1.5)	1.4±0.7	
45 - 49	5.01±1.2	0.4 (-1.4-2.4)	6.3±2.0	-0.5 (-2.5-1.6)	3.9±1.8	2.1 (-2.4-7.1)
50 - 54	10.4±1.2	0.1 (-0.8-0.9)	13.8±4.2	-0.1 (-1.9-1.7)	7.8±1.5	0.5 (-1.2-2.3)
55 - 59	18.7±2.1	0.1 (-0.8-1.1)	24.5±3.9	-0.7 (-1.9-0.5)	13.3±2.4	1.9* (0.6-3.2)
60 - 64	27.8±3.0	1.2* (0.5-1.8)	35.2±5.1	1.1* (0.1-2.2)	20.7±4.1	1.7 (-0.2-3.7)
65 - 69	40.0±5.5	1.7* (1.1-2.4)	50.6±7.4	1.* (1.0-2.5)	30.2±5.8	2.1* (0.6-3.7)
70 - 74	52.2±6.1	1.6* (0.6-2.6)	63.5±9.8	1.6* (0.6-2.6)	42.9±6.6	0.9* (0.01-1.8)
75 - 79	62.1±9.4	2.5* (1.2-3.7)	71.3±14.5	2.1* (0.6-3.6)	53.7±9.0	2.9* (0.3-3.6)
80 - 84	70.4±10.0	1.7* (0.7-2.8)	77.9±18.1	1.5 (-1.1-4.3)	65.2±12.2	2.1* (0.7-3.4)
85+	63.3±11.4	3.1* (1.6-4.5)	68.2±22.9		57.9±13.3	4.4* (2.1-6.4)

[a]AAPC – Average Annual Percent Change
[b]CI – Confidence Interval
* Statistically significant trend

DISCUSION

Despite the rapid progress of advanced medical technology, pancreatic cancer still remains to be one of the deadliest malignant tumors (3). A close parallel that can be drawn between the incidence and mortality rates of pancreatic cancer reflects the fatal nature of this disease. There are several reasons why pancreatic cancer has such a high mortality rate such as the following: late diagnosis, aggressive nature, a lack of effective screening and limited treatment options (11).

The pancreatic cancer-related mortality rates vary significantly, depending on the region and population (12). The highest burden is notified in the regions with a very high and high HDI, despite the fact that these regions were reported to have better medical resources, whereas the reduced burden was reported in regions with a medium and low HDI, which is in accordance with the findings obtained from the previous research studies (12, 13, 14).

In the year of 2022, the highest ASRs were recorded in Western Europe (7.92) and Eastern Europe (7.08), whereas the least mortality rates were registered in South Central Asia (1.12) and Middle Africa (1.50). There are significant differences observed in the mortality rates reported among the countries. In the year of 2022, the highest standardized mortality rates of pancreatic cancer were reported in Hungary (9.61/100,000), Uruguay (9.41/100,000), the Czech Republic (8.55/100,000), Latvia (8.42/100,000) and Finland (8.41/100,000), whereas the lowest mortality rates were registered in Malawi (0.44/100,000), Vanuatu (0.55/100,000), Sierra Leone (0.65/100,000), Pakistan (0.67/100,000) and Rwanda (0.7/100,000) (5). The above-mentioned differences may be explained due to the increased proportions of the ageing population and its unhealthy lifestyle habits, complete with the higher prevalence of metabolic risk factors in countries with a higher HDI. On the other hand, it is possible that the pancreatic cancer-related incidence, mortality rates and risk factors are underestimated in the regions with a lower HDI due to underdevelopment of the mechanism of cancer reporting (7, 15, 16).

The rise in pancreatic cancer-related mortality rates has been noticed in almost all countries worldwide ever since 1991, particularly among women and those older than 50 years (7, 17). Favourable trends in the mortality rates of pancreatic cancer were reported in Canada only (an annual

decline of 0.4% in men and 0.2% in women) and Mexico (an annual decline of 0.7% in men and 0.8% in women). Turkmenistan showed the greatest rise in mortality rates in both sexes (AAPC = +10.0% in men and AAPC = +6.4% in women).

What was shown in a study conducted by Tana *et al*., was a slow, but continuously rising trend in the mortality rates of pancreatic cancer, with an annual increase of 0.23% in the period from 1999 to 2020 in the United States. Possible factors listed as the ones contributing to the rising trend in mortality rates were related to the population ageing and the increasing prevalence of obesity (19).

The findings of our study indicated a significant increase in the trend of pancreatic cancer-related mortality rates in both men and women for the past two decades. One study that was previously conducted in Serbia showed that in the period from 1991 to 2010 pancreatic cancer caused the rise in mortality rates, with an annual increase of 1.6% in men and annual increase of 2.2% in women.

It is estimated that the incidence and mortality rates of pancreatic cancer will continue to grow in the following 20 years in both men and women. By 2030, pancreatic cancer may become the second leading cancer-related death cause (20, 21). The greatest rise in fatal outcomes is expected to be found in regions with a medium and low HDI. It is estimated that the mortality rates of pancreatic cancer in Africa will be increased by 17,744 deaths in the year of 2040, with the growth rate of 114.8%, meaning that its rates will simultaneously be considered the highest ones worldwide. The above-mentioned rates will be followed by Latin America and the Caribbean (a rise of 109%). However, in 2040, the growth of mortality rates of 31.6% will be the lowest in Europe (22). This alarming rising trend in the incidence and mortality rates of pancreatic cancer emphasizes the need to use more advanced diagnostic tools and introduce earlier cancer screening programs along with more effective treatment options.

The prevailing patterns of an increase in the pancreatic cancer incidence and mortality rates are closely associated with the process of globalization, urbanization, economic growth and changes in the age-related population structure (15). Due to epidemiological studies, it is known that geographical differences in the existing pancreatic cancer burden may be explained by variations in the prevalence of some of the essential lifestyle and metabolic risk factors such as the following: tobacco smoking, obesity, alcohol consumption, nutrition factors, type 2 diabetes mellitus (23, 24). Therefore, comprehensive understanding of risk factors for developing pancreatic cancer is of enormous practical importance for the efficient prevention of pancreatic cancer (3).

Meta-analysis provided strong evidence, pointing out that tobacco smoking was one of the main risk factors associated with pancreatic cancer. 11-32% of all death cases caused by pancreatic cancer was primarily attributed to active smoking (25). One large European study that included 2,009 cases and 1,532 controls confirmed the fact that active smokers were at a 72% higher risk of developing pancreatic cancer when compared to those who had never smoked (26). Although there was a decline in the pancreatic cancer-related mortality rates due to tobacco use in the period from 1990 to 2019, with a reduction of 20% reported in men and 6% in women (408), smoking continues to be a public health concern. Bearing in mind the existing differences in the smoking rates reported between men and women, this particular fact can provide at least a partial explanation of the gender-based differences observed in the prevalence of pancreatic cancer.

Globally, there has been a 3.5-fold increase in the number of death cases caused by pancreatic cancer, that can be attributed to metabolic risk factors, ranging from 22,091 death cases reported in 1990, to 77,215 deaths reported worldwide in 2019. North America and Central Europe were reported to have the highest age-standardized mortality rates (ASMRs) caused by pancreatic cancer that can be attributed to the high fasting plasma glucose (FPG) levels and high BMI, estimated in 2019, respectively (25). A pooled analysis of 14 cohort studies demonstrated that the respondents with a higher body mass index (BMI) were at a 40% higher risk of developing pancreatic cancer when compared to those whose BMI was stable (26), whereas the previous epidemiological studies indicated that obesity was associated with approximately 1.5-fold increased risk of developing pancreatic cancer (27, 28).

In Europe, the number of adult patients with diabetes has been increasing with the population growth and ageing (29). Therefore, if no additional measures are to be undertaken, it is expected that poor control of blood glucose levels will give its own increasing contribution to pancreatic cancer-related deaths (30).

The examination of mortality trends according to specific categories, such as gender and age groups, represents a highly significant aspect of comprehensive assessment of pancreatic cancer burden, complete with formulating and enforcing various prevention strategies. Globally, pancreatic cancer incidence and mortality rates are higher in men than in women (9). Our study demonstrated that there was a rise in the pancreatic cancer mortality trends reported in both sexes, with the significant increase in mortality rates among women occurring in a slightly quicker and earlier manner when compared to men. This particular pattern tends to follow the global trend of a greater rise in the female mortality rates (7), ranging from 0.78 to 5.83% when compared to the range of 0.55 to 4.20% detected in men. The most evident rise in female mortality rates is attributable to the greater prevalence of obesity among women, complete with the incidence of metabolic syndrome occurring more frequently with ageing (31).

Our analysis of the age-related distribution confirmed the results of previous studies underlying that the epidemiological pancreatic cancer burden was reported to be gradually increasing with ageing. Concerning the fact that pancreatic cancer is a disease typically found in older people, old age was identified as an independent risk factor (25).

Approximately 90% of newly diagnosed patients belonged to the older age group of 55 years and over, with the majority of them being in the seventh or eighth decade of life (18, 21, 32). Globally, the highest number of death cases was reported in the age group of 65-69 years in men, whereas the peak number of deaths among women was reported in the age group of 75-79 years (12).

CONCLUSION

Our study observed an increase in pancreatic cancer mortality rates during the studied period in Serbia. Taking into consideration the population ageing and an increase in the prevalence of specific risk factors, complete with the fact that pancreatic cancer screening is not currently recommended, pancreatic cancer burden still continues to be a major public health challenge. However, the provision of updated statistics on the occurrence and outcomes of pancreatic cancer, time trends among various population groups, along with a better understanding of its etiology and identification of causal changeable risk factors – are essential when it comes to the primary prevention of this particular disease.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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PSYCHOLOGICAL SAFETY AND BURNOUT AMONG COMMUNITY PHARMACY EMPLOYEES: A LONGITUDINAL STUDY

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ABSTRACT

Burnout is a significant issue among healthcare professionals, including pharmacists and pharmacy technicians working in community pharmacies. This study aimed to examine changes in burnout levels over six months and explore the role of psychological safety and stress management programs. The study included 651 licensed pharmacists and pharmacy technicians employed in community pharmacies. Burnout was assessed using the Shirom-Melamed Burnout Questionnaire (SMBQ), while psychological safety was measured with a validated 15-item scale. All participants completed stress management training. Data were collected at three time points and analyzed using descriptive statistics, repeated measures ANOVA, correlations, and multiple regression. Group differences between pharmacists and technicians were examined through separate analyses and Fisher's r-to-z transformation. The results indicate that burnout levels remained stable across the measurement points, with no significant differences observed. A slight increase in psychological safety was detected over time, suggesting a trend toward improved perceptions of workplace support. No significant overall differences in burnout levels were found between pharmacists and pharmacy technicians. The findings suggest a potential protective effect of stress management training and psychological safety in mitigating burnout. Future research should explore individual and organizational factors influencing these interventions and assess long-term burnout trends. Implementing systematic stress prevention programs may be essential for safeguarding the mental health of pharmacy professionals.

Keywords: *Burnout, psychological safety, pharmacists, pharmacy technicians, stress management, community pharmacies, longitudinal study.*

INTRODUCTION

Burnout is a well-documented occupational phenomenon characterized by emotional exhaustion, physical fatigue, and cognitive weariness, which negatively impact professional performance and overall well-being (1, 2). It is particularly prevalent among healthcare professionals, including pharmacists and pharmacy technicians, due to high job demands, emotional labor, and the need for constant decision-making in patient care settings (3-5). Burnout has been associated with decreased job satisfaction, reduced professional efficacy, and an increased risk of medical errors, underscoring the need for effective prevention and intervention strategies (6, 7).

One of the key factors influencing burnout is psychological safety, defined as an individual's perception that they can express thoughts, concerns, and emotions without fear of negative consequences (8-10). Psychological safety has been linked to increased job engagement, better teamwork, and lower stress levels, suggesting its potential role in mitigating burnout (11-12). However, the relationship between psychological safety and burnout in community pharmacy settings remains underexplored.

To address workplace stress and enhance psychological well-being, many organizations have implemented stress management training programs designed to improve employees' ability to cope with occupational challenges (13, 14). These programs typically focus on cognitive-behavioral strategies, relaxation techniques, and resilience-building exercises aimed at reducing perceived stress and enhancing emotional regulation (15-16). While previous studies have demonstrated the effectiveness of such interventions, there is a need to evaluate their long-term impact on burnout levels among pharmacists and pharmacy technicians.

This study aims to examine the longitudinal relationship between psychological safety and burnout in community pharmacy professionals. Specifically, it investigates whether burnout levels change over time and whether psychological safety and stress management training contribute to burnout prevention. By providing empirical evidence on these factors, this research seeks to inform organizational strategies for fostering a supportive work environment and enhancing employee well-being.

MATERIALS AND METHODS

Study design and sample

This study employed a longitudinal observational design to examine psychological safety and burnout among healthcare professionals in community pharmacies. The sample consisted of 651 licensed pharmacists and pharmacy technicians employed in surveyed healthcare institutions who voluntarily agreed to participate. Inclusion criteria required participants to be actively employed and possess a valid professional license. Informed consent was obtained from all participants, ensuring their voluntary participation and adherence to ethical research standards. The study sample remained intact throughout all measurement points, with no participant attrition observed. All employees were pre-selected based on predefined criteria, ensuring a consistent and motivated sample. Given their engagement in professional development initiatives and workplace programs, participants demonstrated sustained commitment to the study. The absence of sample attrition enhances the reliability of the findings, minimizing the risk of bias associated with dropout effects. This stability suggests that the observed trends reflect genuine patterns rather than artifacts of differential participation.Data collection was conducted at three time points over a six-month period to assess changes in psychological safety and burnout. Prior to study initiation, ethical approval was obtained from the institutional ethics committee, ensuring compliance with ethical research standards (Approval 12/2022, issued in December 2022).

Stress management training

Participants underwent standardized stress management training aimed at increasing awareness of occupational stressors and developing adaptive coping strategies. The training covered evidence-based techniques, including cognitive-behavioral strategies for stress regulation, methods for enhancing emotional resilience, and structured approaches for maintaining well-being in high-demand work environments. Training sessions were conducted in a controlled format, ensuring uniform exposure to the content across all participants. It was conducted in a controlled format to minimize variability, and no distinctions were made between pharmacists and pharmacy technicians in the training approach. Individual engagement and adherence to the acquired techniques were self-reported, which is acknowledged as a study limitation.

However, individual engagement and adherence to the acquired techniques were self-reported and not externally monitored.

The standardized stress management training was conducted by a licensed psychologist with 15 years of experience and formal training in Cognitive-Behavioral Therapy (CBT), Acceptance and Commitment Therapy (ACT), and Transactional Analysis. All 651 participants received the same structured training program, ensuring consistency in content delivery.

Measures

Psychological safety was assessed using a 15-item self-report questionnaire developed for this study. Participants rated each statement on a 5-point Likert scale (1 = strongly disagree, 5 = strongly agree), with higher scores indicating greater perceived psychological safety. The scale demonstrated high internal consistency (Cronbach's alpha = 0.92). The validity of the scale was confirmed through content and face validity assessments, ensuring it effectively captured core aspects of psychological safety. The psychological

safety scale was developed through multiple iterations to ensure its validity and reliability. The initial version (32 items) underwent refinement based on content and face validity assessments, resulting in a 15-item version with high internal consistency (Cronbach's alpha = 0.92). Based on total scores, psychological safety was categorized as Low (15–30), Moderate (31–45), or High (46–75). The detailed validation of the questionnaire was conducted as part of a separate study, which is currently in preparation for publication.

Burnout was measured using the Shirom-Melamed Burnout Questionnaire (SMBQ), a 22-item instrument designed to assess job-related burnout across five dimensions: Emotional Exhaustion, Physical Fatigue, Cognitive Weariness, Tension, and Listlessness. Participants rated each item on a 7-point Likert scale (1 = Never or almost never, 7 = Always or almost always), with higher scores indicating greater burnout severity. The total score was computed as the average of all subdomains, with burnout categorized as No risk (0–3.0), Normal stress (3.1–3.6), or High burnout risk (3.7–4.0). The questionnaire, translated into Serbian and transculturally adapted, was validated for use among pharmacists. The adaptation process included forward and backward translation, expert review, and psychometric evaluation. The Serbian version demonstrated high internal consistency (Cronbach's alpha = 0.91) and a stable one-factor structure (17).

Data collection was conducted at three predefined time points over a six-month period to systematically assess changes in psychological safety and burnout levels. The first measurement (T1) was conducted at baseline, before participants engaged in stress management training. The second measurement (T2) occurred three months after the initial assessment, and the final measurement (T3) was conducted at the six-month mark. The time intervals between assessments were equal for all participants, ensuring consistency in data collection. Each participant completed the assessments at the same respective time points within the study timeline, minimizing variability due to differing exposure durations.

Data analysis

Data analysis was conducted using descriptive statistics, correlation analyses, and multiple regression modeling. Pearson correlation coefficients were calculated to examine relationships between psychological safety and burnout across the three time points. A multiple linear regression model was applied to determine the predictive value of psychological safety on burnout at the final time point (T3). The ANOVA test was used to evaluate model significance. Statistical significance was set at $p < 0.05$. To assess potential differences in the relationship between psychological safety and burnout among pharmacists and pharmacy technicians, separate correlation and multiple regression analyses were conducted for each group, followed by Fisher's r-to-z transformation to compare the correlation coefficients between them.

Statistical analyses were performed using the SPSS software package, version 29.0.1.

RESULTS

The socio-demographic characteristics of the study participants are presented in Table 1. The sample included pharmacists and pharmacy technicians, with variations in age, gender distribution, years of experience, and workload. These characteristics provide essential context for interpreting further findings.

Table 1. Socio-demographic characteristics of study participants

Characteristic	Pharmacists (n=412)	Pharmacy Technicians (n=239)	Total (N=651)
Age (Mean ± SD)	37.4 ± 8.2	35.1 ± 7.6	36.5 ± 8.0
Gender			
- Male (%)	112 (27.2%)	48 (20.1%)	160 (24.6%)
- Female (%)	300 (72.8%)	191 (79.9%)	491 (75.4%)
Years of Experience (Mean ± SD)	12.8 ± 7.3	10.5 ± 6.8	11.9 ± 7.1
Workload (Hours/Week, Mean ± SD)	42.1 ± 5.6	40.7 ± 5.2	41.5 ± 5.5

As presented in Table 2, burnout levels remained relatively stable across the three measurement points, with a slight decrease in the proportion of participants classified as being at high risk for burnout. This trend suggests a potential effect of the applied interventions, including relaxation techniques, in mitigating burnout severity over time. Similarly, psychological safety scores showed a gradual increase, indicating an improvement in perceived psychological safety among both pharmacists and pharmacy technicians. Despite these changes, differences between the two professional groups remained minimal and were not statistically significant, suggesting that both pharmacists and pharmacy technicians experienced similar trends in burnout and psychological safety throughout the study period.

Table 2. Burnout and Psychological Safety across three time points

Measure	Pharmacists T1 (Mean ± SD)	Pharmacy Technicians T1 (Mean ± SD)	Pharmacists T2 (Mean ± SD)	Pharmacy Technicians T2 (Mean ± SD)	Pharmacists T3 (Mean ± SD)	Pharmacy Technicians T3 (Mean ± SD)
Burnout (Total Score)	3.5 ± 0.8	3.4 ± 0.8	3.4 ± 0.7	3.3 ± 0.7	3.3 ± 0.7	3.3 ± 0.7
Burnout Categories (%)						
No Risk (0–3.0)	28.5%	30.1%	30.8%	32.0%	32.2%	33.5%
Normal Stress (3.1–3.6)	42.1%	41.8%	43.4%	43.1%	44.0%	44.2%
High Burnout Risk (3.7–4.0)	29.4%	28.1%	25.8%	24.9%	23.8%	22.3%
Psychological Safety (Total Score)	42.9 ± 6.4	42.6 ± 6.7	44.3 ± 6.6	44.1 ± 6.8	45.2 ± 6.8	45.0 ± 7.0
Psychological Safety Categories (%)						
Low (15–30)	18.3%	18.7%	16.0%	16.4%	14.6%	15.0%
Moderate (31–45)	52.5%	52.1%	50.3%	50.0%	48.8%	48.6%
High (46–75)	29.2%	29.2%	33.7%	33.6%	36.6%	36.4%

Table 3 presents Pearson correlation coefficients between psychological safety and burnout across three time points. A significant positive correlation was observed between psychological safety at T1 and burnout at T2 ($r = .101$, $p = .010$). However, no significant correlations were found between psychological safety at other time points and burnout levels.

Table 3. Correlations between Psychological safety and Burnout for Pharmacists and Pharmacy Technicians

Variable	Psychological Safety T1	Psychological Safety T2	Psychological Safety T3	Burnout T1	Burnout T2	Burnout T3
Pharmacists (n=412)						
Psychological Safety T1	1	-0.004	0.061	-0.029	0.120	-0.015
Psychological Safety T2	-0.004	1	-0.007	-0.045	-0.042	0.015
Psychological Safety T3	0.061	-0.007	1	-0.010	-0.040	-0.068
Burnout T1	-0.029	-0.045	-0.010	1	0.048	-0.070
Burnout T2	0.120	-0.042	-0.040	0.048	1	-0.018
Burnout T3	-0.015	0.015	-0.068	-0.070	-0.018	1
Pharmacy Technicians (n=239)						
Psychological Safety T1	1	-0.003	0.057	-0.035	0.080	-0.019
Psychological Safety T2	-0.003	1	-0.005	-0.048	-0.045	0.012
Psychological Safety T3	0.057	-0.005	1	-0.015	-0.038	-0.075
Burnout T1	-0.035	-0.048	-0.015	1	0.045	-0.076
Burnout T2	0.080	-0.045	-0.038	0.045	1	-0.025
Burnout T3	-0.019	0.012	-0.075	-0.076	-0.025	1

To examine potential differences in the relationship between psychological safety and burnout among pharmacists and pharmacy technicians, separate correlation analyses were conducted for each group. Fisher's r-to-z transformation was then applied to compare the correlation coefficients between the groups.

The results indicate that the correlations between psychological safety and burnout are similar across both groups,

with no statistically significant differences. For instance, the correlation between psychological safety at T1 and burnout at T2 was $r = 0.12$ for pharmacists and $r = 0.08$ for pharmacy technicians. Fisher's test did not show a significant difference between these correlations ($z = 0.73$, $p = 0.46$). Similarly, correlations between psychological safety at later time points (T2 and T3) and burnout remained consistent across groups, with minimal deviations.

Additionally, multiple regression analyses were performed separately for pharmacists and pharmacy technicians, with burnout at T3 as the dependent variable and psychological safety at T1, T2, and T3 as predictors. The model was not statistically significant for either group (pharmacists: $F(3, 407) = 1.312$, $p = 0.270$; pharmacy technicians: $F(3, 235) = 1.014$, $p = 0.388$), indicating that psychological safety was not a significant predictor of burnout at a later time point in either subgroup.

These findings suggest that while there are minor variations in the correlation coefficients between the groups, they are not statistically significant. Therefore, it can be concluded that the relationship between psychological safety and burnout is comparable for both pharmacists and pharmacy technicians.

To further examine the relationship between psychological safety and burnout, a multiple regression analysis was conducted with burnout at T3 as the dependent variable and psychological safety at T1, T2, and T3 as predictors. The model was not statistically significant, $F(3, 647) = 1.218$, $p = .302$, explaining only 0.6% of the variance in burnout at T3.

Table 4 presents the regression coefficients. None of the psychological safety measures significantly predicted burnout at T3. Psychological safety at T3 approached significance ($\beta = -0.072$, $p = .066$), suggesting a potential negative association with burnout over time, but this result did not reach conventional significance levels.

Table 4. Regression coefficients for psychological safety predicting burnout at T3

Predictor	B	SE	β	t	p
Constant	2.148	0.156	—	13.798	<.001
Psychological Safety T1	-0.013	0.040	-0.013	-0.325	.745
Psychological Safety T2	0.010	0.040	0.010	0.261	.794
Psychological Safety T3	-0.074	0.040	-0.072	-1.842	.066

Overall, the results suggest that psychological safety levels remained relatively stable over time, showing no strong association with burnout progression. While relaxation techniques were implemented during the study period, their direct impact on psychological safety and burnout remains inconclusive based on the current findings.

A multiple regression analysis was conducted to examine the relationship between psychological safety (measured at three time points: T1, T2, and T3) and burnout (measured at T3). The overall model was not statistically significant, $F(3, 647) = 1.218$, $p = .302$, indicating that psychological safety did not significantly predict burnout at T3. The model explained only 0.6% of the variance in burnout scores ($R^2 = 0.006$, Adjusted $R^2 = 0.001$), suggesting a very weak predictive relationship. Regarding individual predictors, none of the psychological safety variables (T1, T2, or T3) were significant. Psychological safety at T3 showed a marginal negative association with burnout ($B = -0.074$, $p = 0.066$), but this result was not statistically significant at the conventional $p < 0.05$ level. Psychological safety at T1 and T2 showed no meaningful relationship with burnout ($p > 0.7$ in both cases) (Table 5).

Table 5. Multiple regression analysis: Psychological safety and Burnout

Predictor Variable	B	Std. Error	Beta	t	Sig.
Constant	2.148	0.156	—	13.798	<.001
Psychological Safety T1	-0.013	0.040	-0.013	-0.325	0.745
Psychological Safety T2	0.010	0.040	0.010	0.261	0.794
Psychological Safety T3	-0.074	0.040	-0.072	-1.842	0.066

These findings suggest that variations in psychological safety across time did not significantly impact burnout levels at T3. Further research may be needed to explore other potential factors influencing burnout.

DISCUSSION

The findings of this study indicate that no significant changes in burnout levels were observed across the three measurement points. While mean burnout scores showed a slight increase over time, statistical analyses did not reveal significant differences between individual time points (T1–T2, T2–T3, T1–T3), with low inter-timepoint correlations further supporting the absence of substantial changes. This pattern suggests a relative stability of burnout levels among

participants, which can be interpreted through several potential explanations. In contrast, a slight increase in psychological safety was observed over time. Although the changes were not pronounced, statistical analyses indicated a trend toward improved perceptions of psychological safety among participants, while the correlations between psychological safety and burnout remained low across all time points, suggesting that these two constructs did not exhibit a strong association within the observed timeframe.

One possible explanation is that the stress management training received by participants during the study period had a protective effect, potentially preventing a significant increase in burnout levels. Research has consistently demonstrated that stress management interventions, such as cognitive-behavioral strategies, relaxation techniques, and resilience training, can effectively reduce perceived stress, improve emotional regulation, and enhance coping mechanisms in demanding work environments (12, 14, 18). Given the complex nature of workplace stressors, it is plausible that participants applied the acquired techniques to mitigate the psychological burden associated with their professional responsibilities.

