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