Additionally, the role of psychological safety in this context should be considered. Psychological safety, defined as an individual's perception that they can freely express thoughts, concerns, and emotions without fear of negative consequences, has been linked to lower burnout levels and improved stress management capacity (8, 11). If participants worked in an environment that encouraged open communication and emotional security, they may have been more motivated and able to consistently implement stress management strategies, contributing to the stabilization of burnout levels.

However, given the low correlations between time points, the results may also suggest individual variations in the experience and response to stress. It is possible that some participants did not utilize stress management techniques to the same extent or that other factors, such as workload, interpersonal relationships, or personal traits, played a more significant role in shaping burnout trajectories. Studies have shown that occupational burnout is influenced by a complex interplay of organizational and individual factors, with job demands, perceived control, and social support serving as key determinants (1, 19).

One potential explanation is that participation in stress management training may have contributed to mitigating a more pronounced increase in burnout. Given that the participants underwent stress management training, the results of the multiple regression analysis may indicate that psychological safety was not a significant predictor of burnout because the participants had developed effective stress management skills. These skills could have mitigated the impact of psychological safety on burnout, leading to the observed non-significant relationship. Research has shown that psychological safety is associated with lower burnout levels, as it fosters an environment where employees feel safe to express concerns and seek support (20). However, in this study, the participants' enhanced stress management abilities may have compensated for lower levels of psychological safety, resulting in no significant effect on burnout. These findings suggest that while psychological safety is important, effective stress management training can also play a crucial role in preventing burnout. Future research should explore the interplay between psychological safety and stress management skills to better understand their combined impact on employee well-being.

Prior research has demonstrated that structured interventions focused on stress reduction, emotional regulation, and resilience-building can play a protective role in preventing burnout and improving overall well-being (14, 16, 21). Additionally, the presence of psychological safety within the workplace may have supported employees in effectively applying stress management techniques, fostering a more resilient response to occupational stressors (8, 11). This environment not only enhances well-being but also facilitates the continuous development of professional competencies, enabling employees to refine their skills and adapt to evolving workplace demands (22, 23). Moreover, mentorship plays a crucial role in this process by providing guidance, knowledge transfer, and constructive feedback, further strengthening professional growth and overall job performance (22-25).Future research should consider additional analyses to assess individual differences in the application of stress management techniques, as well as the role of specific organizational factors in fostering psychological safety among employees. Furthermore, a longer-term longitudinal study could provide deeper insights into the sustained effects of stress management interventions on burnout prevention.

In conclusion, the results suggest that burnout levels remained stable throughout the study period, despite the cognitive and emotional demands of the profession, potentially indicating a protective effect of stress management training and psychological safety in the workplace. Additionally, the slight increase in psychological safety over time suggests that targeted interventions may contribute to fostering a more supportive work environment. These findings reinforce the importance of systematic support programs for employees, emphasizing stress prevention and mental health promotion as key components of workplace well-being initiatives.

CONCLUSION

The findings of this study indicate that burnout levels among community pharmacy professionals remained relatively stable over the observed period, despite slight increases in mean scores. No statistically significant differences were found between measurement points, nor were there strong correlations between them. These results suggest that burnout may be a persistent issue rather than one that fluctuates significantly over short periods.

However, the relatively low correlations between burnout measurements suggest that individual differences and external factors may have influenced the results. Variability in the extent to which participants engaged with stress management strategies, as well as other organizational and personal factors such as workload, team dynamics, and coping mechanisms, could have played a role in shaping burnout experiences over time.

Future research should explore these individual and organizational factors in more detail, incorporating a longer follow-up period to assess the factors contributing to burnout stability.. Additionally, studies examining the role of workplace culture, leadership support, and specific psychological safety initiatives could provide valuable insights into effective burnout prevention strategies in pharmacy settings.

In conclusion, this study highlights that burnout levels remained stable despite professional demands, while psychological safety showed a slight increase over time. Although no direct link was established between psychological safety and burnout, these findings suggest the need for further research on its potential role in well-being. These findings reinforce the need for organizations to implement comprehensive stress prevention programs and foster supportive work environments that promote well-being and resilience in healthcare settings.

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CONFLICT OF INTEREST

The author has no confict of interest to declare

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ANXIETY AND DEPRESSION AMONG FINAL-YEAR HIGH SCHOOL STUDENTS IN SERBIA: A CROSS-SECTIONAL STUDY FOLLOWING A NATIONAL SCHOOL TRAGEDY

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ABSTRACT

Anxiety and depression are prevalent among adolescents and may be exacerbated by exposure to traumatic events. This study aimed to assess the levels of anxiety and depression among high school seniors in Kragujevac, Serbia, in the relation with tragic school shooting in Belgrade. A cross-sectional, population-based study was conducted from November to December 2024, including 145 final-year high school students. Participants completed the Hamilton Anxiety Rating Scale (HAM-A) and Beck Depression Inventory-II (BDI-II), along with a detailed sociodemographic survey. Of the participants, 39.3% exhibited mild to severe anxiety, while 31.7% reported mild to severe depressive symptoms. Female students, those living away from parental homes, individuals who consumed alcohol or psychoactive substances, and those with poor peer relationships or experiences of bullying showed significantly higher anxiety and depression levels. Emotional responses to the tragic event were strongly associated with elevated psychological distress. Behaviors such as school refusal, thoughts of transferring schools, and seeking psychological help also correlated with higher symptom severity. This study highlights the acute psychological vulnerability of adolescents following national traumatic event. The findings underscore the importance of early emotional development, social support systems, and school-based mental health programs in mitigating the long-term consequences of trauma exposure during adolescence.

Keywords: *Adolescents, anxiety, depression, school violence, mental health.*

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INTRODUCTION

Anxiety and depression are the most common mental health disorders, affecting over 301 million and 280 million people worldwide, respectively (1). According to the World Health Organization (WHO), one in eight individuals globally is affected by a mental disorder (2), highlighting the urgency of evaluating contributing factors to this growing health concern.

Anxiety and depression are widely present in children and adolescence, with 58 million suffering from anxiety and 23 million suffering from depression (1). While fears in social situations are relatively common among adolescents, diagnostic interviews reveal a high prevalence of social anxiety disorder (3). Symptoms of social anxiety typically emerge during adolescence (4), with some studies indicating a prevalence of 9% among American adolescents (5) and approximately 12% among Dutch adolescents (6). Also, higher lifetime depression prevalence of 12% among American adolescents (5) and 15% among Dutch adolescents (6). Numerous epidemiological studies suggest that, in the past two years, anxiety disorders have become the most prevalent mental health disorders among adolescents. Among these, generalized anxiety disorder is recognized as one of the most common anxiety disorders, with a prevalence ranging from 2.2% to 3.6% (7). Various studies conducted over the past decades reported that the prevalence of stress, anxiety, and depression is higher among university students compared to the general population (8).

Although school violence has been a long-standing issue, its impact has intensified in recent times due to widespread media coverage of dramatic and often deadly incidents. Acts of bullying and mass shootings within educational settings evoke intense emotions such as fear, shock, and a sense of insecurity among students, disrupting what was once considered a safe space. Those who survive these traumatic events, along with individuals exposed to them through media reports, may experience a range of psychological effects, including acute stress disorder, post-traumatic stress disorder, depression, and anxiety (9). There is evidence that gun violence associated with negative mental health outcomes, including anxiety, depression, panic attacks, and post-traumatic stress symptoms (10). Having in mind the occurrence of tragic event in elementary school "Vladislav Ribnikar" in Belgrade, Serbia, which occurred in May 2023, the aim of our study was to determine the levels of anxiety and depression among high school seniors experienced in relation to this tragic event.

MATERIALS AND METHODS

Study design

This was an observational, population-based, cross-sectional epidemiological study which included high school senior students (n=145) attending final year of the high school on the territory of city of Kragujevac, Central Serbia. The study was undertaken during the period of one month, from November 2024 until December 2024. The study protocol was approved by the Institutional Ethical Committee, Faculty of Medical Sciences, University of Kragujevac, Serbia. Before every study intervention, all participants were introduced with the study and volountarly gave their informed consent for participation.

Inclusion criteria for participation in the study were: being 18 years of age or older, enrolled as a final-year student at a high school in Kragujevac, literate, capable of understanding the study, and having provided signed informed consent. Exclusion criteria for participation in the study were: individuals younger than 18 years of age, those who were illiterate or unable to comprehend the study, and individuals with diagnosed mental disorders and/or a history of substance abuse.

Study sample and questionnaries

A random sample was used as the method for selecting students. Only students from the fourth (final) grade of high schools were included. As part of the research instrument, the following standardized questionnaires were used:

The HAM-A is used to assess both somatic and psychological symptoms of anxiety. It includes 14 items, each rated on a 5-point scale (0 = not present; 4 = very severe). Interpretation of the total score is as follows: ≤7: minimal or no anxiety; 8-14: mild anxiety; 15-23: moderate anxiety; ≥24: severe anxiety (11).

The BDI-II is a widely used self-report questionnaire for screening and assessing the severity of depression. It consists of 21 multiple-choice items, each offering four statements ranked by the severity of a particular depressive symptom, scored on a 0-3 scale. Total scores are interpreted as follows: 0-13: minimal or no depression; 14-19: mild depression; 20-28: moderate depression; 29-63: severe depression (12,13).

As part of the study, participants also completed a sociodemographic questionnaire designed to gather comprehensive background information. This included data on gender, age, family history of psychiatric disorders, the presence of comorbidities, whether participants were raised in intact or non-intact families, and their type of accommodation during the school year. The questionnaire also explored academic variables such as timely enrollment in secondary school, enrollment in the school of choice, and overall academic success. Additionally, it addressed lifestyle behaviors, including the use of cigarettes, alcohol, and psychoactive substances. To further contextualize psychological outcomes, participants were asked about their peer relationships, experiences with peer bullying and opinion of increased peer bullying following tragic event, school refusal, consideration of school transfer, seeking psychological support, as well as emotional responses to the recent school shooting incident in Belgrade.

Statistical analysis

All statistical analyses were performed using the commercial standard software package SPSS, version 20.0 (The Statistical Package for Social Sciences software, SPSS Inc., version 20.0, Chicago, IL). All data collected from the survey questionnaires were presented and analyzed using appropriate mathematical and statistical methods, suitable for the type and nature of the data. Descriptive methods were used to present the data, including tabulation, graphical representation, measures of central tendency, and measures of variability. In the statistical data processing, continuous variables were presented as mean ± standard deviation (SD), while categorical variables were presented as proportions of participants with a specific outcome. To compare the means of continuous variables, Student's t-test for small independent samples was used, or alternatively, a nonparametric test if the results did not follow a normal distribution, as determined by the Kolmogorov-Smirnov test. The chi-square (χ^2) test was used to compare differences in the frequency of categorical variables, or Fisher's exact test if the frequency of individual categories was low. Results were considered statistically significant if the probability of the null hypothesis was less than 5% ($p < 0.05$).

RESULTS

Sociodemographic characteristics of study population

This study included 145 high school seniors, of whom 98 (67.6%) were female and 47 (32.4%) were male. All participants were attending the high school in the territory of city of Kragujevac, Serbia. The obtained demographic characteristics of study participants are presented in Table 1.

The level of anxiety and depression in study population

According to HAM-A and BDI-II score, 39.3% students in our study exhibited mild to severe anxiety (Table 2), while 31.7% reported mild to severe depressive symptoms (Table 3).

The prevalence of anxiety and depression in study population in relation to sociodemographic characteristics

In our study, female students reported significantly higher levels of both anxiety and depression compared to male students. The results, as illustrated in Figure 1, showed that the mean scores for both anxiety and depression were notably elevated among female participants.

In addition, we examined the levels of anxiety and depression in relation to specific sociodemographic characteristics. No statistically significant differences in anxiety or depression were found between students raised in intact families and those from non-intact families. Regarding place of residence during the school year, students living in student dormitories or private accommodations exhibited significantly higher levels of anxiety compared to those residing in their parental homes. Conversely, no significant differences were observed in depression levels between these groups. Smoking status did not influence the development of anxiety, but smokers showed higher scores on depression assessment scales compared to non-smokers. When examining alcohol consumption, data analysis showed significantly higher levels of both anxiety and depression among students who consumed alcohol often compared to those who did not. However, there were no statistically significant difference regarding anxiety and depression between non-consumers and those who consume alcohol rarely or on special occasions. Other psychoactive substances have similar effects and consequences. In our sample, students who abused other psychoactive substances also exhibited significantly higher levels of anxiety and depression. When examining high school enrollment, students who enrolled later than their age cohort exhibited significantly higher levels of anxiety and depression. However, no statistically significant relationship was found regarding enrollment in their desired high school.

The influence of peer relationships on anxiety and depression

Students who reported difficulties in peer communication demonstrated higher levels of anxiety and depressive symptoms (Figure 2A). Furthermore, those who had experienced peer bullying exhibited significantly elevated anxiety and depression scores compared to their peers who had not encountered such experiences (Figure 2B).

The influence of national tragic event on anxiety, depression, and coping behaviors

Students who reported school refusal demonstrated higher levels of anxiety and depressive symptoms (Figure 3A). Furthermore, those who considered transferring to another school exhibited significantly elevated anxiety and depression scores compared to their peers who had not encountered such experiences (Figure 3B).

Students who reported that in their opinion there was an increase in peer bullying following a tragic event demonstrated higher levels of depressive symptoms (Figure 4A). Furthermore, those who were seeking for psychological help after tragic event exhibited significantly elevated anxiety and depression scores compared to peers who did not feel the need to seek professional psychological support (Figure 4B).

Emotional responses to a national tragic event and their impact on mental health

In our study, we examined the most common emotional responses following a tragic event, including feelings of sadness, distress, helplessness, anger, neutrality, as well as combinations such as sadness and distress simultaneously, or helplessness and distress together, and their relationship with anxiety and depression.

Our results showed that students who experienced feelings of sadness had lower level of anxiety than those who experienced feelings such as helplessness, anger, sadness combined with distress and helplessness, or sadness

combined with distress. Additionally, students who felt both sad and distressed simultaneously exhibited higher anxiety levels than those who reported feeling only distressed (Figure 5A).

Regarding depressive symptoms, students who felt angry after the tragic event demonstrated significantly higher levels of depression compared to those who felt sad, distressed, helpless, neutral, or combinations of sadness, distress, and helplessness. Furthermore, students experiencing both sadness and distress simultaneously showed increased depression levels compared to those who reported feeling only sad or the combination of sadness, distress, and helplessness (Figure 5B).

Table 1. Sociodemographic characteristics of the study population.

Characteristic	Number (n) / Percentage (%)
Gender (male/female)	47 (32.4%) / 98 (67.6%)
Family history of psychiatric disorders (yes/no)	15 (10.3%) / 130 (89.7%)
Presence of comorbidities (yes/no)	14 (9.7%) / 131 (90.3%)
Growing up in an intact family (yes/no)	128 (88.3%) / 17 (11.7%)
Place of residence during school year (parental home/student dormitory or private accomodation)	133 (91.7%) / 12 (8.3%)
Smoking (yes/no)	34 (23.4%) / 111 (76.6%)
Alcohol abuse (no/rarly/at special occasios/often)	41 (28.3%) / 29 (20%) / 67 (46.2%) / 8 (5.5%)
Psychoacitve substances abuse (yes/no)	7 (4.8%) / 138 (95.2%)
Timely enrollment in secondary school (yes/no)	143 (98.6%) / 2 (1.4%)
Enrollment in secondary school of choice (yes/ no)	142 (97.9%) / 3 (2.1%)
Academic success in secondary school (excellent/very good/good/sufficient(pass)/ insufficient(fail))	88 (60.7%) / 43 (29.6%) / 12 (8.3%) / 2 (1.4%) / 0 (0%)

Table 2. Level of anxiety in study population.

HAM-A score	Number (n) / Percentage (%)
Minimal or no anxiety (≤7)	88 / 60.7%
Mild anxiety (8-14)	33 / 22.8%
Moderate (15-23)	14 / 9.7%
Severe (≥24)	10 / 6.9%

Table 3. Level of depression in study population.

BDI-II score	Number (n) / Percentage (%)
No depression (0-13)	99 / 68.3%
Mild depression (14-19)	22 / 15.2%
Mild to moderate depression (20-28)	14 / 9.7%
Severe depression (29-63)	10 / 6.9%

Figure 1. Mean scores of anxiety (A) and depression (B) among study participants in relation to gender.

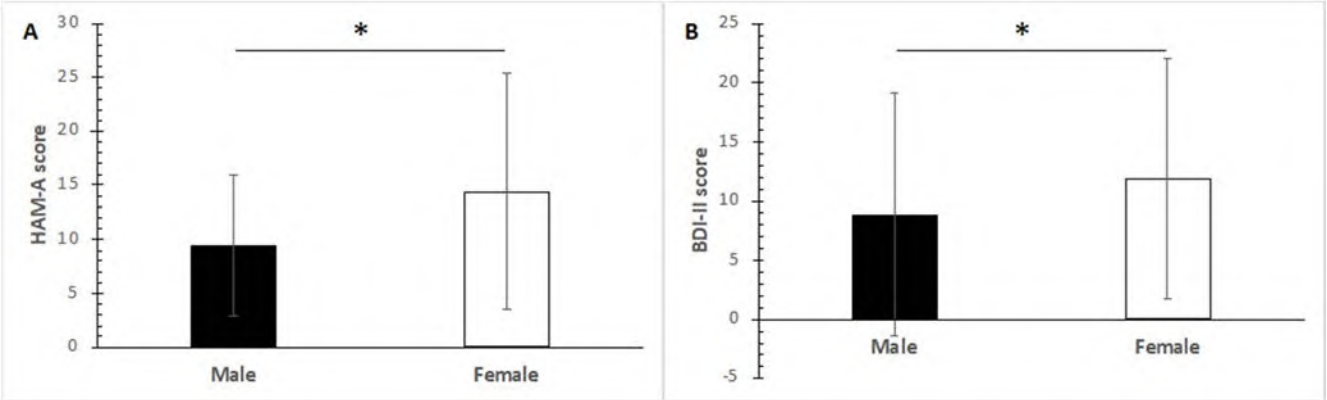


Figure 2. Levels of anxiety and depression among study participants in relation to peer communication (A) and experiences of peer bullying (B).

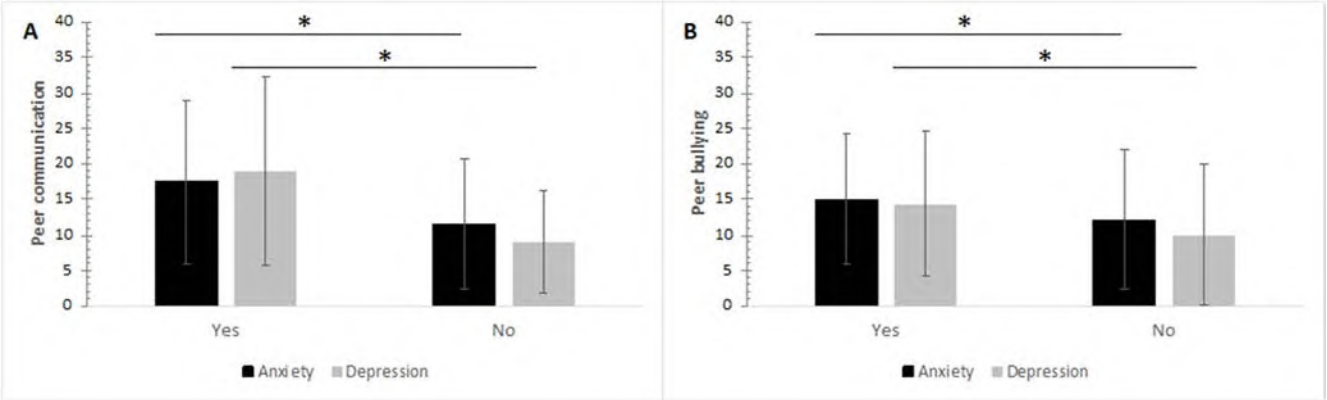


Figure 3. Levels of anxiety and depression among study participants in relation to school refusal (A) and consideration of school transfer (B) following a tragic event.

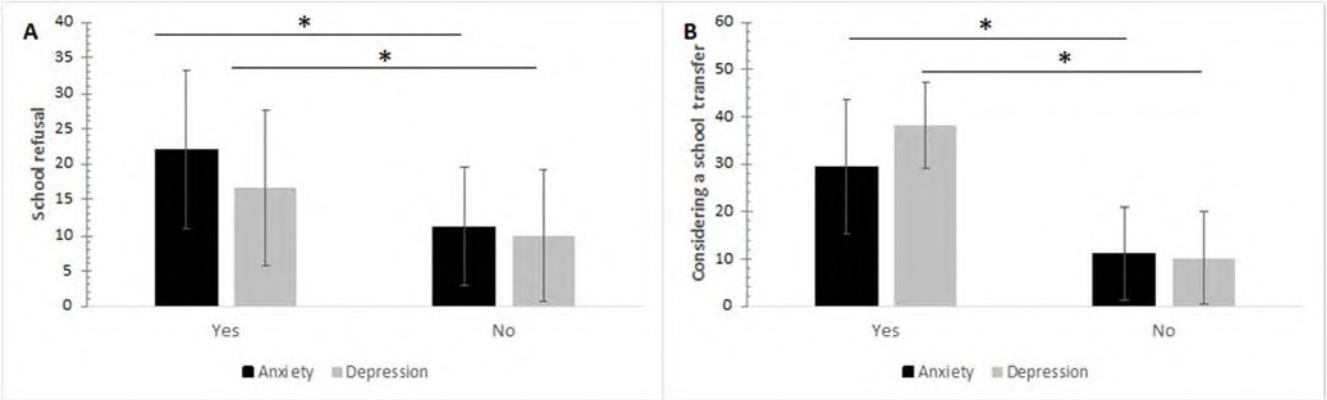


Figure 4. Levels of anxiety and depression among study participants in relation to opinion to the increase in peer bullying (A) and seeking psychological help (B) following a tragic event.

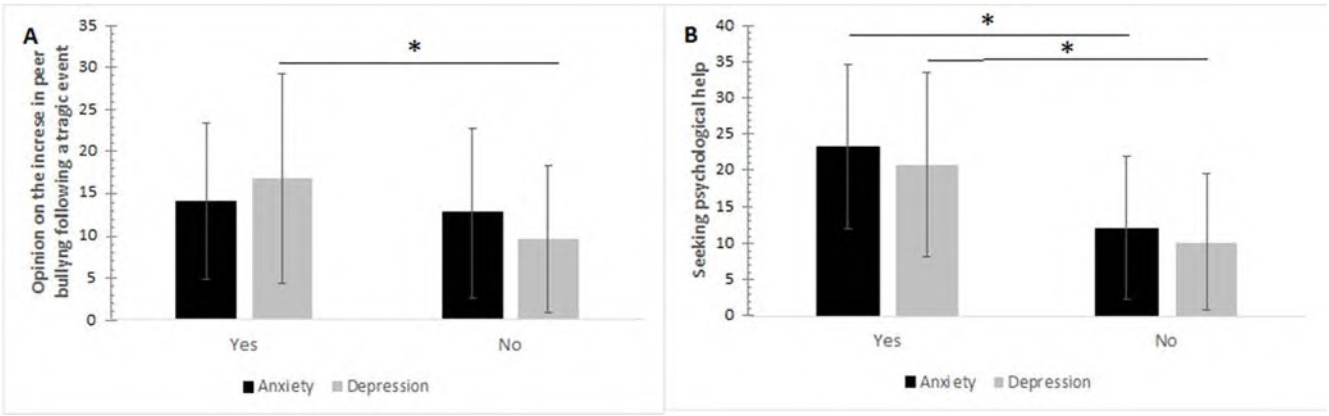
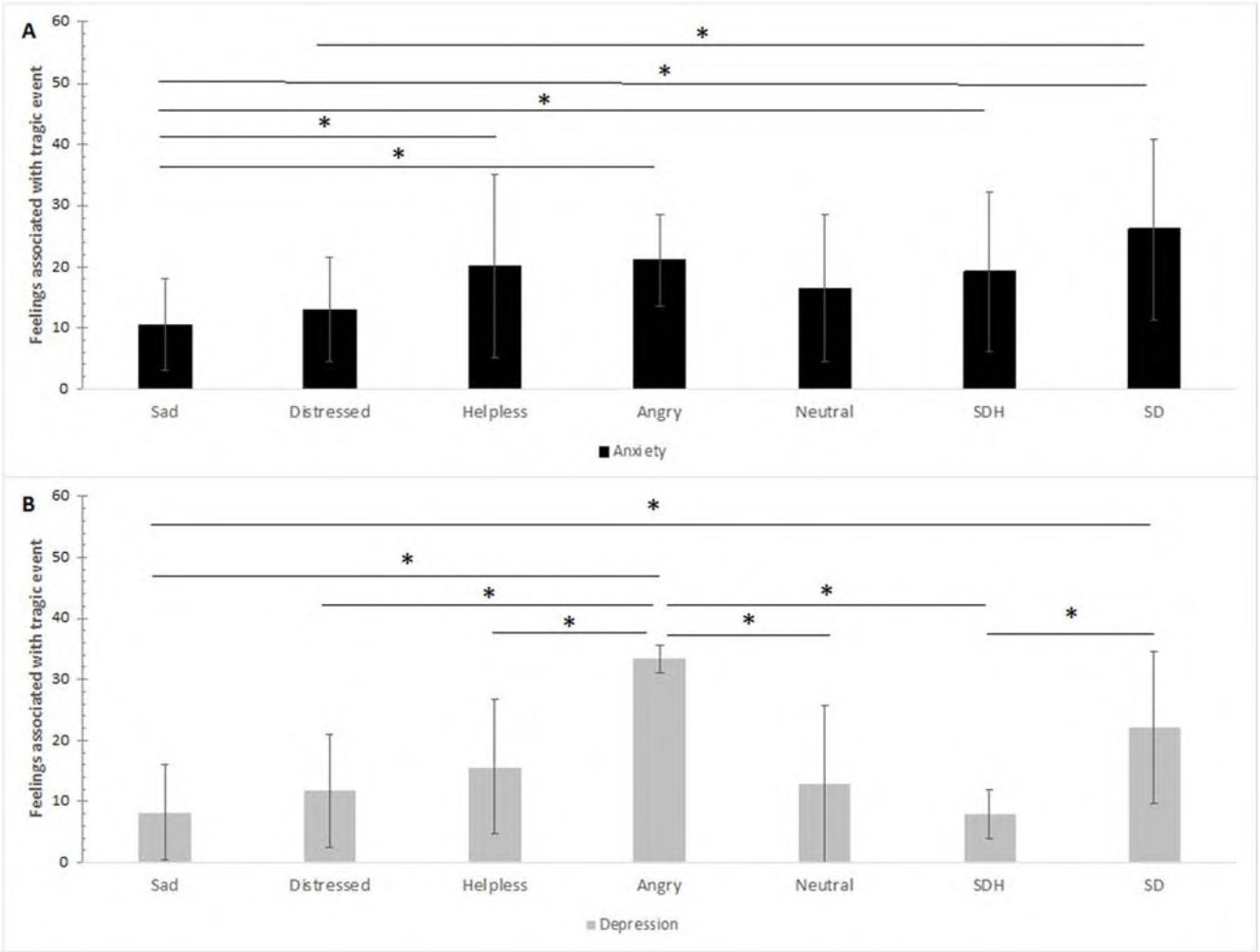


Figure 5. Levels of anxiety (A) and depression (B) among study participants in relation to specific emotional reactions following a tragic event.



DISCUSSION

During development, critical turning points emerge in the maturation of emotion regulation, particularly pronounced during childhood and adolescence. In early childhood, emotional experiences are typically co-regulated by caregivers, who play a central role in helping the child manage and make sense of emotional states. As individuals transition into adolescence, there is a normative shift toward greater emotional autonomy, accompanied by a decreased reliance on parental support. However, this period is also characterized by the ongoing development of internal regulatory mechanisms, which may still lack full maturity and effectiveness. Impairments or delays in the development of emotion regulation capacities are considered central to theoretical models explaining the onset and maintenance of anxiety and depression (14).

Our study reveals a pronounced presence of anxiety and depression among final-year high school students in Kragujevac, following the tragic event at the "Vladislav Ribnikar" elementary school in Belgrade. This finding underscores the heightened sensitivity of adolescents to external stressful events, especially those impacting their peer community and educational environment. Consistent with previous research (15), female participants exhibited significantly higher symptoms of both anxiety and depression, aligning with biological, social, and psychological factors influencing gender differences in mental health during adolescence

Living arrangements also played a role in mental health outcomes. Students residing away from their parental homes, whether in dormitories or private accommodations, reported higher levels of anxiety. This association may be attributed to reduced emotional and social support, increased responsibilities, and uncertainty. However, the difference in depression levels between these groups was not statistically significant. Previous studies have highlighted the role of attachment in shaping responses to separation and anxiety, emphasizing the importance of secure familial relationships. One study showed that family accommodation was more strongly linked to separation anxiety in children with low attachment security, highlighting attachment's role in shaping family responses to child anxiety, while no similar effect was found for other anxiety symptoms (16). Also, it has been demonstrated that social anxiety is closely related to delays in important life steps, like moving out or living with a partner, but most socially anxious teens still reach these milestones in early adulthood. It suggests that adolescence is a key time to offer support, and more research is needed on how social anxiety affects long-term life and health outcomes (17).

Behavioral habits, particularly the consumption of cigarettes, alcohol, and psychoactive substances, demonstrated a clear association with elevated levels of anxiety in relation of smoking habits, and with elevated level of both anxiety and depression in case of alcohol or psychoactive substance abuse in our study. These patterns of self-medication among youth often represent attempts to cope with stress and emotional pain but ultimately exacerbate mental health issues over time. The accessibility and social acceptance of these substances further complicate intervention efforts. A significant association between substance use and mental health disorders has been well established. Notably, cannabis use has been linked to an increased risk of developing depression and anxiety (18). Stimulant use of substances such as cocaine and amphetamines have been associated with a higher risk of developing psychotic symptoms, while chronic alcohol use has been implicated in the exacerbation of attention-deficit/hyperactivity disorder (18). These associations suggest a complex bidirectional relationship, wherein substance use may both contribute to and result from underlying mental health conditions.

Peer relationships emerged as a significant factor in adolescent mental health. Negative peer interactions, including poor communication and peer bullying, were strongly associated with heightened anxiety and depression levels in our study. Adolescence is a period when peer relationships become central to emotional development; thus, problems in this area can have profound psychological impacts. Our findings align with studies indicating that the quality of peer relationships significantly predicts depressive symptoms, with girls being more affected than boys (19).

Following the traumatic event, a significant number of students in our study displayed emotional reactions such as sadness, anger, helplessness, and distress, which were predictors of higher psychological distress. Furthermore, those who reported feelings of anger and combined emotional responses (e.g., sadness + distress) had the highest anxiety and depression scores, supporting theories about the importance of emotional regulation abilities in preventing mental disorders in youth (20). Additionally, school avoidance, thoughts of changing schools, and seeking psychological help emerged as important reactions, indicating an urgent need for systematic psychological support in schools. Emotionally based school avoidance is a growing concern, with recent studies highlighting its increasing prevalence among students experiencing anxiety and emotional distress. *Corcoran* and *Kelly* emphasize the importance of multi-agency approaches to support regular attendance and address the underlying emotional factors contributing to emotionally based school avoidance (21).

The impact of stress on children and adolescents can be understood through different models. The literature frequently emphasizes theoretical models that examine the role of negative life events and chronic stress in the etiology of mental health disorders. Early childhood is a particularly sensitive period for the development of emotional regulation and stress coping capacities, which are largely shaped through interactions with primary caregivers. When caregivers experience high levels of anxiety, these emotional states are often transmitted to the child in non-verbal and diffuse ways - what some theorists describe as "undifferentiated noise" within the

developmental environment. The child's ability to process and make sense of such experiences depends significantly on the caregiver's capacity to contain and transform distressing emotions into manageable, structured feedback, thereby communicating that the world is safe and predictable (22).

Children and adolescents are frequently exposed to a series of negative or even traumatic life events, which may verge on overwhelming stress or existential threat. When such events occur, even in the presence of external support systems, processing the associated fear and emotions requires considerable time and psychological resources. Without adequate support, these experiences may remain unintegrated, increasing the risk for various psychopathologies. Such exposures are recognized as key precipitants in the development of numerous psychiatric disorders and broader dysfunctions in emotional, social, and academic domains. Initial manifestations may present as somatic symptoms - such as headaches, fatigue, or gastrointestinal issues - which often precede or accompany the emergence of psychological symptoms, illustrating the dynamic interplay between the body and mind. Given this, stress must be acknowledged as a critical factor influencing mental health across all developmental stages (23).

In light of these considerations, the implementation of school-based screening and counseling programs is essential, while it can enhance early detection, prevention, and enable appropriate further guidance for those students with more pronounced/chronic symptoms (24). Furthermore, it is crucial to recognize that poor mental health during adolescence not only affects immediate well-being but also has long-term implications (25). Adolescents experiencing depression are at significantly elevated risk for future major depressive episodes, suicidal behavior, anxiety disorders, substance use disorders, interpersonal difficulties, and early parenthood (26).

CONCLUSIONS

This study highlights the significant psychological impact of traumatic events on adolescents, particularly in the form of increased anxiety and depression. Emotional responses such as sadness, helplessness, and school avoidance were common and signal the need for urgent psychological support within educational settings. Female students, those without parental support, and individuals involved in risky behaviors or poor peer relationships were especially vulnerable. Substance use was strongly linked to mental health symptoms, suggesting maladaptive coping strategies among youth. These findings underline the importance of early development, caregiver support, and emotional regulation in shaping resilience to stress. Implementing school-based screening, prevention, and counseling programs is essential to identify students at-risk and reduce long-term consequences. A coordinated response involving schools, families, and mental health professionals is vital to promote healthy adolescent development and well-being.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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CLINICAL AND LABORATORY CHARACTERISTICS OF CHILDREN WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA

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ABSTRACT

The most common primary autoimmune thrombocytopenia in children is immune (idiopathic) thrombocytopenic purpura (ITP) with extremely variable clinical manifestations, from asymptomatic forms to life-threatening bleeding. The latest classification divides ITP into acute (ndITP), persistent (pITP) and chronic form (cITP). This research was designed as a retrospective–prospective study which analyzed basic demographic, clinical and routine laboratory parameters relevant to ITP. There was slight predominance of girls in all forms of ITP. Children of preschool age dominated in ndITP, while adolescents in cITP group. pITP and cITP patients predominantly presented as asymptomatic or with mild haemorrhagic signs, while ndITP patients had moderate or severe bleeding. Skin hematomas are the most common sites of bleeding. Mostly ndITP patients did not have other diseases, while 30% of cITP patient have other autoimmune disease. No hepatosplenomegaly was observed in ndITP patient, but almost a quarter of the cITP patients had splenomegaly. The mean value of platelet count is significantly higher in chronic groups compared to ndITP group, against mean platelet volume values that show an inverse correlation. More than half ndITP patients achieved complete remission after intravenous immunoglobulin and additional 30% experienced spontaneous remission during the persistent disease period and about one third of cITP patients required therapy. The spleen is dominant or only organ of platelet sequestration in cITP patients.

Keywords: *Idiopathic thrombocytopenic purpura, children.*

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INTRODUCTION

The most common primary autoimmune thrombocytopenia or immune (idiopathic) thrombocytopenic purpura (ITP) is an autoimmune disease characterized by binding of autoantibodies (most often IgG, less often IgM class) to specific glycoproteins on platelets (primarily GP-IIb/IIIa and GP-Ib/IX complex) which cause platelet destruction (1, 2). These antibodies are usually directed towards viral antigens, vaccine components, insect toxins, or reproduced in other autoimmune or infectious diseases. Platelets marked in this way aggregate and their extravascular destruction by the cells of the reticuloendothelial system is enhanced. Predominantly, this process occurs in the spleen, but also in the liver. In addition, platelets marked with autoantibodies trigger complement activation and consequent apoptosis is initiated (1–4).

However, regardless of these clear facts and observations, the pathophysiology of autoimmune thrombocytopenia remains unclear. For example, one group of investigators observed oligoclonality in B-lymphocytes of patients with ITP (5). A similar disorder of T-lymphocytes indicates a possible role of T-lymphocyte dysfunction (6). Studies have shown that some patients with ITP have platelet counts close to normal. This indicates the possibility that autoantibodies can also damage megakaryocytes or that only some platelets are a target for antibodies, while others are unaffected (1, 2). Many studies in recent years have shown that abnormalities of dendritic cells, natural killer (NK) cells, cytokine disorders (interleukin 2, interleukin 17 and interferon γ), programmed cell death, oxidative stress, infection, pregnancy and drugs may play a significant role in the pathogenesis of ITP (5–7).

The latest classification divides ITP into a newly diagnosed form (ndITP), which lasts up to 3 months, a persistent form (pITP) from 3 to 12 months, and a chronic form (cITP), when the disease lasts longer than 12 months (4).

Acute ITP is a disease that usually occurs in childhood, most often following a viral infection or vaccination (8, 9). The clinical manifestations of ITP are extremely variable, from asymptomatic forms to life-threatening bleeding. Usually the disease begins suddenly, mainly with skin bleeding (hematomas, petechiae and ecchymoses) and visible mucous membranes (most often epistaxis or gingival bleeding), gastrointestinal (melena or hematemesis) or urogenital tract (usually menometrorrhagia). Apart from the tendency to hemorrhagic syndrome, the other clinical findings in the majority of cases are usually normal, so any presence of lymphadenopathy or organomegaly is not consistent with the diagnosis. The most serious complication is intracranial bleeding, fortunately with a low incidence of <1% (4, 10, 11).

Acute ITP is most often a benign disease and usually resolves spontaneously without any consequences. In 60% of children with ITP, clinical manifestations may disappear spontaneously within 3 months and in an additional 20-30% within a year, when they transition to a persistent form and require more serious monitoring. It is up to the clinicians to decide, with a mandatory consultation and a detailed explanation to the parents, in accordance with the severity of the hemorrhagic syndrome, how the treatment will be carried out. Usually the degree of the hemorrhagic syndrome correlates with the platelet count (4, 11, 12).

Until now, it is not possible to predict the length of remission achieved (either clinical or laboratory), both in newly diagnosed and chronic forms. In about 10-30% of children with acute ITP, thrombocytopenia is present for more than 12 months and then chronic ITP occurs, and it is not yet possible to define the factors that can predict which patients will develop a chronic form of the disease at presentation. It is also not possible to predict which 20% of children with chronic ITP will have a spontaneous recovery (13, 14). Patients with chronic ITP and a history of hemorrhagic syndrome should be treated, especially those with platelet counts <10–20×10^9/L or moderate thrombocytopenia (20–30×10^9/L). Asymptomatic ITP with moderate thrombocytopenia (20–50×10^9/L) or patients with platelet counts greater than 50×10^9/L generally do not require treatment (4, 13, 14).

This study presents a set of the most significant clinical and laboratory characteristics of children with different forms of ITP, which can additionally help clinicians in their daily work, in terms of evaluating therapeutic and prognostic parameters for each individual patient.

MATERIAL AND METHODS

This clinical research was designed as a retrospective - prospective, cohort and observational study in children aged 6 months to 18 years with a diagnosis of various forms of idiopathic thrombocytopenic purpura. A total of 102 children divided into 4 groups were included in the research: 1.) Newly diagnosed ITP (ndITP): 27 children with some of the signs of hemorrhagic syndrome or asymptomatic, with thrombocytopenia in complete blood count; 2.) Persistent ITP (pITP): 22 children whose illness lasts longer than 3 and shorter than 12 months treated with different therapeutic modalities; 3.) Chronic ITP (cITP): 29 children in whom the disease lasts longer than 12 months, in whom a platelet kinetics test was performed with a radioactive tracer, who were treated with available treatment methods in the previous period; 4.) Control groups: a.) 12 healthy children and b.) 12 healthy children who previously suffered from ITP and were in stable complete clinical and laboratory remission for at least 12 months. The research was carried out as academic and non-profit. We used data from the medical records of children hospitalized at the Pediatric Clinic, University Clinical Centre Kragujevac over a 3-year period in retrospective part of study and in 2-year period in prospective part as newly diagnosed cases were hospitalized and in whom standard clinical and laboratory work-up related to ITP was performed.

Study inclusion criteria was: 1.) the diagnosis of newly diagnosed ITP according to the guidelines of the American Society of Hematology (4); 2.) for the persistent and chronic

form of the disease, meeting the conditions regarding the duration of the disease.

Criteria for excluding subjects from the study: 1.) pseudothrombocytopenia (20), 2.) infants younger than 6 months with a diagnosis of neonatal thrombocytopenia; 3.) children with some form of thrombasthenia; 4.) pregnant women up to the age of 18 with ITP; 5.) children with ITP and any haemostatic disorder; 6.) children with Evans syndrome; 8.) children with ITP and other diseases that require the use of chronic therapy (asthma, kidney patients, oncology patients, etc.).

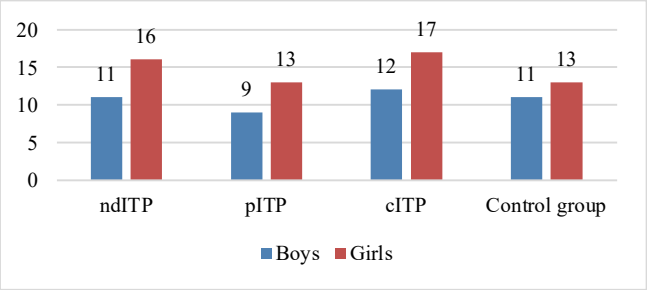
All patients were analyzed for basic demographic and clinical parameters relevant to ITP, as well as all routine laboratory and radiographic tests used in the diagnosis of ITP.

The obtained results were processed and presented using the methods of descriptive statistics. A value of $p<0.05$ was considered statistically significant. All data were analyzed using the statistical program IBM statistics SPSS 21.

RESULTS

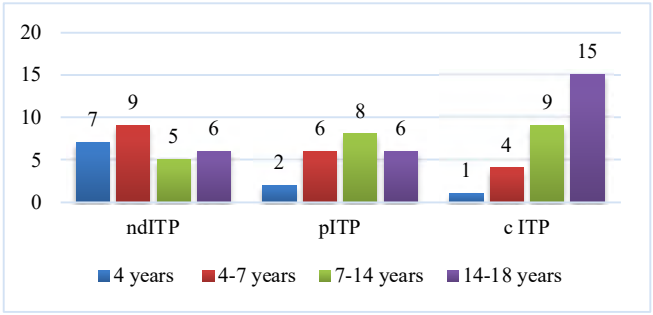
The total number of examined children was 102. The examines were divided into 4 groups: 1) 27 children with newly discovered ITP (ndITP); 2) 22 children with persistent ITP (pITP); 3) 29 children with chronic ITP (cITP) and 4) 24 children in the control group (12 healthy subjects and 12 patients cured of ITP – cITP).

Graph 1 shows gender distribution of examined children. A slight predominance of girls is observed in all forms of ITP (F:M 1.2–1.3:1).



Graph 1. Number of children with ITP according to gender

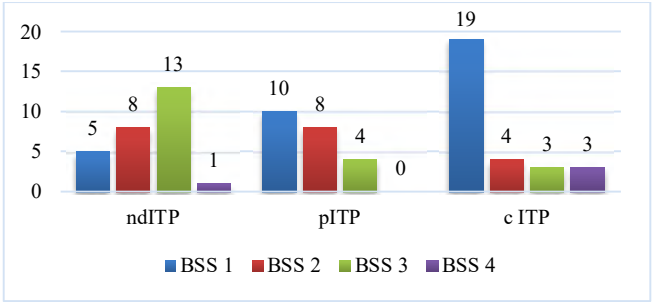
The average age of our subjects was 8.7 ± 3.8 years. However, the most of the patients in cITP group were adolescents (around 55%), while ndITP mainly affects children of preschool age (60%) (Graph 2). The average age of our examinees in the group of children with ndITP is 7.4 ± 2.5 years, while in the group with cITP the average age is 14.5 ± 3.7 years.



Graph 2. Number of children with ITP according to age

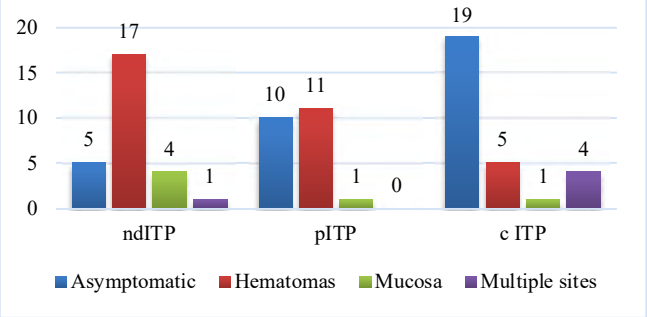
In our cohort, nearly two-thirds of patients with cITP did not have a clear causative factor that triggered the immune process (infection, vaccine, insect bite, etc.) at the time of disease diagnosis. On the other hand, in the ndITP group, almost a third of patients do not have data on a clear causative factor (8/27). In five out of six patients, who developed chronic form of disease during the follow-up period, the disease started without a clear triggering cause.

The severity of the clinical outcome in our patients was assessed according to the degree of bleeding based on the Bleeding Severity Score (BSS), dominant bleeding sites, as well as the need for transfusions of deplasmatized erythrocytes and concentrated platelets during the course of the disease. Among our subjects with ndITP and pITP, there were none one who required transfusions of blood components, while 4 chronic patients had to receive blood transfusions due to extensive bleeding. About half of patients with ndITP had moderate bleeding, on the other hand, 20% were asymptomatic at disease presentation. Persistent and chronic patients predominantly presented with mild signs of hemorrhagic syndrome or were asymptomatic, while 10% of chronic patients had moderate and severe bleeding (Graph 3).

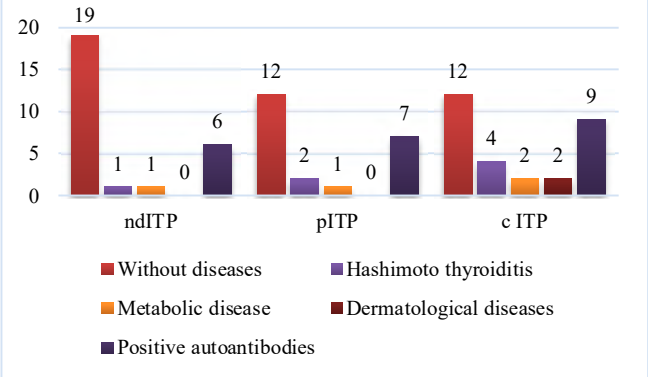


Graph 3. Degree of bleeding according to BSS in children with ITP

The most common sites of bleeding in our patients were skin hematomas, while gingival bleeding, epistaxis or other mucous membranes bleeding was generally less frequent in patients with a more severe clinical picture. There were no patients with intracranial and gastrointestinal bleeding (Graph 4).



Graph 4. Dominant sites of bleeding in children with ITP



Graph 5. Frequency of other autoimmune diseases in children with ITP

The frequency of other autoimmune diseases changes depending on the form of the disease in all examined groups, so 70% of children with ndITP did not have other diseases, while this percentage is significantly lower in pITP (55%), and around 40% in cITP group. On the other hand, there are only individual examples of patients with thyroid diseases or some metabolic disorders in the groups with ndITP and pITP, while the number is more convincing in patients with cITP. Interestingly, in each group, autoantibodies available for routine diagnostics were registered in 25-30% of patients without a clearly manifested autoimmune disease (Graph 5).

Regarding the family history, about 30% of children's family members in all groups did not have any known autoimmune diseases. Also, there were similar percentage of cases where several members of the household expressed autoimmunity, and there were individual cases with metabolic diseases and thyroid gland diseases (Table 1.).

Table 1. Frequency of autoimmune diseases in the family in children with ITP

	ndITP		pITP		cITP	
Family history	**N**	**%**	**N**	**%**	**N**	**%**
ITP in the family	0	0	1	4.5	3	10.3
Thyroid gland diseases	2	7.4	2	9.0	4	13.8
Skin diseases	0	0	1	4.5	1	3,4
Metabolic diseases	6	22.2	3	13.6	5	17.3
Negative family history	10	37.0	7	31.8	8	27.6
More members with autoimmune diseases	9	33.4	8	36.6	8	27.6

N - number

All examined patients had haemostasis screening parameters within reference values, all liver and kidney function parameters were within normal limits, and all had values of immunoglobulin subclasses also within reference values according to age.

No patient in ndITP and pITP had a positive stool antigen for *Helicobacter pylori*, but slightly more than 20% of patients (6/29) in the cITP group tested positive at some point. Three of these patients also had gastric disturbances. Esophagogastroduodenoscopy was performed on them, with definitive confirmation by urease test and PH verification. In one patient, the test was negative in every subsequent control, so eradication therapy was not carried out, while the other 5 were on quadruple therapy, according to the protocol, for a month.

Interestingly, all these patients were boys, with asymptomatic (4/6) and mild clinical presentation (2/6) and platelet count >50 x10^9/L.

Table 2 shows the frequency of hypovitaminosis D and elevated values of lactic dehydrogenase (LDH) in children with ITP. It was observed that about 65–75% of children in all groups with ITP had hypovitaminosis D. 15% of children with ndITP had vitamin D deficiency, while this number is up to 25% in pITP and cITP. Extremely high LDH values, over 750 U/L, were characteristic of children with ndITP (40%), and another half of them had elevated values over 450 U/L. On the other hand, in 65% of patients with pITP and in 40% of patients with cITP, LDH values were within normal limits.

Table 2. Values of lactic dehydrogenase and vitamin D in children with ITP

	ndITP		pITP		cITP	
Vitamin D (ng/mL)	**N**	**%**	**N**	**%**	**N**	**%**
30-40	6	22.2	4	18.2	4	13.8
20-30	3	11.2	2	9.1	4	13.8
10-20	12	44.4	13	59.1	14	48.3
<10	6	22.2	3	13.6	7	24.1
Lactic dehydrogenase (U/L)	**N**	**%**	**N**	**%**	**N**	**%**
<450	3	11.2	14	63.6	12	41.4
450–750	13	48.1	8	36.4	15	51.7
>750	11	40.7	0	0	2	6.9

N - number

No hepatosplenomegaly was observed in any patient with ndITP. On the other hand, in almost a quarter of the patients (5/22 in the pITP and 8/29 in the cITP group), splenomegaly was verified by the echosonographic examination of the abdomen at some point during the follow-up, but in the platelet kinetics test that number was lower, only 4 subjects in cITP group. Also, an accessory spleen was observed in 17% of subjects (5/29) with cITP, while it was not verified in the other groups.

The mean value of platelet count at the time of disease diagnosis in the group with ndITP was $14.7 \pm 3.6 \times 10^9$/L, significantly higher values were registered at pITP $36.4 \pm 7.8 \times 10^9$/L, and at cITP $39.8 \pm 9.1 \times 10^9$/L. This is expected because almost 70% of patients with ndITP had a platelet count $<20 \times 10^9$/L, while only about 20-25% of patients with pITP or cITP had that such a low platelet count, if we exclude clinical relapse of the disease.

Additionally, in the group with ndITP, the highest mean platelet volume (MPV) of 11.2±1.4 fL was registered, while the platelet volume was significantly lower in patients with longer disease duration, 10.3± 1.9 fL in pITP and 10.4±1.7 fL in cITP group. In all children with ITP, a cytological examination of the bone marrow aspirate was performed and no dysplastic changes were found in any of them. The interesting result was that 20-25% of the patients in all three groups with ITP had decreased megakaryocytes in the bone marrow, in contrast to others who had the expected normal bone marrow or hyper production of megakaryocytes (Table 3).

Table 3. Number of platelets, mean volume of platelets and percentage of megakaryocytes in children with ITP

	ndITP		pITP		cITP	
Platelet count ($\times 10^9$/L)	**N**	**%**	**N**	**%**	**N**	**%**
>50	2	7.4	10	45.4	9	31.1
20-50	6	22.2	8	36.4	13	44.8
10-20	9	33.3	2	9.1	5	17.2
<10	10	37.1	2	9.1	2	6.9
Mean platelet volume (fL)	**N**	**%**	**N**	**%**	**N**	**%**
7–10	6	22.2	8	36.4	10	34.5
10-12	10	37.1	14	63.6	15	51.7
>12	11	40.7	0	0	4	13.8
Percentage of megakaryocytes	**N**	**%**	**N**	**%**	**N**	**%**
Hyper production	15	55.5	14	63.6	16	55.2
Normal	5	18.5	3	13.6	7	24.1
Decreased	7	26.0	5	22.8	6	20.7

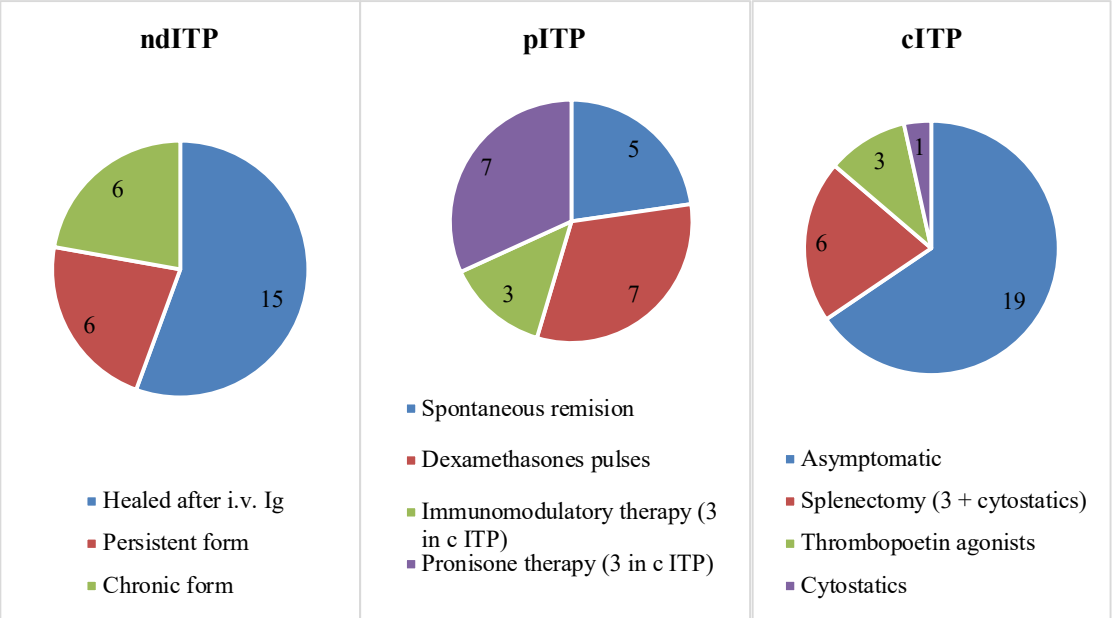
N - number

Of the 27 children with newly diagnosed ITP who were followed for at least one year, more than half (15/27) achieved complete clinical and laboratory remission after intravenous (i.v.) immunoglobulin therapy. The others mostly had a laboratory relapse of the disease, but transitioned to a persistent form (6/27), and the same number of patients had illness which lasted longer than a year, and they were in the chronic phase of ITP.

In patients with pITP, about 30% experienced spontaneous remission, and those who required the use of therapy, same number of patients treated with Prednisone and pulse doses of Dexamethasone(30% = 7/22). Three patients did not respond to Prednisone, thus they received immunomodulatory therapy with Mycophenolate mophetil. In all three patients on immunomodulatory therapy, as well as in 3 patients who were only on Prednisone therapy, the disease progressed to a chronic form. Patients treated with pulse doses of

Dexamethasone and the remaining 4 on Prednisone therapy are in complete remission.

About 35% of patients with cITP (10/29) required therapy: 3 patients were splenectomized and achieved complete remission; 3 patients were treated with thrombopoietin receptor agonists and achieved clinical remission; 4 patients were treated based on different cytostatic protocols and/or Rituximab (anti CD20 antibody) and 3 of them were also splenectomized, but without success (Graph 6.).



Graph 6. Applied therapy in children with ITP

Platelet kinetics test was done in all patients with chronic disease. The average lifespan of platelets in our study group was 0.94 ± 0.47 days (about 22h). It is striking that the largest number of patients (almost 70%) with cITP had a mean platelet lifespan of less than one day, but on the other hand, 15% of patients had an almost normal platelet lifespan. 35% of patients had normal, and another 35% very low level of platelet production in the bone marrow. In almost 60% of patients, the spleen was dominant or only organ of platelet sequestration, and in 30% there was mixed sequestration in the liver and spleen. In 10%, the spleen was not involved in the platelet sequestration process at all (Table 4).

Table 4. Presentation of parameters from the platelet kinetics test in children with cITP

Platelet life span (days)	**<0.5**	**0.5–1**	**1–1.5**	**1.5–4**	**>4**
N	6	13	5	2	3
%	20.7	44.7	17.2	6.9	10.3
Production index	**<0.5**	**0.5–1**	**1–1.5**	**1.5–2**	**>2**
N	5	6	7	6	5
%	17.2	20.7	24.3	20.7	17.2
Place (index) of sequestration	**Liver + other organs**	**Mixed (liver + spleen)**	**Predominantly the spleen**	**Spleen**	
N	3	9	3	14	
%	10.3	31.1	10.3	48.3	

N – number

DISCUSSION

In our representative sample of 90 children suffering from ITP (102 in total and 12 healthy children), it was found that the gender distribution of patients corresponds to data from the literature (F:M ~ 1.2–1.3:1) and that the highest percentage of affected children with the chronic form of ITP were adolescents, with a prevalence of about 35%, while the newly diagnosed form of ITP mostly affected children of preschool age (about 60%) (4, 8, 10). One of the most documented epidemiological differences between ITP in childhood and in adults is certainly a more pronounced predominance of women among adults with the disease in a ratio of ~2:1, while in children it is ~1.3:1, which is certainly associated with an

increased incidence of autoimmune diseases in adult women (15, 16). This fact was also confirmed in our group, because almost 70% of all patients with an associated autoimmune disease were female.

Regarding the anamnestic data, a detail that is often mentioned in the literature as one of the markers that can indicate the chronic form of the disease in patients with newly diagnosed ITP is the absence of a clear causal factor that led to the onset of the disease, such as a previous infection, vaccination, insect bite, etc. (13, 14). In our cohort, almost two-thirds of patients with chronic ITP at the time of diagnosis did not have data on a clear causative factor that triggered the immune process. On the other hand, in our ndITP group, almost a third of patients did not have information about a clear causative factor, and of the patients who transited to chronicity during the follow-up period, four out of six did not have this information. Although it is a small sample, literature data were confirmed in our group as well.

About half of patients with ndITP had moderate bleeding at diagnosis, while 20% were asymptomatic. Among persistent and chronic patients, asymptomatic clinical presentations or forms with mild signs of haemorrhagic syndrome predominate, while 10% of chronic patients had moderate and severe bleeding. By far the dominant sites of bleeding in our patients were skin hematomas, while gingival bleeding, epistaxis or bleeding from other mucous membranes was generally less frequent in patients with a more severe clinical outcome. We had no patients with intracranial and gastrointestinal bleeding. Certainly, the degree of bleeding has been left up to the individual clinicians to assess, although attempts have been made to form scales for the assessment of bleeding (17, 18), but there is still a large discrepancy among researchers in this field. Certainly, asymptomatic patients in ndITP range in most studies around 20%, in contrast to cITP where the results are in different series of 10–70% (4, 10–14, 19). It is expected that patients with a newly diagnosed disease have the most pronounced hemorrhagic syndrome, and according to the clinical experience so far, as well as according to the literature data, chronic patients with ITP have a consistent pattern of alterations of the number of platelets, and even a consistent bleeding pattern over the years, which is disturbed in infections or when using nonsteroidal anti-inflammatory drugs. We considered our chronic patients asymptomatic only if they had a clinical remission after the first acute attack of the disease, with maintenance of thrombocytopenia. After one year, almost 75% of patients were in a stable asymptomatic phase of the disease, which again correlates with literature data (4, 8, 10–14, 19). About 35–40% of patients with pITP have a milder BSS of 2, and only 10% with cITP have severe, sometimes life-threatening bleeding with a score of 4 (Graph 3).

In accordance with the previous data, among our subjects with ndITP and pITP there were none who required transfusions of blood components, while 4 of our chronic patients had to receive transfusions of concentrated platelets and/or deplasmatized erythrocytes due to extensive bleeding. And these percentages are similar to other researchers, where the number of transfused patients ranges between 5–15% (8, 10–14, 19).

Another major historical difference between paediatric and adult ITP is the incidence of comorbid medical conditions. The presence of 1 or more comorbidities was proven in only 5% of children and >30% of adults at disease presentation and in 10–15% of children and 35–50% of adults during 2 years of follow-up (15, 16). In our sample, in all examined groups with ITP, children predominantly do not have other autoimmune diseases, but the frequency changes, so 70% of children with ndITP are free of other diseases, while this percentage is lower in pITP (55%) and in cITP (about 40%). On the other hand, there are only individual examples of patients with thyroid diseases (predominantly Hashimoto's thyroiditis) or some metabolic disorders (polycystic ovary syndrome, insulin resistance, obesity) in the groups with ndITP and pITP, while the number is more convincing in patients with cITP. Interestingly, 25-30% of patients with positive autoantibodies available for diagnosis, without a clearly manifested autoimmune disease, were registered in each group (Graph 5). In most cases, that was the presence of anti-thyroglobulin, anti-nuclear and/or lupus anticoagulant antibodies, as well as a positive Coombs test (either direct or indirect) without any elements for haemolytic anemia, individually or in combination. In other studies, the presence of positive autoantibodies is often mentioned, but so far their importance has not been established(1, 2, 4, 8, 10, 20) and while some authors recommend their monitoring, others question whether they should be done at all (21, 22).

Looking at the family history, in almost the same percentage (about 30-35%) in the families of children with ITP in all groups, either there were no known autoimmune diseases or there were several affected members of the household. There were also individual cases with metabolic diseases and thyroid gland diseases (Table 1.). It must be noted, however, that patients with a chronic form of the disease have a richer family history in terms of autoimmune diseases. Only 4 subjects had relatives who had or still have ITP. Several studies have tried to determine the genetic predisposition and the importance of autoimmune diseases in the family on the occurrence of ITP itself, as well as on the development of the chronic form of the disease, and they all agree that a positive family history contributes to both occurrences, but so far there are no consistent results related specifically to the genetic predisposition to the development of ITP (4, 7, 8, 21–23).

No patient in the ndITP and pITP groups had a positive stool antigen for *Helicobacter pylori*, but slightly more than 20% of patients (6/29) in the cITP group tested positive at some point. Three of these patients also had gastric symptoms, thus esophagogastroduodenoscopy was performed on them, with definitive confirmation of infection by urease test and pathohistological verification. In one patient, the test was negative in every subsequent control, so eradication therapy was not carried out, while the other 5 were on quadruple

therapy for a month, according to the protocol. Interestingly, all these patients were boys, with asymptomatic (4/6) and mild clinical outcome (2/6) and platelet count >50 x10^9/L. So far, no mechanisms have been established that would explain how *Helicobacter pylori* can influence the pathogenesis of ITP and all results are still very contradictory. There is no clear evidence whether these are patients in whom remission would occur in any case or whether eradication therapy and elimination of the causative agent led to remission, and the exact connection between these two entities is still unknown (10, 24).

Our study showed that about 65–75% of children in all groups with ITP had hypovitaminosis D, of which 15% of children with ndITP had vitamin D insufficiency, while this number in pITP and cITP was up to 25% (Table 3). In recent years, the connection between these two entities has been in the sphere of interest. In accordance with our study, some studies have shown the connection between ITP and hypovitaminosis D in over 80%. Still, there is evidence that not only a reduced level of vitamin D contributes to the disease to such an extent, but above all vitamin D receptor gene polymorphism. However, more extensive research is still to be conducted on this topic (25, 26). The fact is that vitamin D supplementation cannot harm patients with ITP.

Extremely high LDH values over 750 U/L are characteristic of children with ndITP (40%), and another half of them had elevated values over 450 U/L. On the other hand, in 65% of patients with pITP and in 40% of patients with cITP, LDH values were within normal limits. This is expected, given that LDH is an intracellular enzyme, released by increased destruction of platelets. On the other hand, viral infections that are frequent in childhood may also cause an increase in LDH. That is why, unlike in adults, LDH is not an adequate parameter for evaluating disease activity in children (4, 8, 10).

No hepatosplenomegaly was observed in any patient with ndITP. On the other hand, splenomegaly was verified at some point during the follow-up by echosonographic examination of the abdomen in almost a quarter of chronic patients (5/22 in the pITP and 8/29 in the cITP group). Nevertheless, on the platelet kinetics test that number was lower, only 4 of respondents in the cITP group. It is true that any appearance of organomegaly automatically excludes the diagnosis of ITP. However, this is not applicable when it comes to chronic forms, given that the long-term destruction of platelets in the reticuloendothelial system of the spleen inevitably leads to their hyperplasia and causally to occasional organ enlargement depending on the activity of the immune process. A similar percentage of splenomegaly was reported by other researchers, especially those who focused on splenectomy as a therapeutic option for ITP (3, 27, 28). Also, either by ultrasound, during the performance of the kinetics test or during splenectomy, an accessory spleen was observed in 17% of subjects (5/29) with cITP, while it was not verified in the other groups. There are only individual case reports of subsequent removal of the accessory spleen, in case of worsening thrombocytopenia after splenectomy, with a good therapeutic effect (29).

The mean platelet count in the group with ndITP was 14.7 ± 3.6 ×10^9/L, significantly higher values were registered in pITP 36.4 ± 7.8 ×10^9/L, and in cITP 39.8 ± 9.1 × 10^9/L. This is expected because almost 70% of patients with ndITP had a platelet count <20 ×10^9/L, while this number is significantly lower in patients with pITP and cITP, only about 20-25% of patients and only in some controls, if we exclude the clinical relapse of the disease (Table 3). On the other hand, about 30% of patients with ndITP had platelet counts >20 ×10^9/L. A higher platelet count of 30–50×10^9/L (depending on the author) at the time of diagnosing the disease is one of the important criteria for the development of a chronic form (4, 10–14). Despite the enormous progress in clarifying the pathophysiology of ITP, both our study and other studies as well as a large number of facts and results from clinical practice still support the fact that the number of platelets is still the leading factor that determines the severity of the clinical outcome in patients with all forms of ITP (4, 8, 10–14, 19). It is relatively rare that patients with a platelet count >20–30 ×10^9/L, which we can consider a satisfactory number, especially in the chronic form, bleed more than patients with a lower platelet count, which brings us back to the beginning and the complicated pathophysiological mechanism of the disease (4, 8, 19).

In the group of patients with ndITP, the highest mean platelet volume (MPV) of 11.2 ± 1.4 fL was registered, while the platelet volume was significantly lower in patients with longer disease duration, 10.3 ± 1.9 fL in pITP and 10.4 ± 1.7 fL in cITP group. This phenomenon suggests that larger platelets are probably more active, both in the metabolic sense and in the process of haemostasis. Larger platelets try to compensate their paucity, which is dominant in the acute form of the disease, with their size. Certainly, in the later course of the disease, other factors are also involved, especially the bone marrow microenvironment (30). Over time in the chronic form, the bone marrow gets used to producing somewhat larger platelets, so this could become the subject of some future research. Numerous studies highlight the importance of MPV in patients with ITP. Some authors even proposed elevated MPV in the newly diagnosed form as one of the markers of the chronic form of the disease (4, 12, 31).

A cytological examination of the bone marrow aspirate was performed in all children with ITP and no dysplastic changes were found in any of them. The interesting result was that 20-25% of the patients with ITP had decreased megakaryocytes in the bone marrow, unlike the others who had expected normal bone marrow or hyper production of megakaryocytes (Table 4). This indicates the fact that autoantibodies, in addition to having a peripheral effect on platelets, can bind to megakaryocytes and inhibit their maturation or lead to their destruction, so that thrombopoietin cannot perform its role. This is particularly evident in patients with ITP who are bleeding and have a platelet count close to normal. This indicates the possibility that autoantibodies directly damaged

megakaryocytes or that they act on cytokines necessary for the growth and proliferation of megakaryocytes or that only some platelets are a target for antibodies, while others are not affected (1, 2, 4, 8, 22, 30, 32).

Five asymptomatic patients in the newly diagnosed group underwent a watch and wait approach, none of them experienced a complete remission, so all of them were administered IV immunoglobulins. Of the 27 children with ndITP who were monitored for at least one year, more than half (15/27) experienced a complete clinical and laboratory remission after IV immunoglobulin therapy. The rest had a mostly laboratory relapse of the disease, which is why a new watch and wait approach was tried. This approach had no effect, and after patients transitioned to a persistent form, they were given systemic corticosteroid therapy – Prednisone and pulse doses of Dexamethasone (7/22 pITP patient). A relapse 4-6 weeks after the drugs was considered a short-term response to the initial treatment with IV immunoglobulins, and a relapse after reducing the dose of Prednisone or inadequate reactivity to Dexamethasone was considered a short-term response to systemic corticosteroids. About 20% (6/27) of our ndITP patients were resistant to the applied therapy (absence of remission or relapse in a shorter period). In these patients the disease lasted longer than 12 months and after that they entered the chronic phase of the disease.

In patients with pITP, about 25% (5/22) experienced spontaneous remission. Those who required therapy, in addition to patients treated with systemic corticosteroid therapy, three patients were treated with immunomodulatory therapy with Mycophenolate mophetil due to the failure of therapy. In all three patients on immunomodulatory therapy, as well as in 3 patients who were only on Prednisone therapy, the disease progressed to a chronic form. Patients on pulse doses of Dexamethasone and the remaining 4 on Prednisone therapy are in complete remission. About 35% of patients with cITP (10/29) required the use of therapy, the rest were asymptomatic. 3 patients were splenectomized and achieved complete remission, 3 patients are on therapy with thrombopoietin receptors and achieved clinical remission, in 4 patients, in addition to other therapies, various cytostatic protocols and/or Rituximab (anti CD20 antibody) were tried, in 3 out of 4, splenectomy was also performed but without success. All of these patients have from 1-5 episodes per year of severe hemorrhagic syndrome requiring transfusions of blood products (Graphs 6.).

In addition to the mentioned standard therapeutic options for the treatment of ndITP, other approaches have appeared in recent years with the use of individual second-line therapy, but most researchers who examined the effects of standard therapy have results similar to these (4, 10 – 14, 19). The vast majority of authors previously believed that the therapy in children's acute ITP may be overdone and that whenever the degree of bleeding allows it, an observational approach should be tried (33). However, in addition to the certainly longer time to achieve remission as a downside, such an approach did not have any effect in terms of longer duration of achieved remission or less occurrence of chronicity compared to the treated group of children (4, 10 – 14, 19, 33). In our cohort, we did not use any currently available experimental drugs.

Performing a platelet kinetics test with a radioactive tracer is recommended in all patients with acute ITP who have an inadequate response to the initial therapy or in whom the disease progresses to a chronic form. A platelet kinetics test was performed in all examined patients within a period of about a year from the diagnosis of ITP, and in some after that, for the purpose of re-evaluation due to inadequate therapeutic response. The average lifespan of platelets in our study group was about 22h. It is striking that the largest number of patients (almost 70%) with cITP have a mean platelet lifespan of less than one day, but on the other hand, 15% of patients had an almost normal platelet lifespan (Table 4). This points to the already mentioned fact that the pathophysiological concept of the origin of the disease in individual patients is quite complex (1–4, 8, 10, 27).

However, earlier studies have shown that platelet lifespan, and especially the platelet production index, were the least reliable parameters for any interpretation, but these studies primarily focused on the evaluation of the therapeutic success of splenectomy (3, 27, 34, 35). In our group, almost the same percentage (35%) of patients with chronic ITP had either a normal, or reduced or very low level of platelet production in the bone marrow. On the other hand, regarding the therapeutic success evaluation, most authors agree that the most significant factor is the index (site) of platelet sequestration (34, 35). In our cohort, in more than half of the patients (60%), the spleen was the dominant or only organ of platelet destruction, and in about 30% there was mixed destruction, almost equally in the liver and spleen. In about 10%, the spleen was not involved at all in the process of platelet destruction, but the liver took over, while in only 1 patient, in addition to the liver, it was found that platelet sequestration also took place in the thymus, stomach, testicles and lungs (Table 4.). The platelet kinetics test once again confirms that the pathophysiological substrate affects megakaryocytes in the bone marrow and plateletsin the peripheral blood equally. The above results contribute to the conclusion that the place of sequestration of platelets can largely determine the prognosis of the chronic disease (3, 4, 10, 19, 27, 34, 35).

CONCLUSION

There was slight predominance of girls in all forms of ITP. Children of preschool age dominated in ndITP, while adolescents in cITP group. Persistent and chronic patients predominantly presented as asymptomatic or with mild haemorrhagic signs, while newly diagnosed patients had moderate or severe bleeding. The most common sites of bleeding are skin hematomas. Mostly ndITP patients did not have other diseases, while 30% of chronic ITP patient have other autoimmune disease. Similar number of ITP children's family members in all groups did not have any known

autoimmune diseases or expressed multiple autoimmune disorders. Supplementation of Vitamin D is strictly recommended for ITP patients. No hepatosplenomegaly was observed in ndITP patient, but almost a quarter of the chronic patients had splenomegaly. The mean value of platelet count is significantly higher in chronic groups compared to ndITP group, against MPV values that show an inverse correlation. More than half ndITP patients achieved complete remission after intravenous immunoglobulin and additional 30% experienced spontaneous remission during the persistent disease period and about one third of patients with chronic forms of ITP required therapy. The spleen is dominant or only organ of platelet sequestration in chronic form of disease.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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ARTIFICIAL INTELLIGENCE IN IMPROVING STROKE DIAGNOSIS: FOCUS ON MACHINE LEARNING MODELS AND EXPLAINABLE AI APPLICATION

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ABSTRACT

Stroke is a major and deadly health concern on a global scale, requiring fast and precise methods for effective management. The current research explores six machine learning models: logistic regression, k-nearest neighbors (KNN), support vector machine (SVM), decision tree, random forest, and eXtreme Gradient Boosting (XGBoost), to improve stroke diagnosis. By applying Local Interpretable Model-agnostic Explanations (LIME), this work bridges the gap of interpretability in conventional machine learning models, making it easier for healthcare experts to understand generated model predictions. 5,109 clinical cases with features including age, gender, hypertension, heart disease, average glucose level, and details of patient lifestyle as risk variables, were used to train the applied algorithms. As a result, Logistic Regression had an accuracy of 77%, whereas KNN and SVM had accuracies of 92% and 89%, respectively. The decision tree classifier achieved high precision and accuracy of 95%; however, the random forest and XGBoost models achieved the highest accuracy (97%) and AUC (99%), respectively, outperforming all applied classifiers. The importance of various attributes for each prediction was assessed using LIME, supporting a clear and transparent understanding of the model predictions. Case-based analyses revealed that age, gender, BMI, average glucose level, as well as stressful lifestyle conditions were the major risk factors for stroke. This study highlights the importance of explainable artificial intelligence in assisting healthcare professionals and offering transparent, reliable, and effective personalized treatments relative to specific patient needs.

Keywords: *Stroke, machine learning, data analysis, diagnosis, prediction, LIME.*

INTRODUCTION

Globally, stroke is among the most common causes of death and long-term disability, whereby each year, it affects over thirteen million people and kills over five million people [1]. A stroke is a neurological condition characterized by blood vessel obstruction, also known as a brain clot, or the bursting of a blood vessel, causing bleeding, damage to brain cells, or even death [1]. It is mainly classified into two primary categories, which are ischemic stroke, the most prevalent form of stroke, accounting for 87% of all stroke incidents [2]. The prevalence of ischemic infarctions grew significantly between 1990 and 2016 [3]. In this category, a portion of the brain cannot receive blood or oxygen because of a blood clot [1]. Secondly, a hemorrhagic stroke, this type of stroke results when a blood vessel ruptures, frequently caused by aneurysms or venous abnormalities [4].

While prevention relies on managing risk factors, identifying stroke symptoms is essential for early diagnosis, urgent triage, and immediate medical support. The symptoms include stuttering or the prodromal symptoms of basilar artery blockage, especially in people with intracranial artery blockage [5]. Furthermore, stroke incidence has been reported to rise with age, whereby after age 55, the incidence of stroke doubles. Between 1990 and 2016, the percentage of stroke cases worldwide among adults aged 20 to 54 surged from 12.9% to 18.6%. Conversely, throughout the same period, age-standardized related mortality rates lowered by 36.2% [5]. Although stroke is controllable and treatable, there is a high possibility of significantly reducing its prevalence as well as its long-term effects, because according to predictions, stroke continues to be one of the top causes of mortality and impairment [6]. Therefore, it is crucial to focus on early detection and prevention to control the severity of this condition. Frequently, traditional risk assessments depend on parameters like clinical records and epidemiology. Still, these do not cover how several aspects of life and health contribute to the cause of a stroke. For this reason, robust data-based strategies that consider diverse factors such as demographics, clinical, and lifestyle factors are crucial to identify early signs of stroke, thereby effectively assessing possible risks to improve its diagnosis and treatment as well as minimize its mortality rates.

Over the last couple of years, machine learning (ML), an artificial intelligence (AI) subfield, has emerged as a powerful tool in clinical decision support systems. ML algorithms are capable of recognizing unknown patterns in large datasets and complex health conditions, and they offer predictive approaches that can outperform traditional statistical techniques. Such algorithms help to accurately determine the risk of diseases based on complex multidimensional data, such as demographic, radiological, omics, clinical records, and lifestyle-related metrics [7]. However, regardless of their predictive strength, most ML algorithms lack the explainability concerning how input parameters affect the model output. This gap causes challenges to healthcare facilities, where transparency, trust, and accountability play significant roles. As a result, to address such issues, explainable ML models, such as SHAP (SHapley Additive exPlanations) and LIME (Local Interpretable Model-agnostic Explanations), have been developed to balance model complexity and model interpretability [8]. These techniques provide explanations of individual predictions by modeling the behavior of the system over every input variable, thus allowing physicians to understand the specific features impacting a prognosis, making doctors more informed before making a clinical decision, and gaining confidence in AI-driven systems [9].

The application of explainable AI models in stroke prediction is still a developing field. Recent studies have applied decision trees, random forests, support vector machines (SVM), and deep learning algorithms in predicting stroke occurrences based on electronic health records and structured data [10], [11], [12]. Bentley et al. [13] assessed the performance of SVM for the prediction of acute ischemic stroke, whereby 116 instances were considered. The training dataset was composed of 106 instances, whereas the testing dataset was composed of 10 instances. From their findings, the area under the curve (AUC) of SVM was found to be 0.744, which showed a better performance than the compared prognostic scores. Furthermore, Han et al. [14] employed ML methods to build a classification model that is more accurate in predicting short-term stroke probability than the conventional scores. The optimum result in the testing set was accomplished by ensemble algorithms, random forest, and a convolutional neural network (CNN), which outperformed CHA2DS2-VASc by up to 0.14 in the AUC. These results demonstrate that the atrial fibrillation burden signature technique can add benefits for risk prediction, particularly for the short-term risk of stroke. AI has played an essential role in areas like radiology, pathology, and genetics, and has also reduced errors and allowed more personalized treatment [15], [16], [17]. With this, the incorporation of AI in the healthcare field is revolutionizing diagnostics, treatment, and overall disease management.

Stroke management is demanding and advanced, thus requiring a new technology that may support a quick decision-making process and personalized treatment plans. Though quite effective, the traditional methods of diagnosing and treating strokes are constrained by the availability of specialists and the speed at which necessary information is processed. ML offers the possibility to overcome such limitations by enhancing the accuracy of radiological images, predictive power, and tailoring treatment plans to the individual patient level in a short period. Even though these models hold some potential concerning their ability to predict with high sensitivity and specificity, they still tend to lack the ability to explain specific results. The current study aims to perform stroke risk prediction using six ML algorithms (logistic regression, k-nearest neighbors (KNN), SVM, decision trees, random forests, and extreme gradient boosting (XGBoost)) and incorporating LIME to generate local predictions of different stroke cases and interpret the effects of each variable (gender, age, hypertension, heart disease, marital status, work type, residence type, average glucose level in blood, BMI

(body mass index), smoking status) on stroke cases. This is especially needed in healthcare, where patients, their families, and practitioners have to understand the outputs of a model, especially in life-threatening scenarios. Moreover, LIME provides a more accurate evaluation of risk factors in diverse populations when integrated with ML models, as heterogeneous features can be easily explained using this approach. The creation of robust models depending on specific pathophysiology is essential for directing diagnosis and treatment, as well as setting reasonable expectations for patients, considering the multitude of parameters involved in the decision system and their divergent connections with the final result [18].

The remaining parts of the manuscript are arranged as follows: the 2nd part elaborates more on the methodology applied as well as the dataset used in this study, the 3rd part provides the results generated after applying the considered ML models, which are then discussed in the 4th part of the text, and the whole study is concluded in part 5.

MATERIALS AND METHODS

The current study applied a data-driven approach to develop prediction tools for diagnosing stroke using a publicly available dataset (https://www.kaggle.com/datasets/fedesoriano/stroke-prediction-dataset) from Kaggle. The dataset consists of 5,109 cases of patients with information on their age, gender, medical history (hypertension and heart disease), lifestyle habits (smoking and type of work), and clinical test records (glucose level and BMI), with a binary value for the target variable (stroke) indicating if the patient had a stroke (1) or not (0). Ethical approval was not required because the data is anonymized and available online for research purposes.

The data was preprocessed using basic analytical methods to confirm its quality and the possibility of using it in ML processes. This analysis included checking the dataset for missing data values, replications, and clinically irrelevant instances. Consequently, some data points for BMI were missing and were thus replaced using the median value found within the range. There were no duplicate patient entries; thus, each entry represented a unique patient case. All the categorical variables were encoded to ensure a good fit with the ML models while maintaining the original information so as not to affect the performance of the algorithms. As a result, binary variables, such as “gender”, “ever married”, “Residence type”, were encoded using the one-hot encoder technique into 0 and 1, whereas all multiclass categorical variables in the data, including “work type”, “smoking status”, were processed using the label encoding technique. The converted dataset consists only of numbers, which allows for easier application of ML tools.

The data was split into training and testing sets in an 80:20 ratio after preprocessing. With the class imbalance in the dataset, 4,860 patients without stroke and only 249 patients with stroke, it is essential to use a strong resampling process to ensure equal representation of each class during training as such a situation may cause classifiers to give more weight to the majority and ignore the minority class, which can be less effective clinically since patients with stroke are the minority group in the dataset used. As a result, the synthetic minority over-sampling technique (SMOTE), which helps increase the number of instances from the minority category, was applied.

SMOTE fills in the gaps between minority class instances and their nearest neighbors in the feature space by generating new samples to make the boundary between classes more distinct. With SMOTE, the non-stroke and stroke classes were made equal, leading to a 1:1 class balance. Various ML models, including logistic regression, KNN, SVM, decision trees, random forests, and XGBoost, were tested to predict stroke diagnostic cases. Furthermore, the measures used in the evaluation of the models were accuracy, precision, recall, F1-score, and area under the curve (AUC). LIME, along with medical knowledge from health experts, was applied to select the important features and confirm their relevant contribution to the model outcomes.

RESULTS

Exploratory data analysis (EDA) was performed on a dataset of 5,109 people to examine how important stroke risk-related clinical factors were distributed. The average age of the patients was found to be 43 ± 23 years, and the glucose level test showed an average of 106.1 ± 45.3 mg/dL, pointing to a higher than average glucose reading. In addition, the BMI variable had an average of 28.9 ± 7.7 kg/m^2, which is overweight for the population, and a few outliers showed severe obesity. Figure 1 displays a donut plot illustrating a roughly even gender breakdown in the dataset (Male: 50.06%, Female: 49.94%). Gender equilibrium is important for model training stability and plausibility, reducing the chance of gender bias that is likely to affect the prediction. This distribution ensures that the results and the used models can be applied across genders, adding to the dependability of the current study.

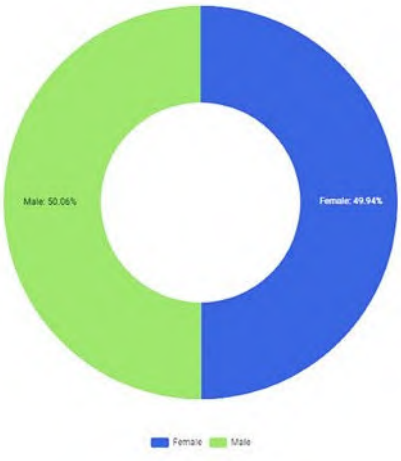


Figure 1. The gender distribution in the stroke dataset

Figure 2 compares people with and without hypertension based on three stroke risk factors: age, average glucose level, and BMI. Approximately, the data showed that patients with hypertension are 62 years old on average, while those who do not have hypertension are 41 years old on average, which aligns with the relationship between ageing and hypertension risk reported in previous studies [19]. Furthermore, the majority of hypertensive patients had higher BMI (32.59 kg/m^2), which is classified as obese, and was associated with higher average glucose level (130.19 mg/dL) compared to non-hypertensive cases (glucose levels 103.54 mg/dL, BMI = 28.47 kg/m^2). Clinically, patients having a high BMI with hypertension may have poor cardiovascular health [20], which is why different risk factors should be examined when assessing stroke risk [21]. By referring to these descriptive data, we gained basic knowledge about the population within the dataset, which was later explored through ML modeling.

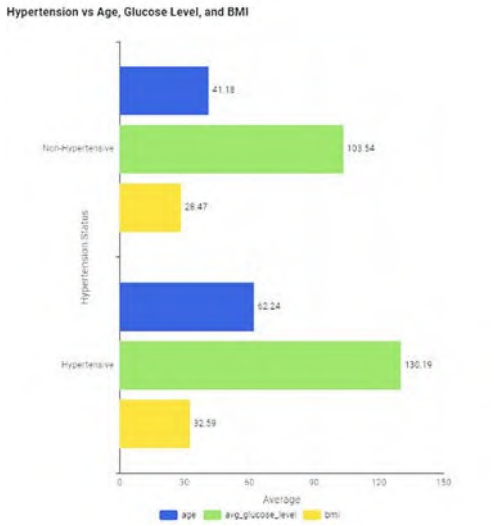


Figure 2. Bar chart illustrating the association between hypertension, age, glucose level, and BMI

Figure 3 shows a heatmap matrix that clearly illustrates the relationship between demographic and health factors associated with stroke diagnosis. The matrix displays a Pearson correlation coefficient for each pair of variables, ranging from -1 (a strong negative correlation) to 1 (a strong positive correlation). A strong positive relationship is observed between "age" and "BMI" (r=0.33), and "ever married" and "work type" (r=0.38). Likewise, a strong negative correlation is seen between "work type" and "age" (r=−0.41) as well as "age" and "ever married" (r=−0.68). The small correlation coefficients indicate that there is not much multicollinearity among features, which is likely to speed up training and reduce the risk of models learning from noise, which is likely to lead to overfitting. Identifying how different variables are linked is vital during feature selection, especially for stroke prediction, because related variables can influence the overall performance of the models as well as their interpretability.

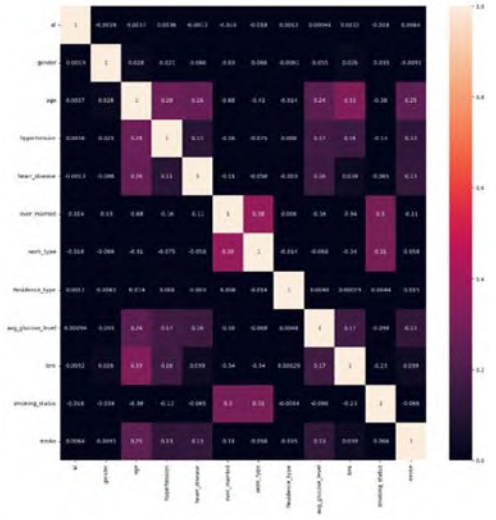


Figure 3. Correlation Matrix of the features within the stroke dataset

Different models were used and tested using an 80% training and 20% test set, as well as SMOTE to address the unequal distribution of classes, for predicting stroke outcomes. Logistic Regression achieved an accuracy of 77%, with precision, recall, AUC, and F1-scores of 76%, 81%, 85%, and 78%, respectively. KNN and SVM were more accurate than Logistic Regression, with KNN reaching 92% accuracy and 87% precision, and SVM achieving 89% accuracy and a precision of 90%. Furthermore, the decision tree model had an accuracy rate of 95%, a precision and recall of 95% and 94%, respectively. However, even though KNN achieved the highest recall score (refer to Table 1), both Random Forest and XGBoost ensemble models outperformed all the algorithms with a 97% accuracy.

Table 1. The overall performance of the six applied ML models

Models	Accuracy	Precision	Recall	F1	AUC
Logistic Regression	0.775	0.757	0.809	0.782	0.846
KNN	0.920	0.874	0.981	0.925	0.973
SVM	0.894	0.906	0.878	0.892	0.968
Decision Tree	0.947	0.952	0.941	0.947	0.947
Random Forest	0.970	0.992	0.947	0.969	0.994
XGBoost	0.970	0.988	0.952	0.970	0.992

As illustrated in Figure 4, the applied ML methods show strong performance on the ROC curve, with AUC ranging from 0.85 to 0.99. The XGBoost and random forest, being the best classifiers, have an AUC of 0.99, followed by KNN and SVM with an AUC of 0.97. All the curves located in the top-left part signify that each model is highly sensitive in detecting false positives by effectively classifying stroke patients from non-stroke patients. Clinically, tree-based ensemble approaches such as XGBoost and random forest can

detect complex, non-linear relationships [22], [23]. Most of the AUC values are fairly high, suggesting that the used algorithms can be effective in improving clinical decisions for assessing stroke risk.

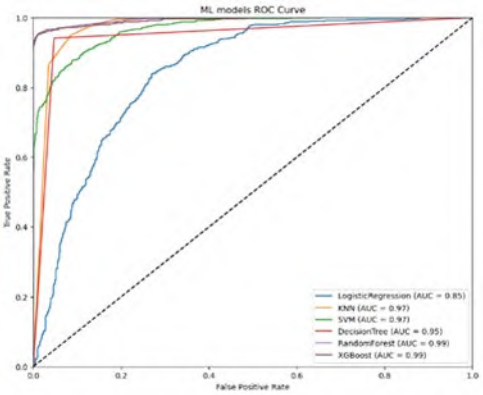
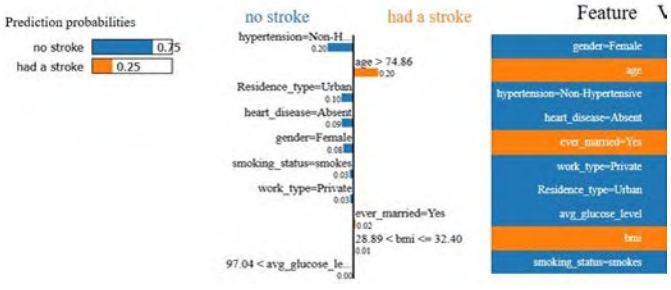


Figure 4. The Area Under the Curve for all applied models for stroke prediction

Using LIME during stroke classification helps discover how specific features affect the model predictions. As illustrated in Fig. 5. (a), an obese female patient of 77 years old with a 25% probability of having a stroke demonstrates that some factors have a high chance of lowering the risk of having a stroke. Even though the patient is old and smokes, and has a high BMI of 31.0 kg/m^2, LIME highlights that in such a case, it is unlikely to have a stroke when associated with having normal blood pressure, absence of heart diseases, as well as a normal lifestyle. On the other hand, Fig. 5. (b), an 80-year-old male patient has a 99% probability of having a stroke. Though he was non-hypertensive, it was found that advanced age, gender (being a male patient), BMI, and a severe spike in glucose level were the major reasons behind the increased risk, as well as being self-employed, which is likely to trigger stress. According to these results, age, gender, BMI, and glucose level majorly increase stroke risk, especially when associated with a complex lifestyle that prompts stress. Additional health conditions and lifestyle factors that are not too demanding mitigate the risk, suggesting several variables can interact and impact the overall stroke prediction.

(a)



(b)

Figure 5: LIME results of two different scenarios (patient at low risk vs patient at high risk) and the factors influencing the outcome. The higher the absolute amount of weight, the more significant the impact on the projected result.

Discussion

Stroke prediction using ML has been quite promising, especially in situations where early detection and sorting out higher-risk patients can impact the results of treatment. The use of ML methods on structured health records revealed that using ensemble models, like random forest and XGBoost, effectively delivers accurate predictions than single models in stroke detection. Even though such models offer strong performance, they are not easy to explain, thus not favorable for inexperienced users. To solve this, LIME, an explainable AI method, was used to identify the features that affect model outcomes and are valuable in clinical practice. Consistently, feature significance analysis across diverse patient cases revealed that age is the top predictor of stroke. Subsequently, the next health-related factors addressed were average glucose levels, BMI, and hypertension, known to be related to cardiovascular and cerebrovascular diseases [24]. This aligns with recent clinical findings [25], [26] and enhances the model's transparency, encouraging professional and inexperienced users to find it more suitable. Moreover, while most studies target a small population, the current study focuses on a larger dataset and relies on common hospital information, making it more generalizable and easily applicable in health systems, including medical facilities with limited resources.

AI models, ML algorithms in particular, contribute greatly to making clinical decisions and the early detection of disease risk. The current study expands the evidence that ML models and ensemble models, in particular, work better than standard analytical methods in handling complex medical analysis. Additionally, computational algorithms that can analyze how clinical factors work in combination with other factors, like demographic characteristics and patient lifestyle, are highly needed in the medical field. The findings of the current study highlight that advanced computer models can find weak but essential patterns that normal methods may overlook, subsequently making their predictions about stroke risk highly sensitive and specific. Another essential goal of this study was to take medical data and refine it for easy

interpretation. Through the use of LIME, the contribution of each feature was assessed, which is necessary for physicians to understand the rationale behind the outcome of the applied models, as many healthcare professionals are not yet confident in AI algorithms, as they are hard to understand. Therefore, being able to see the results of a model boosts confidence and encourages its use in healthcare settings. In clinical work, it is important to detect patients at risk promptly, as this can strongly affect treatment outcomes. Therefore, choosing the right algorithm in medical applications involves weighing how accurate, generalizable, and reliable it is and how explainable it must be for critical medical choices.

Our research findings indicate that ML-based stroke risk prediction is a promising application; however, it has numerous limitations. The data used doesn't include some critical vascular and metabolic risk factors, such as diabetes mellitus, dyslipidemia, atrial fibrillation, and chronic kidney disease, which have been linked to influencing stroke rates and clinical outcomes. This can lead to unmeasured confounding, therefore, reducing the predictive power and generalizability of the used models. Moreover, the etiological subtyping based on the classification of the TOAST was not used in the study, which can limit the ability to identify specific ischemic stroke processes. As a result, future research can include more types of biomarkers, other comorbidities associated with stroke, and more detailed background variables, such as sociodemographic and behavioral factors, to enhance the understanding of lifestyle and clinically related risks of stroke.

Furthermore, this study relies on structured tabular data and does not use multimodal sources (neuroimaging, voice/gait analysis, and genetic analysis), thus limiting its prognostic performance. This could significantly reduce the quality and clinical value of stroke risk assessments, especially concerning the early detection of patients at high risk. Therefore, longitudinal or real-time datasets of hospitals can be incorporated in future research to test how variations of risk factors affect stroke onset. By addressing these limitations and improving the input features, further studies could develop more effective explainable AI models that align computational intelligence with realistic medical decision-making, thus promoting the initial diagnosis and personalized stroke treatment.

CONCLUSION

This study highlights the use of explainable AI algorithms such as LIME in stroke risk prediction. Even though ML algorithms, especially ensembled models, offer accurate predictions, their structure limits their application in healthcare, particularly in disease diagnosis and/or treatment. Therefore, LIME allowed personalized stroke predictions by understanding the influence of clinical features such as demographic parameters, BMI, average glucose level, presence or absence of some other health conditions, and patient lifestyle on model decision-making. As a result, healthcare providers can better explain such computationally generated decisions to patients with confidence and understanding, thus improving patient satisfaction. Furthermore, our findings reveal that ML algorithms associated with explainable AI models can be helpful in medical diagnostics and can contribute to more precise stroke risk categorization. More studies should be done to confirm these results in diverse populations and guide medical decision-making by using real-time data.

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None

CONFLICT OF INTEREST

None

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SELECTED GENETIC POLYMORPHISMS AND THEIR ASSOCIATION WITH PRE-ECLAMPSIA: A META-ANALYSIS AND POWER ANALYSIS

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ABSTRACT

This meta-analysis investigates the association between four gene polymorphisms - IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 and the risk of preeclampsia (PE). Case-control studies published between 2005 and 2025 were retrieved from Scopus, PubMed, Web of Science and Google Scholar. The inclusion of newly available data enhances statistical power and offers an updated, reliable synthesis of evidence. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated using MetaGenyo software across various genetic models. Power analysis validated statistical strength, and protein-protein interaction (PPI) networks were constructed using the database, STRING. A total of 14 research articles, including 3,151 PE cases and 6,101 controls data were analysed. The IL1A rs17561 polymorphism was significantly linked to preeclampsia (PE) susceptibility, demonstrating a protective effect under the recessive model (OR = 0.67) and an elevated risk for heterozygous carriers in the over-dominant model (OR = 1.49). Subgroup analyses were feasible for IFN-γ, STOX1, and PPAR-γ, but no significant associations were identified. Power analysis confirmed an adequate sample size, and PPI network analysis revealed interactions involving 8 nodes and 7 edges. The findings suggest that IL1A rs17561 has a variant-specific influence on preeclampsia risk, supporting the role of IL-1–mediated inflammation in its pathogenesis, while IFN-γ, STOX1, and PPAR-γ polymorphisms showed no significant associations.

Keywords: *Pre-eclampsia, Gene Polymorphism, IL1A, IFN-γ, STOX1, PPAR-γ.*

INTRODUCTION

Preeclampsia (PE) is a hypertension-related condition that occurs during pregnancy and continues to be a major contributor to maternal and fatal complications and deaths worldwide. Contributing to over 50,000 maternal deaths annually, recent estimates indicate that PE complicates approximately 4.6% of pregnancies globally [1,2]. PE occurs after 20 weeks of pregnancy with symptoms of proteinuria and elevated blood pressure, presenting a major risk to the mother as well as the fetus, such as preterm birth, intrauterine growth retardation, and long-term cardiovascular complications [3]. Although preeclampsia's exact cause is unknown, an intricate interplay of environmental variables, immunological, and genetic factors is thought to be a reason. [4]. Furthermore, there is growing evidence that the pathophysiology of PE is profoundly influenced by both poor placental development and dysregulation of the maternal immune response. Gene polymorphisms about trophoblast function and immune response are now being studied as possible factors contributing to PE risk. Among the genetic factors under investigation, key polymorphisms in cytokines and transcription factors, including Interleukin-1 Alpha (IL1A rs17561), Interferon-gamma (IFN-γ rs2430561), Storkhead Box 1 [STOX1 rs1341667 (Y513H)], and Peroxisome Proliferator-Activated Receptor Gamma [PPAR-γ rs1801282 (Pro12Ala)], have been identified as potential contributors to PE risk. IL1A rs17561 is a strong pro-inflammatory cytokine that modulates placental growth and trophoblast invasion during pregnancy. However, high IL1A rs17561 activity causes endothelial damage and persistent inflammation, both essential for developing PE [5]. The IL1A rs17561 polymorphism, a missense variant resulting in an amino acid change, has been linked to increased PE risk and altered cytokine production. The protein's stability and function may be affected by this polymorphism, which could worsen the inflammatory response at the maternal-fetal interface and impair placentation. Like IL1A rs17561, IFN-γ rs2430561 is a pro-inflammatory cytokine that shows a dual role in pregnancy by regulating immune responses and placental development. However, dysregulated IFN-γ rs2430561 has been associated with impaired trophoblast function and increased inflammation, which are hallmarks of PE [6]. The IFN-γ rs2430561 polymorphism, which comprises a T to A substitution, is located in the gene's first intron. It is speculated that this alteration affects IFN-γ rs2430561 transcriptional activity, which could result in an imbalance between pro- and anti-inflammatory responses that aid in the pathophysiology of PE. STOX1 rs1341667 (Y513H), which is a transcription factor expressed in the placenta, has also been identified as a candidate gene for PE. It is involved in the regulation of trophoblast proliferation, invasion, and differentiation, which are essential for successful placentation. Dysregulation of STOX1 rs1341667 expression has been associated with defective spiral artery remodelling, which is a characteristic feature of PE [7]. The STOX1 rs1341667 polymorphism has been linked to altered trophoblast function and increased susceptibility to PE [8]. This polymorphism may disrupt the normal development of the placenta, leading to inadequate blood supply to the foetus and the subsequent development of PE. Similarly, PPAR-γ rs1801282 (Pro12Ala) is a nuclear receptor involved in lipid metabolism and inflammation and plays a key role in angiogenesis and immune tolerance. The PPAR-γ rs1801282 polymorphism, a missense variant, has been associated with altered receptor activity and increased PE risk [9]. This polymorphism can affect the transcriptional activity of PPAR-γ rs1801282, altering its ability to regulate genes involved in lipid metabolism and inflammation, which are critical for maintaining a healthy pregnancy. While individual studies have explored the associations between polymorphisms in these genes and the risk of preeclampsia, findings have often been inconsistent, likely due to variations in study design, population demographics, and sample sizes. A comprehensive meta-analysis is therefore essential to synthesize the available evidence and clarify the strength and consistency of these associations. Therefore, the current meta-analysis aims to assess the association between IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 gene polymorphisms and PE in pregnant women.

RATIONALE OF THE STUDY

This meta-analysis aims to explore the genetic factors contributing to PE, focusing on IL1A, IFN-γ, STOX1, and PPAR-γ as key candidate genes. The year 2005 was chosen as the starting point for our search because large-scale, peer-reviewed genetic association studies on preeclampsia became available only after this time. Earlier publications often lacked standardized genotyping methods or uniform diagnostic criteria for preeclampsia, which could introduce methodological heterogeneity. Restricting inclusion from 2005 onward ensured the consistency and reliability of the data analysed. The development of PE is influenced by the roles of these genes in inflammation, placental function, and metabolic control. IFN-γ affects trophoblast activity and immune tolerance, while IL1A encodes IL-1α, a cytokine associated with the elevated inflammatory response in PE. Trophoblast development and spiral artery remodelling depend on STOX1, while PPAR-γ controls lipid metabolism and vascular health. This meta-analysis aims to expand the available data, evaluate ethnic differences, and elucidate the link among these polymorphisms and PE risk by combining data from several studies. The findings could advance our knowledge of the genetic foundation of PE, encourage tailored preventative strategies, and advance the integration of genetic screening into prenatal care, which can help to improve the health outcomes of both the mother and the fetus by identifying potential genetic risks early and enabling personalized interventions.

MATERIALS AND METHODOLOGY

The protocol for this review was registered with PROSPERO (ID NO: CRD420251130314). The review process was conducted in accordance with the PRISMA 2020

guidelines, and the search policy and study selection procedure are detailed in Figure 1.

Literature search

Related articles were obtained by widespread electronic searches conducted by using Web of Science, Scopus, PubMed, and Google Scholar from 22nd April 2005 till 15th February 2025. From the Scopus database, we have searched the articles using the keywords "IL1A and Pre-eclampsia", "IFN-γ and Pre-eclampsia", "STOX1 and Pre-eclampsia", "PPAR-γ and Pre-eclampsia, were identified; IL1A (rs17561) – 84 articles; IFN-γ (rs2430561) – 114 articles; STOX1 (rs1341667) – 43 articles; PPAR-γ (rs1801282) – 114 articles.

From the PubMed database, we have searched the articles using the keywords "IL1A and Pre-eclampsia", "IFN-γ and Pre-eclampsia", "STOX1 and Pre-eclampsia", "PPAR-γ and Pre-eclampsia, were identified IL1A (rs17561) – 7 articles; IFN-γ (rs2430561) – 112 articles; STOX1 (rs1341667) – 42 articles; PPAR-γ (rs1801282) – 53 articles.

From the Web of Science database, we have searched the articles using the keywords "IL1A and Pre-eclampsia", "IFN-γ and Pre-eclampsia", "STOX1 and Pre-eclampsia", "PPAR-γ and Pre-eclampsia, were identified; IL1A (rs17561) – 102 articles; IFN-γ (rs2430561) – 144 articles; STOX1 (rs1341667) – 77 articles; PPAR-γ (rs1801282) – 117 articles.

From the Google Scholar, we have searched the articles using the keywords "IL1A and Pre-eclampsia", "IFN-γ and Pre-eclampsia", "STOX1 and Pre-eclampsia", "PPAR-γ and Pre-eclampsia, were identified IL1A (rs17561) – 1370 articles; IFN-γ (rs2430561) – 5520 articles; STOX1 (rs1341667) – 685 articles; PPAR-γ (rs1801282) – 848 articles.

The examination policy employed the Boolean operator "AND" to achieve precise results. English-language articles were used exclusively throughout the search. In addition to the above, all databases, Google Scholar was also used to identify the relevant case vs. control studies using the following keywords ("IL1A", "IFN-γ", "STOX1", "PPAR-γ, Pre-eclampsia) and the filters (22nd April 2005 till 15th February 2025). Rayyan software was employed as an automation tool to facilitate duplicate removal and initial screening of articles during the literature search. Additionally, we ensured data reliability by removing duplicates within the updated database, including reviews, and existing meta-analyses as well. The year 2005 was chosen as the starting point for our literature search because large-scale, peer-reviewed genetic association studies on preeclampsia became increasingly available only after this time. Earlier publications often lacked standardized genotyping methods, consistent diagnostic criteria for preeclampsia, or adequate sample sizes, which could introduce methodological heterogeneity and reduce comparability across studies. Restricting inclusion from 2005 onward, therefore, ensured that the studies analyzed were conducted with improved methodological rigor, enhancing the reliability and reproducibility of the meta-analysis findings. We have analysed and extracted data from the two authors (Sharon Benita Stephen and Rozario Cyril), independently selected the titles, abstracts, and full texts of all selected studies. Discrepancies regarding eligibility were resolved through discussion, and when consensus could not be reached, a third author (Gowtham Kumar Subbaraj) adjudicated.

Inclusion criteria

Articles are cautiously selected for the meta-analysis based on selection criteria. To fulfil the inclusion criteria, the article needed to examine the IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 gene polymorphisms association with PE. The criteria were considered for the inclusion of the data: Full text in the English language is available; Case-control studies using gene polymorphism association studies on PE and variations in IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282. Study examines how allele and genotype data are distributed among cases and controls.

Exclusion criteria

The following articles were excluded: Reviews or prior meta-analyses about IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 with PE; Investigations not associated between the genes IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 and pre-eclampsia risk; Articles with duplicated data; Case studies and research on animals that overlapped with different areas of study. Studies in which ethnic subgroups were reported but genotype frequencies were not separable according to our predefined classification criteria were excluded from subgroup analyses to avoid misclassification bias.

Data extraction and Quality assessment

Extracting key data from each study, including name of the author, publication year, ethnicity of the study population, country, allele frequencies, and genotype of IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282. The individual characteristics like gender, age, and sample size were taken into consideration. The HWE p-value has also been determined. To assess the risk of studies, we assessed their methodological quality using the Newcastle–Ottawa Scale. Because all eligible studies were observational case-control genetic association studies, we assessed study quality using the Newcastle-Ottawa Scale (NOS; case-control version). Two authors independently evaluated each study across the NOS domains (Selection, Comparability, Exposure). Disagreements were resolved by consensus with a third reviewer. We categorized the overall risk as low (NOS 7–9), some concerns/moderate (NOS 5–6), or high (NOS ≤4).

Power analysis

The acquired metadata was subjected to power analysis with a 95% CI (0.05 α error). By using the GPower 3.1 software, the sample size power of each study (case and control) was aggregated and analysed individually for each selected gene.

Protein-to-protein interactions

To understand the gene variations linked to PE and the protein function of three genes, such as IL1A rs17561, STOX1 rs1341667, and PPAR-γ rs1801282, was analyzed. The IFN-γ rs2430561 gene couldn't be identified in the database. The STRING database (version 11.0) was utilized to predict functional changes and protein-protein interactions (PPIs) with a confidence score of ≥ 0.4.

Statistical analysis

Statistical analysis was done in analyzing the importance of IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 gene polymorphism with PE susceptibility. The association between IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 polymorphism and PE susceptibility was estimated, along with the 95% confidence interval (CI) range of values lying within the degree of confidence. A p-value of <0.05 was considered statistically significant. The Index of Inconsistency (I^2) was utilized to assess the consistency of findings across all studies. I^2 quantifies the percentage of the total variability in observed effect estimates that is attributable to true between-study heterogeneity rather than sampling error. I^2 value of 0% indicates no observed heterogeneity, and larger values indicate increasing heterogeneity. A heterogeneity value less than 50% led the study group for a fixed effect model, and a heterogeneity value above 50% led the study group to use a random effect model. A Chi-square test was conducted to determine the heterogeneity, using the Q statistic. A Z-test was used to calculate the odds ratios (ORs) for multiple comparisons. A combined Odds Ratio (OR) was calculated across all studies to evaluate the overall impact of genetic factors. When the Z-test p-value was less than 0.05, the combined effect was deemed statistically significant. MetaGenyo, a robust programme used to conduct statistical analysis.

RESULTS

Search results

The present research observed 14 studies [5, 9-21], out of which studies comprising four genes, namely IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282, with a sum of 3151 PE cases and 6101 normal controls, were selected for this meta-analysis. Figure 1 represented the data of the selected case vs. control studies.

Risk bias

Based on the NOS assessment, studies were assigned overall risk classifications (low, moderate, or high) according to the total NOS score. In line with our prespecified rule, the pooled evidence was considered to present a low overall risk of bias.

Quantitative data analysis of IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 and PE

Across four candidate gene polymorphisms, significant associations with preeclampsia (PE) risk were only identified for the IL1A (rs17561) gene polymorphism, while IFN-gamma (rs2430561), STOX1 (rs1341667), and PPAR-gamma (rs1801282) showed no significant link in any tested genetic model. Exposure to IL1A rs17561 was significantly associated with preeclampsia risk across multiple genetic models. Specifically, the recessive model (AA vs. AC+CC, OR = 0.67, 95% CI 0.52-0.87, p = 0.002) indicated a significant protective effect, whereas the dominant model (AA+AC vs. CC, OR = 1.03, 95% CI 0.56-1.88, p = 0.92) suggested there is no significant association with PE. In contrast, the allelic model (A vs. C, OR =0.88, 95% CI 0.76-1.0, p = 0.07) suggested that there is no significance with PE and the over-dominant model (AC vs. AA+CC, OR = 1.49, 95% CI 1.16-1.91, p = 0.001) indicated that heterozygous carriers (AC) may have an increased risk. These findings highlight a complex genetic influence of IL1A rs17561, consistent with the variable roles of IL-1 signalling in inflammatory responses during pregnancy, as shown in Figure 2. The results of IFN-γ rs2430561 for the all the four models had no significant link with PE [the allelic model (T vs. A OR 1.05, 95% CI 0.59-1.86, p=0.8); the recessive model (TT vs. TA+AA OR 0.86, 95% CI 0.32-2.32, p=0.7); the dominant models (TT+TA vs. AA OR 1.18, 95% CI 0.66-2.11, p=0.5); and the over-dominant models (TA vs. TT+AA OR 1.26, 95% CI 0.98-1.61, p=0.06)] as shown in Figure 3.

The results of STOX1 rs1341667 for the all the four models had no significant link with PE [the allelic model (T vs. C OR 0.96, 95% CI 0.87-1.05, p=0.3); the recessive model (OR 0.86, 95% CI 0.62-1.20, p=0.3); the dominant model (TT+TC vs. CC OR 0.98, 0.86-1.12, p=0.7); the overdominant model (OR 1.08, 95% CI 0.83-1.39, p=0.5)] as shown in Figure 4.

The results of PPAR-γ rs1801282 for all the four models had no significant link with PE [the allelic model (C vs. G OR 1.03, 95% CI 0.74, 1.42, p=0.8); the recessive model (CC vs. CG+GG OR 1.00, 95% CI 0.69-1.44, p=0.9); the dominant model (CC+CG vs. GG OR 1.06, 95% CI 0.12-9.41, p=0.9); and the over-dominant model (CG vs. CC+GG OR 1.04, 95% CI 0.71-1.50, p=0.8) as shown in Figure 5.

Sensitivity analysis

To evaluate the robustness of our pooled estimates, the sensitivity analysis was done by sequentially omitting individual studies. The pooled ORs for several gene polymorphisms proved sensitive to the removal of single studies, suggesting a lack of complete robustness in these specific models (Figures 6–9). The combined effect size for the IL1A

(rs17561) gene polymorphism was found to be dependent on the inclusion of the study by Li et al. (2014). Omitting this study resulted in a loss of statistical significance for both the recessive model (OR = 0.75; 95% CI 0.52–1.07, becoming non-significant) and the over-dominant model (OR = 1.36; 95% CI 0.95–1.96, becoming non-significant). This sensitivity indicates that the statistical significance observed in the main analysis for these two models is not robust (Figure 6). Similarly, the results for the IFN-γ (rs2430561) polymorphism showed instability. The combined effect size for the recessive model was significantly altered by the removal of Pinheiro et al. (2015), which resulted in the combined effect size shifting from non-significant to significant (OR = 0.57; 95% CI 0.35–0.85). The dominant model was also sensitive: omitting Daher et al. (2006) resulted in a significant combined effect size (OR = 1.36; 95% CI 1.03–1.80) (Figure 7). For the STOX1 (rs1341667) gene polymorphism, the pooled estimate for the recessive model was likewise sensitive. Removal of the study by Fenstad et al. (2010) changed the result from non-significant to significant (OR = 0.77; 95% CI 0.61–0.98) (Figure 8). This sensitivity analysis suggests that the observed associations, including the significant IL1A findings and the non-significant findings for IFN-gamma and STOX1, are not entirely robust and must be interpreted with extreme caution. Therefore, we must downgrade the confidence in the significant associations found for the IL1A recessive and over-dominant models, as their significance was lost upon the removal of one study.

Similarly, the non-significant findings for IFN-gamma and STOX1 cannot be definitively concluded, as removing other studies caused them to become significant. Future significant, well-designed studies are critically needed to independently validate the associations for the recessive and over-dominant models of IL1A (rs17561), and to confirm the true lack of association for IFN-gamma (rs2430561) and STOX1 (rs1341667). Until such replication occurs, these particular pooled findings should be considered preliminary and highly conditional.

Construction of PPI network and Power analysis

The STRING database was used to build and examine the Protein-Protein Interaction (PPI) network for polymorphic proteins, namely for PPAR-γ rs1801282, STOX1 rs1341667, and IL1A rs17561. The network consists of 8 nodes and 7 edges, where IL1A rs17561 and PPAR-γ rs1801282 exhibit a direct interaction, while STOX1 rs1341667 does not directly interact with any of the genes, as shown in Figure 10. Power analysis was used to further assess each study's significance level and validate the sample sizes within the necessary threshold (α error prob < 0.05). Table 5 provides specifics on the power analysis findings. Furthermore, the Circos plot, as shown in Figures 11(a), (b), (c) & (d), visually depicts the chromosomal locations of the genes under study, transcriptional regulators, and histone modifications, providing information about possible gene interactions and risk allele grouping.

Table 1. Characteristics of the studies for the association of IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282 gene polymorphism with PE

Gene	Study	Ethnicity	Country	PE Cases			Controls			HWE P-value	HWE adjusted P-value	NOS Score
				AC_Cases	CC_Cases	AA_Cases	AC_Controls	CC_Controls	AA_Controls			
IL1A rs (17561)	Li et al., 2014 [5]	Asian	China	53	344	5	111	436	7	0	0	8
	Andraweera et al., 2016 [10]	Asian	Sri Lanka	77	79	16	92	60	16	0.1865	0.1865	7
	Wu et al., 2017 [11]	Asian	China	15	76	0	39	192	1	0	0	7
				TT_Cases	TA_Cases	AA_Cases	TT_Controls	TA_Controls	AA_Controls			
IFN-γ (rs2430561)	Kamali-Sarvestani et al., 2006 [12]	Asian	Iran	21	66	42	30	78	53	0.8903	0.8903	6
	Daher et al., 2006 [13]	Mulatto	Brazil	6	25	24	22	38	29	0.1842	0.8776	7
	Daher et al., 2006_1 [13]	Black	Brazil	9	52	34	17	43	36	0.5075	0.8776	8
	de Lima et al., 2009 [14]	Caucasian	Brazil	7	46	32	18	45	33	0.7021	0.8776	8
	Pinheiro et al., 2015 [15]	Caucasian	Brazil	33	51	32	5	40	62	0.6497	0.8776	7
				TT_Cases	TC_Cases	CC_Cases	TT_Controls	TC_Controls	CC_Controls			
STOX1 (rs1341667)	Kim et al., 2009 [18]	Asian	Korea	155	42	5	150	53	1	0.1041	0.4164	7
	Fenstad et al., 2010 [19]	Caucasian	Norway	124	450	412	245	931	840	0.5981	0.9707	7
	Pinarbasi et al., 2020 [20]	Asian	Turkey	108	280	112	143	249	108	0.9839	0.9839	7
	Bildirici et al., 2023 [17]	Asian	Turkey	10	31	9	18	23	9	0.728	0.9707	8
				CC_Cases	CG_Cases	GG_Cases	CC_Controls	CG_Controls	GG_Controls			
PPAR-γ (rs1801282)	Laasanen et al., 2002 [16]	Caucasian	Finland	95	36	2	76	34	5	0.6334	0.6334	8
	Ghorbani et al., 2021 [21]	Asian	Iran	75	25	0	77	23	0	0.1938	0.5814	6
	Liu et al., 2021 [9]	Caucasian	France	26	8	1	1297	301	15	0.5922	0.6334	8

*HWE-Hardy-Weinberg equilibrium

Table 2. Subgroup analysis for the association of IFN-γ rs2430561 gene polymorphism with PE

Model	Ethnicity	Number of studies	Test of association			Test of heterogeneity		
			OR	95% CI	p-value	Model	p-value	$I^{\wedge 2}$
Allele contrast (A vs. a)	Overall	5	1.0490	[0.5910; 1.8618]	0.870201	Random	0	0.8985
	Asian	1	0.96	[0.6891; 1.3373]	0.80928	Fixed	NA	NA
	Black	1	0.8712	[0.5767; 1.3161]	0.512451	Fixed	NA	NA
	Caucasian	2	1.5813	[0.3650; 6.8515]	0.540133	Random	0	0.9596
	Mulatto	1	0.5934	[0.3623; 0.9718]	0.038098	Fixed	NA	NA
Recessive model (AA vs. Aa+aa)	Overall	5	0.8575	[0.3165; 2.3233]	0.76239	Random	0	0.8531
	Asian	1	0.8491	[0.4599; 1.5675]	0.600945	Fixed	NA	NA
	Black	1	0.4863	[0.2050; 1.1536]	0.101889	Fixed	NA	NA
	Caucasian	2	1.7678	[0.0901; 34.6947]	0.70757	Random	0	0.9484
	Mulatto	1	0.3729	[0.1407; 0.9886]	0.047374	Fixed	NA	NA
Dominant model (AA+Aa vs. aa)	Overall	5	1.1796	[0.6586; 2.1129]	0.578522	Random	0.0005	0.7998
	Asian	1	1.0165	[0.6205; 1.6653]	0.948084	Fixed	NA	NA
	Black	1	1.0765	[0.5974; 1.9396]	0.806235	Fixed	NA	NA
	Caucasian	2	1.7805	[0.4395; 7.2133]	0.418949	Random	0.0007	0.9128
	Mulatto	1	0.6243	[0.3121; 1.2486]	0.182821	Fixed	NA	NA
Overdominant (Aa vs. AA + aa)	Overall	5	1.2604	[0.9840; 1.6146]	0.066959	Fixed	0.9406	0
	Asian	1	1.1148	[0.7014; 1.7718]	0.645821	Fixed	NA	NA
	Black	1	1.4905	[0.8428; 2.6360]	0.17001	Fixed	NA	NA
	Caucasian	2	1.3245	[0.8918; 1.9670]	0.163757	Fixed	0.9665	0
	Mulatto	1	1.1184	[0.5684; 2.2007]	0.745874	Fixed	NA	NA
pairw1 (AA vs. aa)	Overall	5	0.9602	[0.2962; 3.1128]	0.945089	Random	0	0.8763
	Asian	1	0.8833	[0.4434; 1.7596]	0.724229	Fixed	NA	NA
	Black	1	0.5606	[0.2203; 1.4265]	0.224533	Fixed	NA	NA
	Caucasian	2	2.2588	[0.0759; 67.2004]	0.63785	Random	0	0.9551
	Mulatto	1	0.3295	[0.1151; 0.9429]	0.03869	Fixed	NA	NA
pairw2 (AA vs. Aa)	Overall	5	0.7764	[0.3299; 1.8271]	0.562168	Random	0.0011	0.78
	Asian	1	0.8273	[0.4332; 1.5797]	0.5656	Fixed	NA	NA
	Black	1	0.4378	[0.1774; 1.0804]	0.073093	Fixed	NA	NA
	Caucasian	2	1.3946	[0.1680; 18.0693]	0.798861	Random	0.0003	0.9241
	Mulatto	1	0.4145	[0.1474; 1.1661]	0.095159	Fixed	NA	NA
pairw3 (Aa vs. aa)	Overall	5	1.2652	[0.9630; 1.6623]	0.091184	Fixed	0.1303	0.4372
	Asian	1	1.0678	[0.6342; 1.7977]	0.805154	Fixed	NA	NA
	Black	1	1.2804	[0.6896; 2.3774]	0.433633	Fixed	NA	NA
	Caucasian	2	1.6266	[0.7052; 3.7468]	0.253147	Random	0.0553	0.7277
	Mulatto	1	0.795	[0.3794; 1.6658]	0.54322	Fixed	NA	NA

*OR- Odds Ratio, *I^2 – Heterogeneity, *CI – Confidence Interval.

Table 3. Subgroup analysis for the association of STOX1 rs1341667 gene polymorphism with PE.

Model	Ethnicity	Number of studies	Test of association			Test of heterogeneity		
			OR	95% CI	p-value	Model	p-value	$I^{\wedge 2}$
Allele contrast (A vs. a)	Overall	4	0.9573	[0.8739;1.0486]	0.347607	Fixed	0.3168	0.1503
	Asian	3	0.8704	[0.7456;1.0162]	0.079072	Fixed	0.5181	0
	Caucasian	1	1.0067	[0.8994;1.1268]	0.907561	Fixed	NA	NA
Recessive model (AA vs. Aa+aa)	Overall	4	0.8644	[0.6250;1.1955]	0.378488	Random	0.0343	0.6532
	Asian	3	0.7751	[0.4843;1.2405]	0.288338	Random	0.0621	0.6401
	Caucasian	1	1.0398	[0.8255;1.3098]	0.740104	Fixed	NA	NA
Dominant model (AA+Aa vs. aa)	Overall	4	0.9803	[0.8559;1.1229]	0.774395	Fixed	0.5249	0
	Asian	3	0.9317	[0.7009;1.2385]	0.626105	Fixed	0.354	0.0371
	Caucasian	1	0.9951	[0.8527;1.1614]	0.950762	Fixed	NA	NA
Overdominant (Aa vs. AA + aa)	Overall	4	1.0762	[0.8331;1.3902]	0.574082	Random	0.0561	0.603
	Asian	3	1.1554	[0.7391;1.8061]	0.526314	Random	0.0609	0.6426
	Caucasian	1	0.9784	[0.8397;1.1400]	0.779761	Fixed	NA	NA
pairw1 (AA vs. aa)	Overall	4	0.9004	[0.7371;1.0998]	0.303905	Fixed	0.1801	0.3863
	Asian	3	0.69	[0.4893;0.9731]	0.034376	Fixed	0.4948	0
	Caucasian	1	1.0319	[0.8068;1.3198]	0.802505	Fixed	NA	NA
pairw2 (AA vs. Aa)	Overall	4	0.8681	[0.6008;1.2544]	0.451454	Random	0.0171	0.7051
	Asian	3	0.7741	[0.4391;1.3646]	0.376068	Random	0.0244	0.7308
	Caucasian	1	1.0471	[0.8208;1.3359]	0.711047	Fixed	NA	NA
pairw3 (Aa vs. aa)	Overall	4	1.0029	[0.8692;1.1572]	0.968297	Fixed	0.3458	0.0946
	Asian	3	1.0639	[0.7889;1.4347]	0.684766	Fixed	0.2102	0.3588
	Caucasian	1	0.9855	[0.8373;1.1599]	0.860306	Fixed	NA	NA

*OR- Odds Ratio, *I^2 – Heterogeneity, *CI – Confidence Interval.

Table 4. Subgroup analysis for association of PPAR-γ rs1801282 gene polymorphism with PE.

Model	Ethnicity	Number of studies	Test of association			Test of heterogeneity		
			OR	95% CI	p-value	Model	p-value	I^2
Allele contrast (A vs. a)	Overall	3	1.0252	[0.7404; 1.4197]	0.880652	Fixed	0.2569	0.2642
	Asian	1	0.9096	[0.4974; 1.6634]	0.758349	Fixed	NA	NA
	Caucasian	2	1.0768	[0.7316; 1.5849]	0.707445	Fixed	0.1135	0.6008
Recessive model (AA vs. Aa+aa)	Overall	3	1.0002	[0.6944; 1.4408]	0.999026	Fixed	0.4203	0
	Asian	1	0.8961	[0.4681; 1.7156]	0.740601	Fixed	NA	NA
	Caucasian	2	1.0523	[0.6769; 1.6358]	0.820909	Fixed	0.2098	0.3641
Dominant model (AA+Aa vs. aa)	Overall	2	1.0613	[0.1197; 9.4081]	0.957354	Random	0.0973	0.6364
	Asian	0	NA			Fixed	NA	NA
	Caucasian	2	1.0613	[0.1197; 9.4081]	0.957354	Random	0.0973	0.6364
Overdominant (Aa vs. AA + aa)	Overall	3	1.0367	[0.7141; 1.5050]	0.849601	Fixed	0.7199	0
	Asian	1	1.1159	[0.5829; 2.1365]	0.740601	Fixed	NA	NA
	Caucasian	2	0.9999	[0.6343; 1.5762]	0.999641	Fixed	0.4449	0
pairw1 (AA vs. aa)	Overall	2	1.0518	[0.1067;10.3726]	0.965492	Random	0.0835	0.6662
	Asian	0	NA			Fixed	NA	NA
	Caucasian	2	1.0518	[0.1067;10.3726]	0.965492	Random	0.0835	0.6662
pairw2 (AA vs. Aa)	Overall	3	0.9773	[0.6723; 1.4208]	0.904274	Fixed	0.6343	0
	Asian	1	0.8961	[0.4681; 1.7156]	0.740601	Fixed	NA	NA
	Caucasian	2	1.0203	[0.6456; 1.6127]	0.931304	Fixed	0.3687	0
pairw3 (Aa vs. aa)	Overall	2	1.2702	[0.3345; 4.8237]	0.725342	Fixed	0.1755	0.4553
	Asian	0	NA			Fixed	NA	NA
	Caucasian	2	1.2702	[0.3345; 4.8237]	0.725342	Fixed	0.1755	0.4553

*OR- Odds Ratio, *I^2 – Heterogeneity, *CI – Confidence Interval.

Table 5. Emphasizing the importance of determining an appropriate sample size is crucial for accurately evaluating statistical significance and ensuring the reliability of findings in genetic association studies, especially when investigating specific polymorphisms. The estimation of sample size plays a pivotal role in determining the statistical power of these studies

Gene	SNP	No. of Studies	Cases	Control	α err prob	Power (1-β err prob)
IL1A	*rs17561*	3	665	954	0.02303	0.9
IFN-γ	*rs2430561*	5	480	549	0.05343	0.9
STOX1	*rs1341667*	4	1738	2770	0.000495	0.9
PPAR-γ	*rs1801282*	3	268	1828	0.06146	0.9

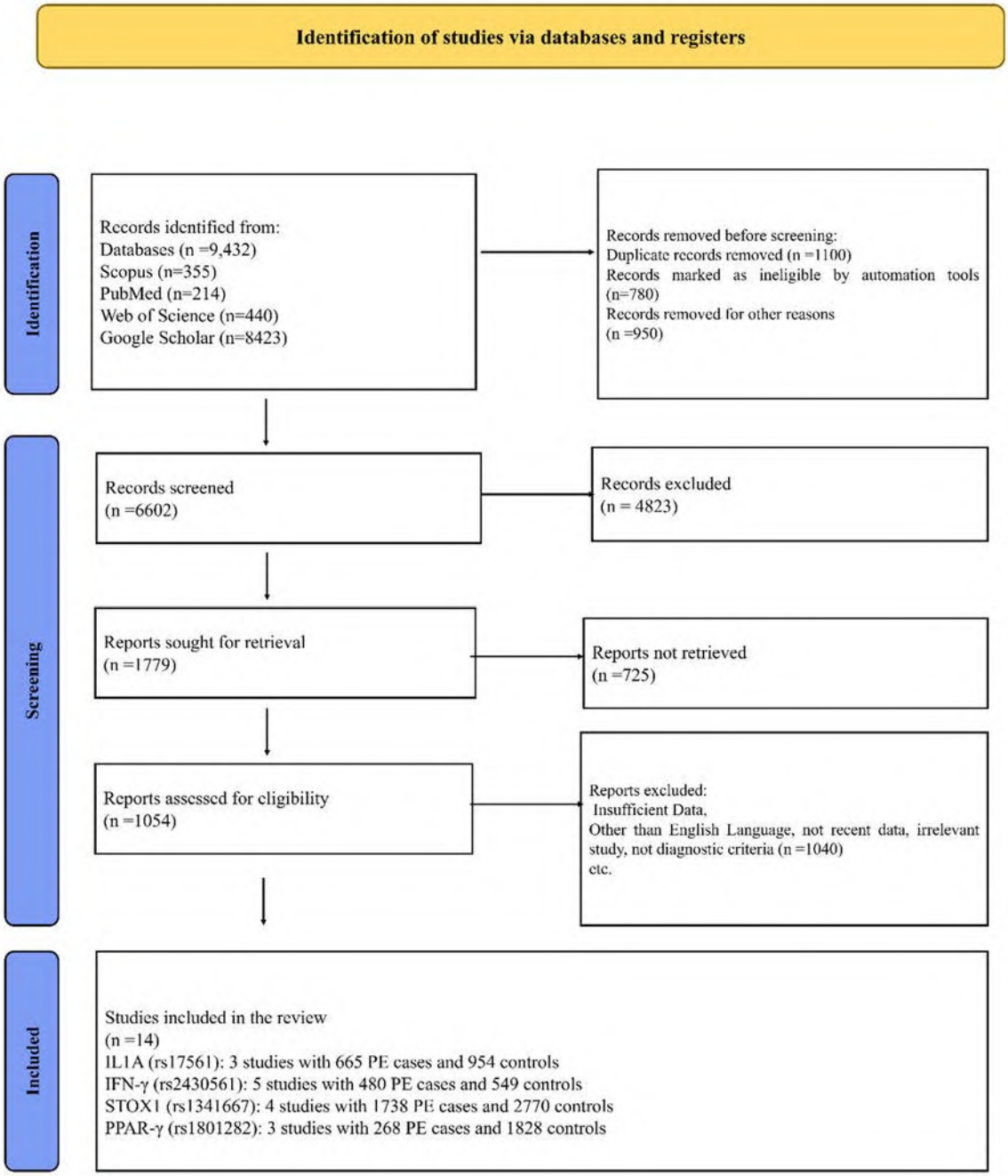

Figure 1. PRISMA

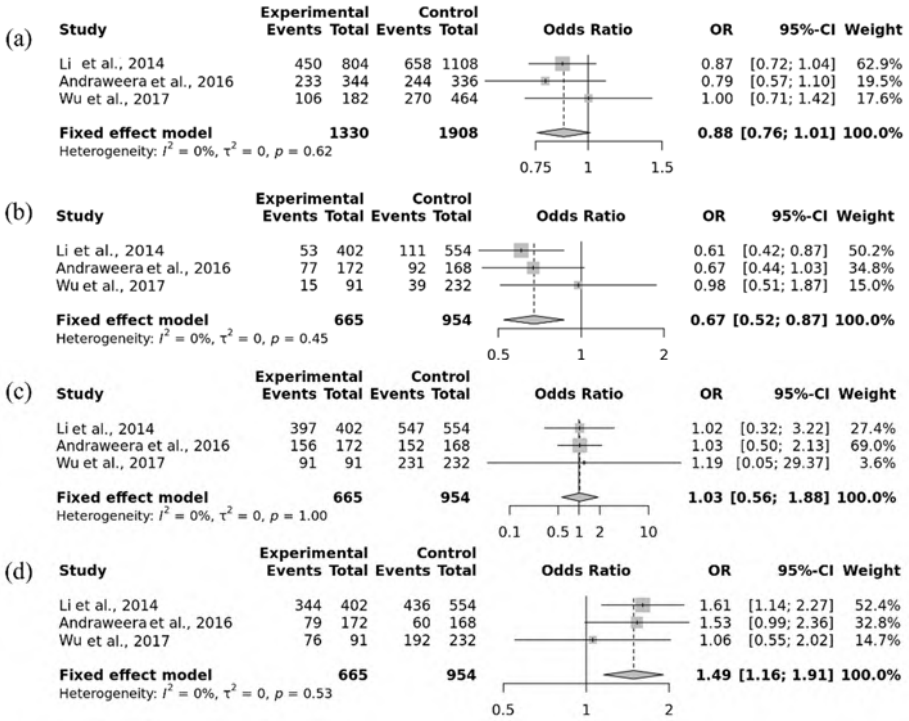

Figure 2. Forest plot for the association of IL1A (rs17561) gene polymorphism with PE risk (a) allelic, (b) recessive, (c) dominant, and (d) over-dominant model.

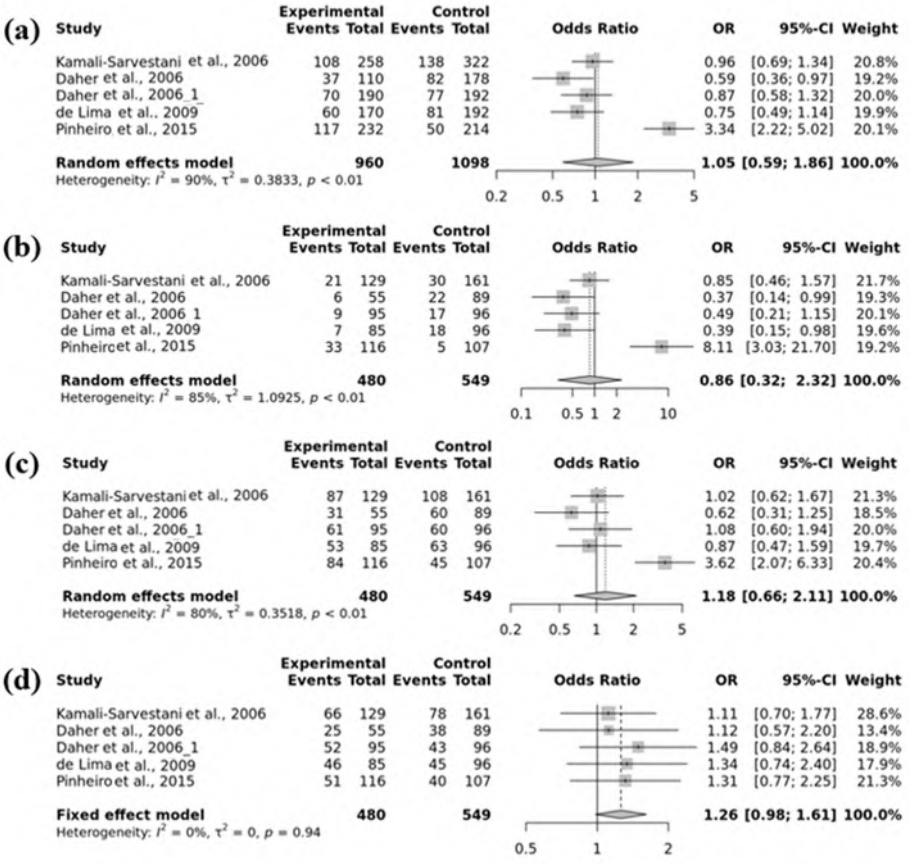

Figure 3. Forest plot for the association of IFN-γ (rs2430561) gene polymorphism with PE risk (a) allelic, (b) recessive, (c) dominant, and (d) over-dominant model.

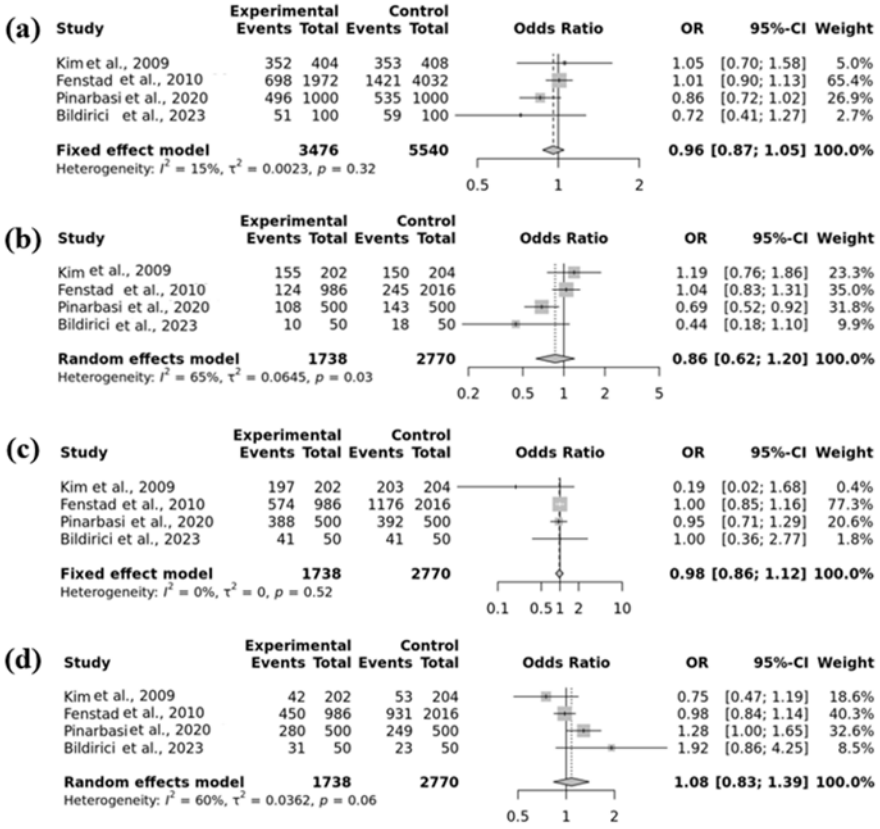

Figure 4. Forest plot for the association of STOX1 (rs1341667) gene polymorphism with PE risk (a) allelic, (b) recessive, (c) dominant, and (d) over-dominant model.

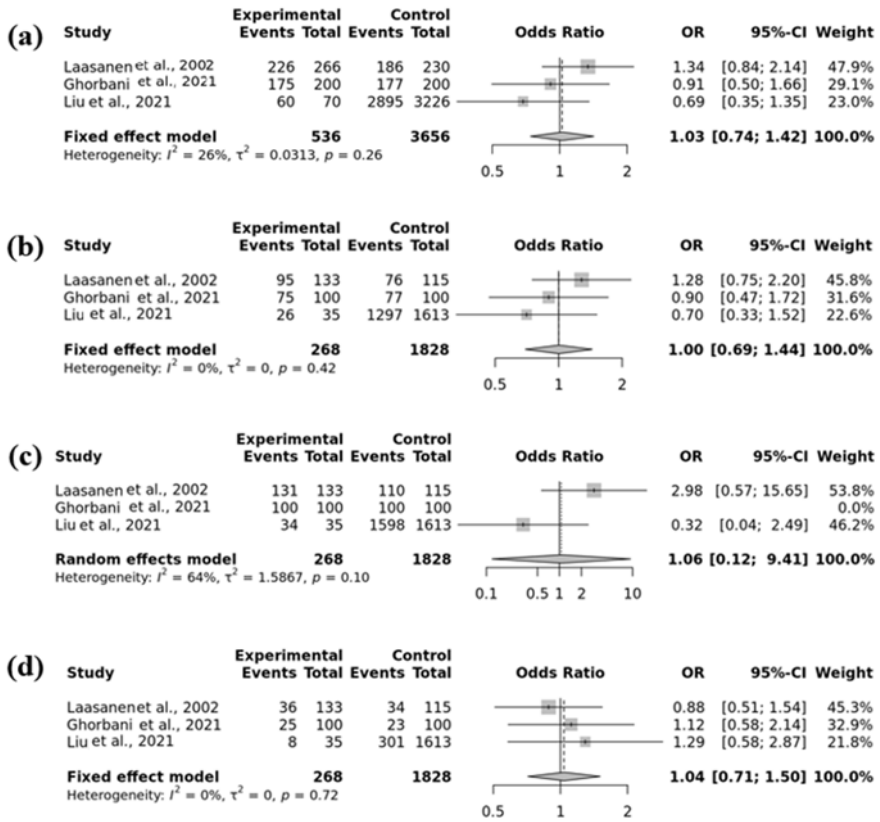

Figure 5. Forest plot for the association of PPAR-γ (rs1801282) gene polymorphism with PE risk (a) allelic, (b) recessive, (c) dominant, and (d) over-dominant model.

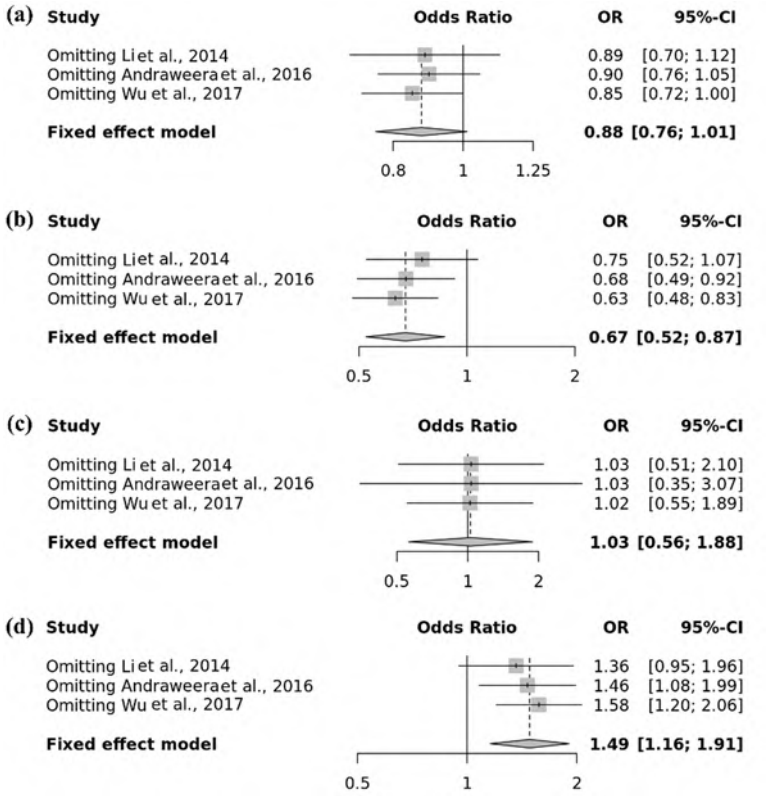

Figure 6. Sensitivity analysis for IL1A (rs17561) among PE cases and controls in all genetic models.

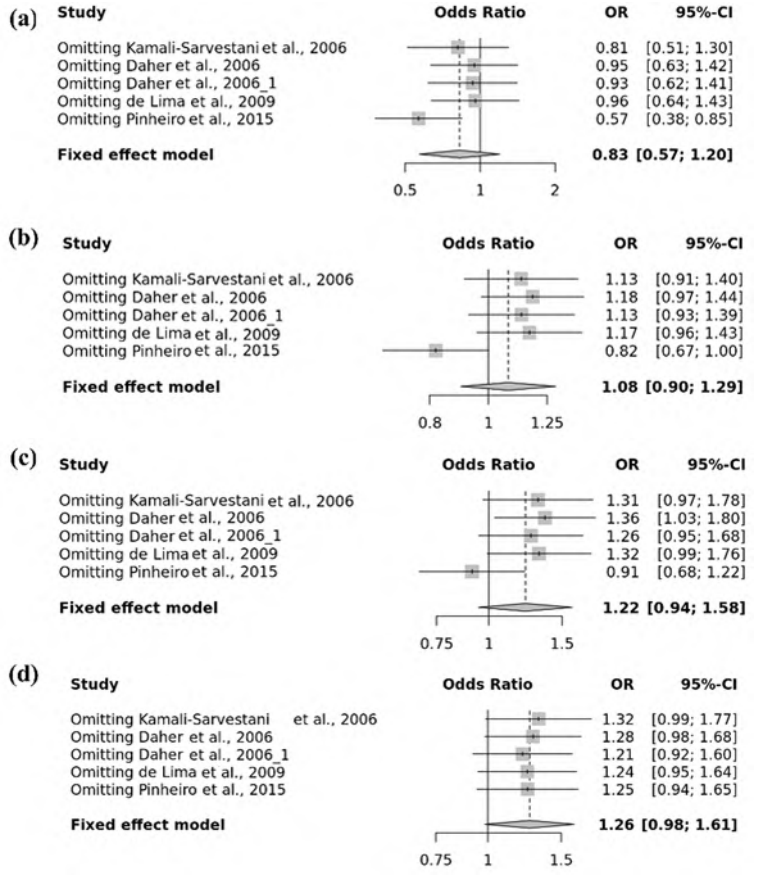

Figure 7. Sensitivity analysis for IFN-γ (rs2430561) among PE cases and controls in all genetic models.

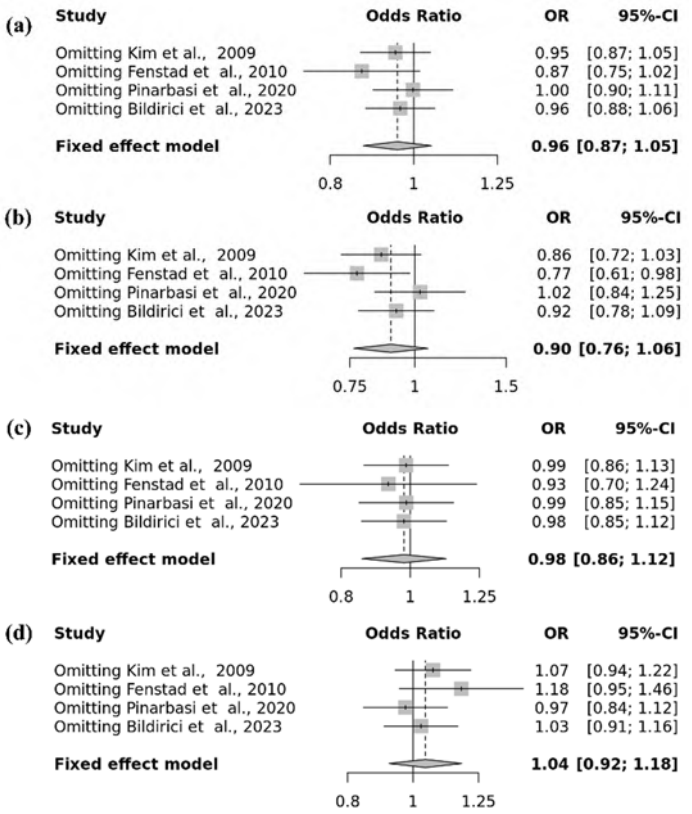

Figure 8. Sensitivity analysis for STOX1 (rs1341667) among PE cases and controls in all genetic models.

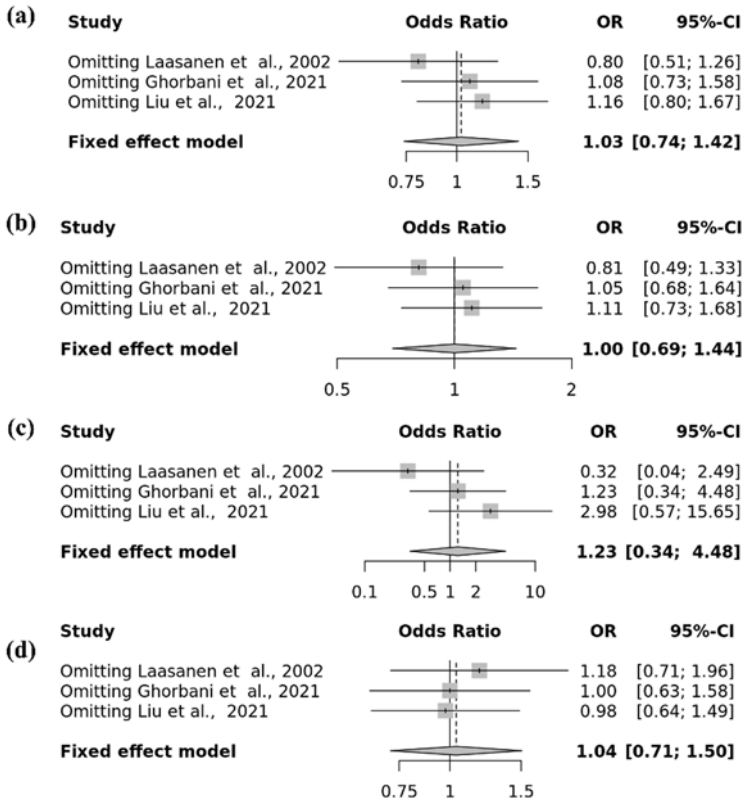

Figure 9. Sensitivity analysis for PPAR-γ (rs1801282) among PE cases and controls in all genetic models.

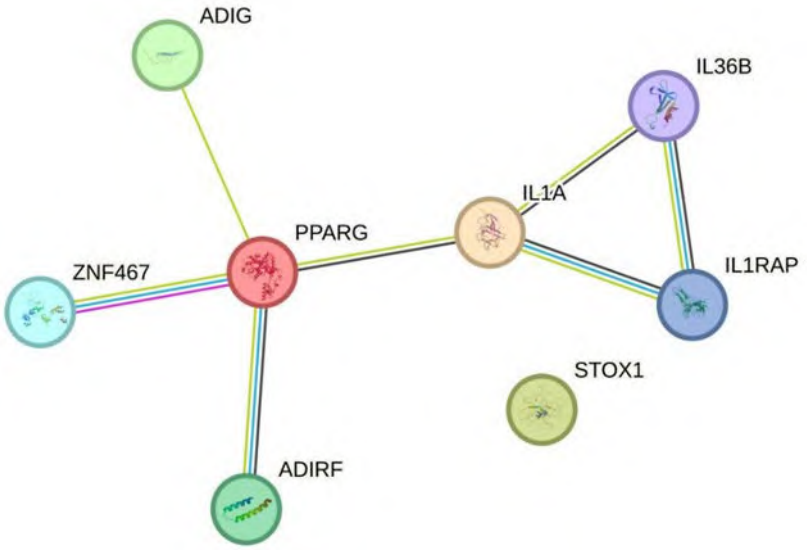

Figure 10. The Protein-Protein Interaction (PPI) network of differentially expressed genes (DEGs) among the selected genes associated with PE. We present the total clusters of the PPI network, f eaturing 8 nodes and 7 edges.

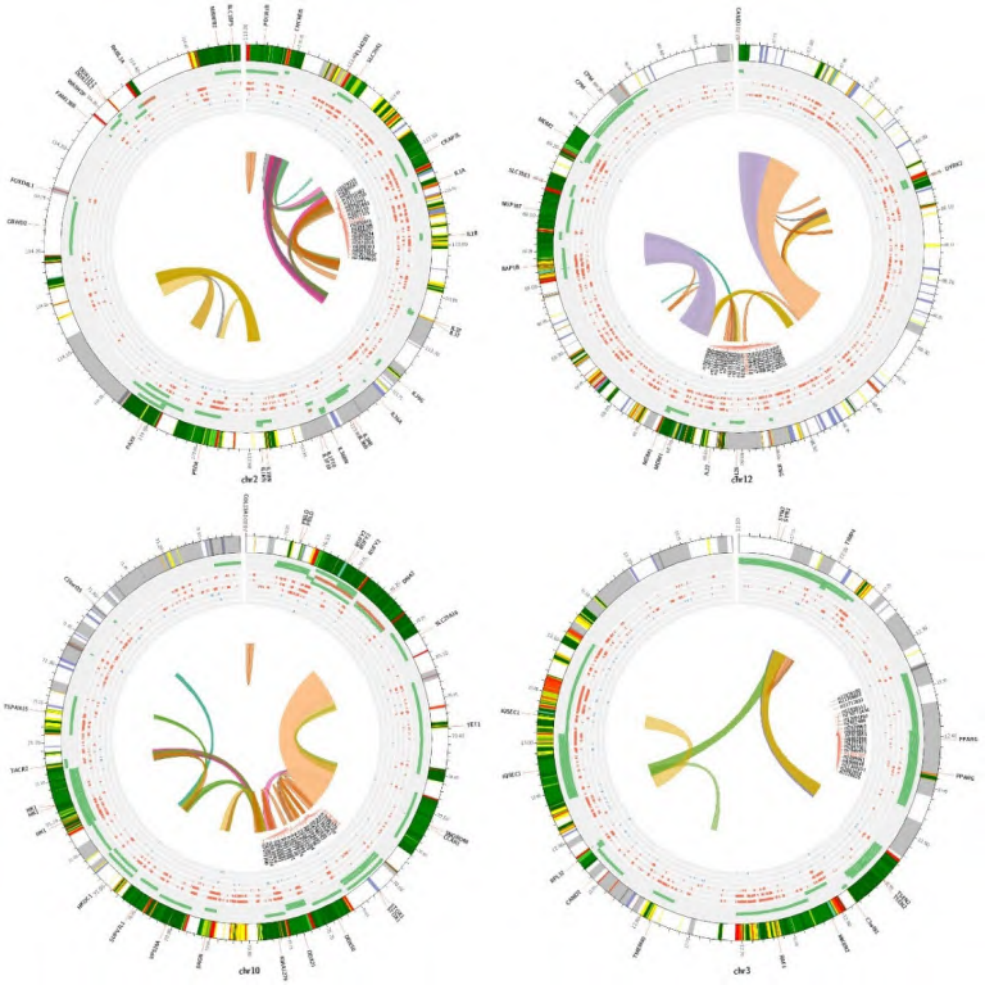

Figure 11. Circos plot visually represents the chromosomal relationships among the selected SNPs, focusing on IL1A (rs17561).

DISCUSSION

Pre-eclampsia (PE) remains a major obstetric complication with a multifactorial etiology involving complex interactions among genetic, environmental, and behavioural determinants [22]. Despite decades of research, the precise mechanisms underlying susceptibility remain elusive. While numerous studies have evaluated individual gene variants, the strength and direction of their associations with PE risk have varied considerably, underscoring the challenges of disentangling the genetic contribution to this condition [23]. The current meta-analysis explored the association of four gene polymorphisms-IL1A rs17561, IFN-γ rs2430561, STOX1 rs1341667, and PPAR-γ rs1801282-with the risk of PE by integrating evidence from 14 independent studies encompassing 3151 PE cases and 6101 healthy controls.

In this meta-analysis, a significant association was observed for the IL1A rs17561 polymorphism, which showed divergent effects across genetic models. The recessive model suggested a protective effect, while the over-dominant model indicated an increased risk of PE. In contrast, the dominant and allelic models showed no significant associations. This pattern suggests a context-dependent role of IL1A in inflammatory regulation during pregnancy, where different genotypic backgrounds may yield contrasting outcomes.IL1A encodes interleukin-1α, a key proinflammatory cytokine that contributes to trophoblast invasion, vascular remodeling, and immune modulation at the maternal–fetal interface [24,25]. The absence of a strong association may reflect the multifactorial contribution of IL-1 signaling, influenced by gene–environment interactions and compensatory mechanisms within the IL-1 family, including IL1B and IL1RN polymorphisms [26,27]. Moreover, population heterogeneity and differences in gestational age at disease onset may obscure genotype–phenotype correlations.

Similarly, no significant correlation was observed for IFN-γ rs2430561 polymorphism with PE risk in any genetic model. IFN-γ is a Th1 cytokine pivotal in immune activation, and elevated IFN-γ levels have been linked to defective placentation and exaggerated inflammation in PE [28,29]. The rs2430561 variant, located in the first intron, influences IFN-γ expression levels by modulating transcriptional activity [30]. However, our findings suggest that the variant alone may not confer a measurable risk, supporting the hypothesis that immune dysregulation in PE arises from polygenic effects and epigenetic regulation rather than single-locus polymorphisms [31].

The results of STOX1 rs1341667 for the all the four models had no significant link with PE. STOX1, a transcription factor expressed in trophoblasts, regulates placental growth and differentiation, and its dysregulation has been reported in familial and sporadic cases of PE [32,33]. Functional studies have shown that STOX1 interacts with the ENG and FLT1 gene pathways involved in endothelial dysfunction and anti-angiogenic signaling, key mechanisms underlying PE [34]. The genetic association observed in our analysis does not support these mechanistic insights and does not confirm STOX1 as a plausible genetic determinant of PE. Further functional validation is warranted to elucidate the biological significance of STOX1 gene polymorphism.

For the PPAR-γ rs1801282 polymorphism, no significant relationship was detected with PE across all genetic models, consistent with several prior meta-analyses [35,36]. PPAR-γ, a nuclear receptor involved in lipid metabolism and placental trophoblast differentiation, modulates vascular tone and anti-inflammatory responses. The rs1801282 (Pro12Ala) variant has been associated with improved insulin sensitivity and metabolic outcomes, which could theoretically influence PE pathogenesis [37]. However, the lack of association in this study may be attributed to ethnic variability in allele frequency, differences in diagnostic criteria, or gene–nutrient interactions affecting PPAR-γ activity during pregnancy.

The protein-protein interaction (PPI) network analysis further revealed that IL1A and PPAR-γ exhibit direct interactions, suggesting potential crosstalk between inflammatory and metabolic pathways in PE pathogenesis. The lack of a direct connection for STOX1 within this network supports the notion that STOX1-related effects might occur through transcriptional regulation rather than direct protein interactions. These findings emphasize the multifactorial and polygenic architecture of PE, wherein cumulative minor genetic effects and pathway-level interactions contribute more significantly to disease susceptibility than single variants [38,39].

The pooled ORs for several gene polymorphisms were sensitive to the removal of individual studies, indicating that some results lack complete robustness. Moreover, the power analysis verified that the included sample sizes were sufficient to detect moderate genetic effects, ensuring the reliability of the conclusions. The Circos plot provided additional insight into the genomic organization and potential epigenetic regulation of the studied genes, suggesting that histone modifications and transcriptional co-regulators might modulate their expression in placental tissue.

Moreover, population-specific allele frequency differences, as observed in prior studies [40,41], further complicate interpretation and suggest that the effect of IL1A variants may vary across ethnic backgrounds. The apparently opposite effects of IL1A rs17561 under different genetic models may also reflect epigenetic and environmental modulation. Dietary influences, obesity, and maternal metabolic status can shape the inflammatory and vascular responses in pregnancy, potentially amplifying or attenuating the genetic effects [42]. Likewise, epigenetic regulation of cytokine genes has been shown to impact PE risk, indicating that IL1A-related genetic susceptibility likely operates within a broader regulatory framework. Our findings reinforce the importance of IL1A as a candidate gene in PE but also underscore the multifactorial nature of disease susceptibility, where

protective and risk effects may co-exist depending on genetic model, ethnicity, and environmental context. This complexity highlights the need for integrative approaches that combine genetic, epigenetic, and clinical risk factors. Future research incorporating systems biology and multi-omics analyses may provide deeper insights into how IL1A polymorphisms interact with other determinants to influence PE outcomes. Ultimately, these advances could contribute to individualized risk prediction, early screening, and targeted interventions for women at risk of developing PE.

LIMITATIONS OF THE STUDY

First, although this meta-analysis focused on four key polymorphisms, numerous other candidate genes, as well as non-genetic environmental factors, may also contribute to PE susceptibility but were not considered here. Second, variations in study design, population characteristics, and sample size likely contributed to heterogeneity across the included studies. While this was partly mitigated using a random-effects model, residual heterogeneity may still have influenced the results. Third, reliance on published studies and the exclusion of non-English articles may have introduced selection bias. Fourth, publication bias could not be formally assessed, as fewer than 10 studies were available for each polymorphism. According to the Cochrane Handbook, statistical tests for funnel plot asymmetry (e.g., Egger's test) are underpowered with small sample sets; therefore, the potential influence of publication bias cannot be entirely ruled out. Finally, the statistical power of some findings may have been limited by the relatively small number of eligible studies included for each gene.

CONCLUSION

The present meta-analysis significantly identifies the IL1A rs17561 polymorphism as a crucial genetic susceptibility factor for preeclampsia, however its impact is highly depending upon the underlying genetic model. Collectively, these findings demonstrate a complex, model-dependent association between the IL1A rs17561 polymorphism and the risk of preeclampsia (PE) except dominant model. In contrast, the IFN-γ, STOX1, and PPAR-γ polymorphisms showed no significant link with preeclampsia across any of the four tested genetic models (allelic, recessive, dominant, or over-dominant), suggesting that its influence on PE risk may be negligible in this cohort.

Abbreviation

PE	Pre-eclampsia
IL1A	Interleukin-1 Alpha
INF-γ	Interferon-gamma
STOX1	Storkhead Box 1
PPAR-γ	Peroxisome Proliferator-Activated Receptor Gamma
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROSPERO	International Prospective Register of Systematic Reviews
HWE	Hardy-Weinberg equilibrium
CI	Confidential interval
OR	Odds Ratio
NOS	Newcastle-Ottawa scale
ROB	Risk of Bias
PPI	Protein-protein interaction
RPL	Recurrent Pregnancy Loss

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This study did not receive funding in any form.

ETHICAL APPROVAL

No ethical approval was required because this study was obtained from already published case vs. control studies. Since the present study was categorized as a meta-analysis, patient consent forms are not required.

CONFLICT OF INTEREST

The authors declare that there is no competing interest regarding the publication of this article.

ACKNOWLEDGMENTS

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ECTOPIC INTRAVESICAL PROSTATIC TISSUE- CASE REPORT

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ABSTRACT

So far in the reviewed literature, less than 50 cases of ectopic intravesical prostate tissue has been published. Possible complications are obstruction of urine and malignant alterations. We present a 48-year-old asymptomatic male patient, who was diagnosed with a bladder lesion around 3 cm in diameter, incidentally by ultrasound scan. It was presumed to be a bladder tumor. Following cystoscopy and computed tomography scan, the entire tumor positioned in the proximity of the left ureteral orifice was removed by transurethral resection. The pathohistological report showed that it was ectopic prostate tissue. Ectopic prostate tissue is very rare in the urinary bladder and mimics bladder tumor. The diagnosis is established after transurethral resection of tissue and histological examination.

Keywords: *Prostate ectopic tissue, benign tumor, urinary bladder.*

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INTRODUCTION

In the up to date reviewed literature, less than 50 case reports of patients with ectopic intravesical prostate tissue have been published [1]. Ectopic prostate tissue is frequently found in the urinary bladder close to the ureteral orifice or at the bladder trigone. It is important to mention that ectopic prostate tissue is clearly separated from the orthotopic prostate tissue. It can also be localised in the urethra, retroperitoneum or infrequently in the gastrointestinal tract [2,3]. The most common cause of ectopic prostate tissue is the migration of undifferentiated prostate tissue during embryogenesis, but some data suggest that metaplasia of transitional cell epithelium and hyperplasia of Mullerian remnants tissues are possible mechanisms [3].

CASE REPORT

During an ultrasound examination in a 48-year-old male patient without urological difficulties, a lesion was seen close to left orifice in the bladder. It was approximately 27 mm in diameter defined as a urinary bladder tumor by the radiologists with a normal ultrasound report of the other urogenital organs. The patient denied macroscopic hematuria and urine analysis did not show microscopic hematuria, neither. PSA value was 1,1 ng/ml and free/total ratio of PSA was 24%. MSCT confirmed the presence of a solid lesion in the bladder in the proximity of the left ureteral orifice 27x24x27mm in diameter (Figure 1a, 1b). Cystoscopy described this urinary bladder tumor as a solid, smooth, nonpapillary tumor with a narrow base with epithelium similar to the normal surrounding bladder epithelium. The position of the tumor caused that the left orifice could not be visualized. The patient underwent transurethral resection of the tumor. Following the resection, the intact left ureteral orifice was identified next to the base of the tumor. The pathohistological report confirmed that it was ectopic prostate tissue in the urinary bladder (Figure 2). There were no signs of inflammation, cellular atypia or adenocarcinoma in the analyzed resected tissue (Figure 3,4). Six months following transurethral resection the patient was without complaints. The urological ultrasound examination and cystoscopy confirmed no residual of ectopic prostate tissue.

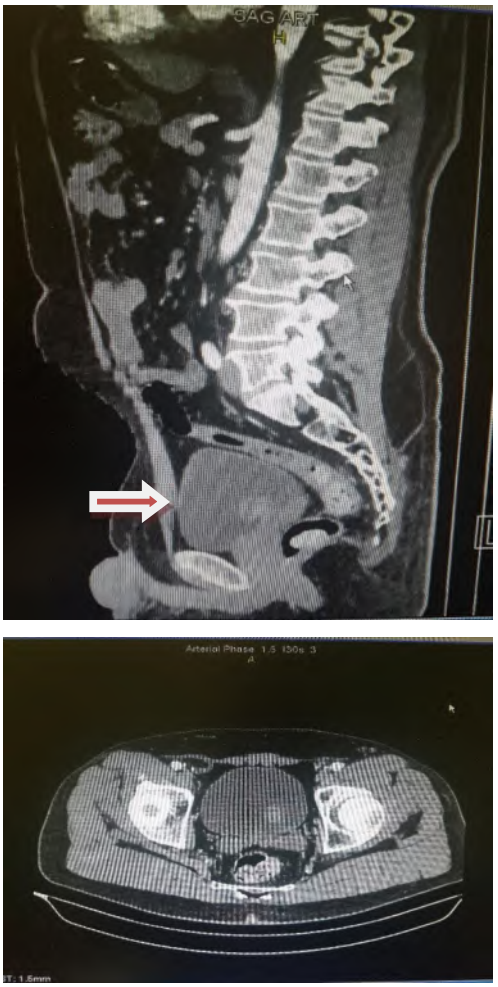

Fig. 1a and 1b - MSCT of abdomen and pelvis minoris shows intravesical mass (arrows).

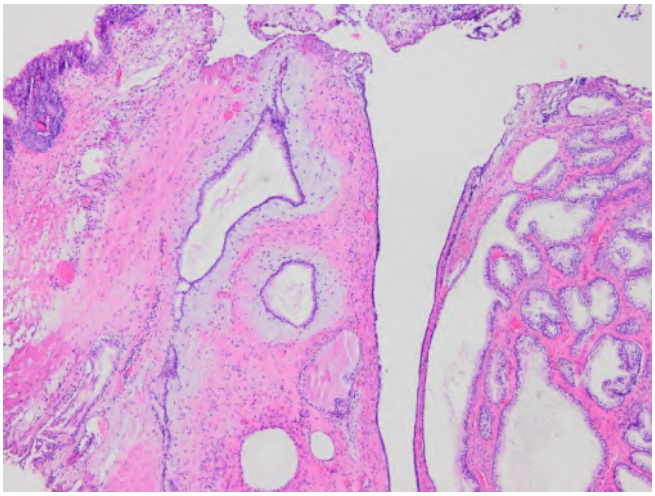

Fig.2 - Ectopic prostate tissue in the subepithelial tissue of the urinary bladder. The bladder mucosa seems to moderately proliferative and intact (arrow). Prostate glands showing irregular dilated lumens with flattened epithelial cells (H&E, ×40).

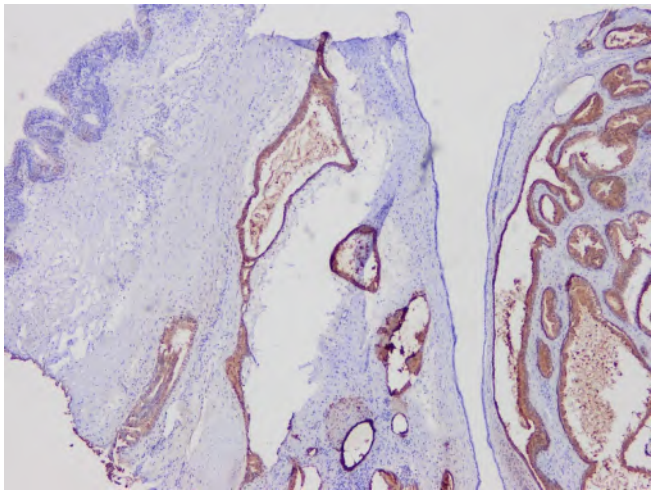

Fig. 3 - Immunopositive staining of ectopic prostate tissue for prostate-specific antigen (original magnification ×40).

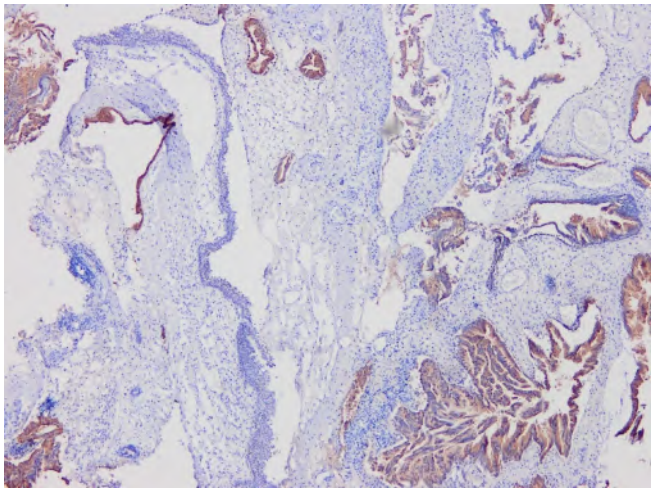

Fig.4 - Microscopic sections showed a dilated glandular structures surrounded by a dense fibrovascular stroma. Immunoperoxidase stain shows strong and diffuse mmunopositivity of the glandular epithelium with antibody to prostate-specific antigen (original magnification ×40).

DISCUSSION

Ectopic prostate tissue is most commonly incidentally detected during diagnostic procedures for hematuria or dysuria [3,4]. In this case, the patient underwent an ultrasound examination without complains on some urological difficulties, so it was incidentally detected. Diagnostic differentiation based on images obtained by imaging techniques (ultrasonography, intravenous urography and MSCT) most frequently indicate the possible bladder tumor. Due to its location near the ureteral orifice, it may cause obstruction and ureterohydronephrosis. Preoperative imaging of whole urological tract is therefore needed, not only in this case, but in any case of bladder intraluminal proliferation. This is due to not only possibility of ureteral obstruction caused by ectopic prostate tissue in urinary bladder (as in this case), but also because of possibility of multifocal localization of urothelial carcinomas. On the other hand, cases of malignant alteration of the ectopic prostate tissue towards the appearance of adenocarcinoma have been reported [5,6]. Therefore, a preoperative PSA assessment is needed and also a postoperative analysis of CD 10 markers (as indicators of possible mesonephric origin), p63, 34βE12 and α-methylacyl-CoA-racemase for revealing possible presence of adenocarcinoma [2,7]. Most commonly two different types of lesions can be present: polypoid or "flat" [8]. In our case, the lesion was presented as polypoid. Patients in whom the ectopic prostate tissue is surgically removed have an excellent prognosis if the tissue was without the presence of malignancy [2]. If prostate adenocarcinoma is found in the resected specimen, the further diagnostic pattern should include rigorous postoperative follow up that includes more frequent check-up of serum PSA levels and cystoscopy, as well as imaging diagnostic such as PSMA PET scan. Incompletely removed ectopic prostatic tissue can continue to grow and can cause dysuria, hydronephrosis and malignant transformation of ectopic prostatic tissue is a possibility. Therefore, in any case of resection of prostatic tissue from urinary bladder postoperative assessment that includes ultrasound and cystoscopy is essential and represents a diagnostic minimum.

CONCLUSION

Benign lesions of the bladder as ectopic intravesical prostatic tissue are extremely rare. In preoperative diagnostic differentiation, these lesions most commonly resemble transitional cell carcinoma of the bladder. Transurethral resection with a pathohistological report is the method of choice for definitive diagnosis and treatment of these cases. Following complete removal of the ectopic intravesical prostate tissue patients have excellent prognosis.

CONFLICT OF INTEREST

None declared.

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NEONATAL MEDIASTINAL TERATOMA: A CASE REPORT

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ABSTRACT

Mediastinal teratomas are the second most common extragonadal teratomas in children. They can be detected as mediastinal mass during routine prenatal ultrasound screening. Our case report shows neonates (female) with severe respiratory distress syndrome and cardiogenic shock due to pressure on the lungs, heart, aorta and systemic veines. Early surgical intervention is important but outcome depends on the stage of development of the mediastinal organs and complications in the postoperative period.

Keywords: *Neonatal teratoma, Respiratory distress, Cardiogenic shock, Obstructive shock, Mediastinal mass.*

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INTRODUCTION

Mediastinal teratomas are the second most common extragonadal teratomas in children[1]. Some cases are detected prenatally via ultrasound[2]. Rapid tumor growth during the fetal period exerts compression on the lungs, heart, and major blood vessels, leading to lung hypoplasia and heart dysplasia. If a fetus with a mediastinal teratoma survives until birth, respiratory distress syndrome develops at birth.

Respiratory distress at birth results from tumor compression on mediastinal structures. Early surgical intervention is crucial; however, the outcome depends on multiple factors, such as the development of mediastinal organs (lung hypoplasia, heart hypoplasia, and tracheomalacia may occur) and perioperative and postoperative complications[3]. This case report presents a newborn with a mass in the anterior mediastinum who survived the neonatal period.

CASE REPORT

A 29-year-old pregnant woman gave birth via cesarean section at GAK Narodni Front (gynecology obstetrics clinic). The term newborn, a female, had an Apgar score of 3/5 and a birth weight of 3300 g. The baby was intubated immediately after birth. Initial blood gas analysis indicated respiratory acidosis (pH = 6.91, pCO_2 = 13.9 kPa, pO_2 = 6.6 kPa). Prenatal suspicion of a diaphragmatic hernia led to the newborn being transferred to our clinic within two hours of birth. Upon admission, a chest X-ray revealed a soft tissue mass occupying the upper and middle thirds of the left lung (Figure 1A). The heart ultrasound shows a tumor mass pushing the heart completely to the right, compressing the left ventricle and both atria. CT imaging demonstrated a massive tumor in the left hemithorax with visible calcifications and prominent vascularization, exerting significant compressive effects on the heart, lungs, and major blood vessels (Figure 1B). The trachea and right main bronchus were shifted to the right (Figure 1D). Upon returning to the neonatal intensive care unit, the newborn was respiratory and hemodynamically unstable and cyanotic. Blood gas analysis confirmed respiratory and metabolic acidosis. The newborn baby was placed in a left lateral position, and a thoracic drain was inserted on the right side. This resulted in minimal hemodynamic and respiratory improvement but further compression of the inferior vena cava, leading to edema in the lower half of the body. High-frequency mechanical ventilation was attempted but did not yield satisfactory results, so the newborn was placed back on pressure-controlled mechanical ventilation. Hypotension developed, necessitating vasoactive support with dobutamine and later adrenaline. Due to signs of pulmonary hypertension, fentanyl was introduced into therapy. This led to relative stabilization of hemodynamic and respiratory conditions, maintained through continuous inotropic stimulation and aggressive pressure-controlled mechanical ventilation settings.

On the first day of life, a surgical intervention was performed via median sternotomy. The tumor was completely excised. The left lung was hypoplastic and expanded manually through ventilation. The right lung was normally developed, and the heart was located in the right hemithorax within an intact pericardium. The tumor weighed 230 g and was histologically classified as an immature teratoma, grade III. Postoperatively, the newborn remained on controlled mechanical ventilation, showing respiratory and hemodynamic stability under inotropic support. In the following days, mechanical ventilation parameters and inotropic drug doses were gradually reduced, with inotropes discontinued six days after surgery. The newborn was extubated on the twelfth postoperative day and placed on nasal CPAP, but due to respiratory deterioration, reintubation was required. Chylothorax developed, but after somatostatin therapy was introduced, drainage gradually decreased. Fiberoptic bronchoscopy revealed tracheomalacia and left bronchomalacia. The heart gradually returned to its central position over four weeks (Figure 1C). In the following days, an extubation attempt was unsuccessful. A chest X-ray showed left hemidiaphragm elevation. After plication of the left diaphragm, performed 28 days after the initial surgery, the newborn was successfully extubated and placed on nasal CPAP. The baby was transitioned to an oxygen mask 35 days postoperatively and was transferred to the regular ward 39 days after surgery.

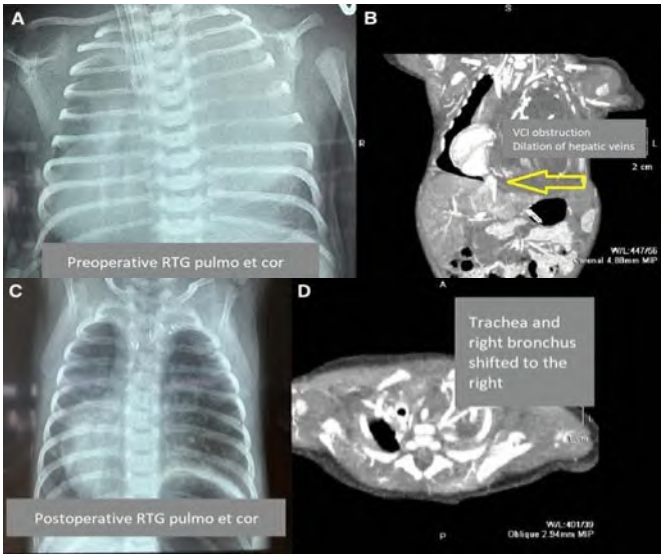

Figure 1: **(A)** Preoperative chest X-ray, **(B)** Chest CT showing obstruction of the inferior vena cava (IVC), **(C)** Postoperative chest X-ray, **(D)** Chest CT showing displacement of the trachea and right bronchus to the right.

DISCUSSION

A teratoma is a tumor originating from a germ cell with the potential to differentiate into tissues from all three embryonic germ layers, with the tissues and structures being more or less mature. Its histological structure is heterogeneous, consisting of both cystic and solid components. The incidence of neonatal tumors is estimated at 7.2 per 100,000 live births, although many benign tumors go undiagnosed, leading to

imprecise incidence estimates. The most common neonatal tumors are teratomas (approximately 25%), with thoracic teratomas accounting for about 8-16%[4].

Radiological diagnostics, both prenatal and postnatal, play a key role in disease evaluation. Mediastinal tumors can be detected prenatally through routine fetal echocardiography in the second and third trimesters of pregnancy. Fetal MRI provides more precise information about the tumor itself and its relationship with adjacent structures. In our patient, the mediastinal teratoma was not diagnosed prenatally; instead, there was a suspicion of a diaphragmatic hernia. After birth, a chest CT scan revealed a massive tumor occupying the left hemithorax. The pathophysiological effect of a mediastinal tumor results from its compression of mediastinal structures, lungs, heart, esophagus, aorta, and systemic veins. Clinically, this manifests as edema, ascites, pleural effusion, and hepatomegaly.

The presence of a tumor mass in utero within the thoracic cavity can lead to cardiac dysplasia and pulmonary hypoplasia. Cardiac dysplasia may present in various ways, depending on the tumor's growth rate and the stage of cardiac development. This can include myocardial wall hypertrophy with atrial and ventricular dilation, abnormal positioning of the pulmonary artery leading to pulmonary hypertension. Additionally, polyhydramnios develops due to esophageal compression and reduced swallowing of amniotic fluid[5]. In our patient, the presence of a mediastinal mass led to respiratory distress, cardiogenic and obstructive shock due to compression of the myocardium, pulmonary systemic veins, and aorta. The echocardiogram showed that the tumor had displaced the heart to the right, compressing the left ventricle and both atria, with minimal systemic venous return. No structural abnormalities of the heart were observed.

The outcome of treatment depends on the tumor's size, location, and the timing of prenatal or postnatal intervention. The presence of a tumor mass in such a small space, like the neonatal thorax where vital structures are located, represents a malignant localization of the lesion, accompanied by a severe clinical manifestations. The effects of the tumor are proportional to its size and pressure on vital structures. Literature reports describe four cases diagnosed prenatally as non-immune hydrops fetalis caused by a mediastinal teratoma, and who survived the neonatal period after surgery, i.e. tumor extraction(Table 1).

Table 1. Clinical neonatal successful outcomes of fetal mediastinal teratomas.

Author	Sex	Imaging	Prenatal Procedure	Outcome
Takayasu[6]	M	US (23WG): Cystic formation in right anterior mediastinum MRI (29 WG): Cystic/solid mass NIHF, polyhydramnion	Aspiration of the fetal tumor cyst fluid. Amniocentesis	Hydrops fetalis subsided No RD after birth Resection 30 days after birth NED
Giancotti[8]	M	US (29WG): Anterior mediastinal mass MRI (31WG): Anterior mediastinal mass, NIHF, polyhydramnion US (32WG): Rapid growing mass	No	Elective cesarean section (32 WG) RD after birth, resection 1day after birth Left vocal cord/left diaphragm paralysis 18th day after birth diaphragm plication No respiratory problem
Merchant[7]	ND	US (21WG): Anterior mediastinal mass MRI(22WG): Anterior mediastinal mass with displacement of the heart, calcification, NIHF	In utero resection of the tumor	Preterm labor (25 WG) Bronchopulmonary dysplasia Well at home
M. Simoncic[3]	M	US (33WG): NIHF, polyhydramnion	No	Urgent cesarean section (33WG) RD after birth, resection 7 days after birth Chronic respiratory insufficiency 8 months after procedure

Author	Sex	Imaging	Prenatal Procedure	Outcome
Present case	F	US (prenatal): diaphragmatic hernia US (first day after birth): homogenous mass occupying the whole thorax CT (first day after birth): anterior mass involving the whole chest and extreme displacement and compression of the heart at great vessels	No	Chylothorax Tracheomalacia Bronchomalacia Left diaphragma paralysis 28th day after birth diaphragm plication No respiratory problem

M – Male, F – Female, MRI – Magnetic Resonance Imaging, CT – Computer Tomography Imaging, ND – No data, NED – No evidence of disease, NIHF – Nonimmune hydrops fetalis, RD – respiratory distress, US – ultrasound, WG – weeks gestation.

Different treatment approaches have resulted in survival, depending on the tumor's characteristics. These approaches can be classified as prenatal and postnatal interventions. Prenatal interventions include aspiration of the tumor cyst[6], resection of the tumor *in utero* before the 30th week of gestation to allow lung and airway development[7], while postnatal intervention involves tumor resection on the first day of life[8]. In our case, a sternotomy with complete tumor resection was successfully performed on the first day of life. Postoperatively, expected complications include sepsis, tracheomalacia, bronchopulmonary dysplasia, diaphragmatic and vocal cord paralysis, chronic respiratory insufficiency, and cardiac dysplasia. In our patient, chylothorax and left diaphragm paralysis developed, leading to diaphragmatic plication surgery, after which the newborn was successfully extubated.

CONCLUSION

Mediastinal teratoma in a neonate is a life-threatening condition.

Patients with mediastinal teratoma and similar pathology are in a state of respiratory and vascular insufficiency which worsens if not treated, and leads to respiratory distress, cardiogenic and obstructive shock.

Prenatal diagnostics, size and localization of the tumor, appropriate intensive care measures and emergency surgical intervention greatly determine the disease outcome.

This case represents an extreme example of a newborn with an anterior mediastinal mass that survived.

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INFORMATION FOR AUTHORS

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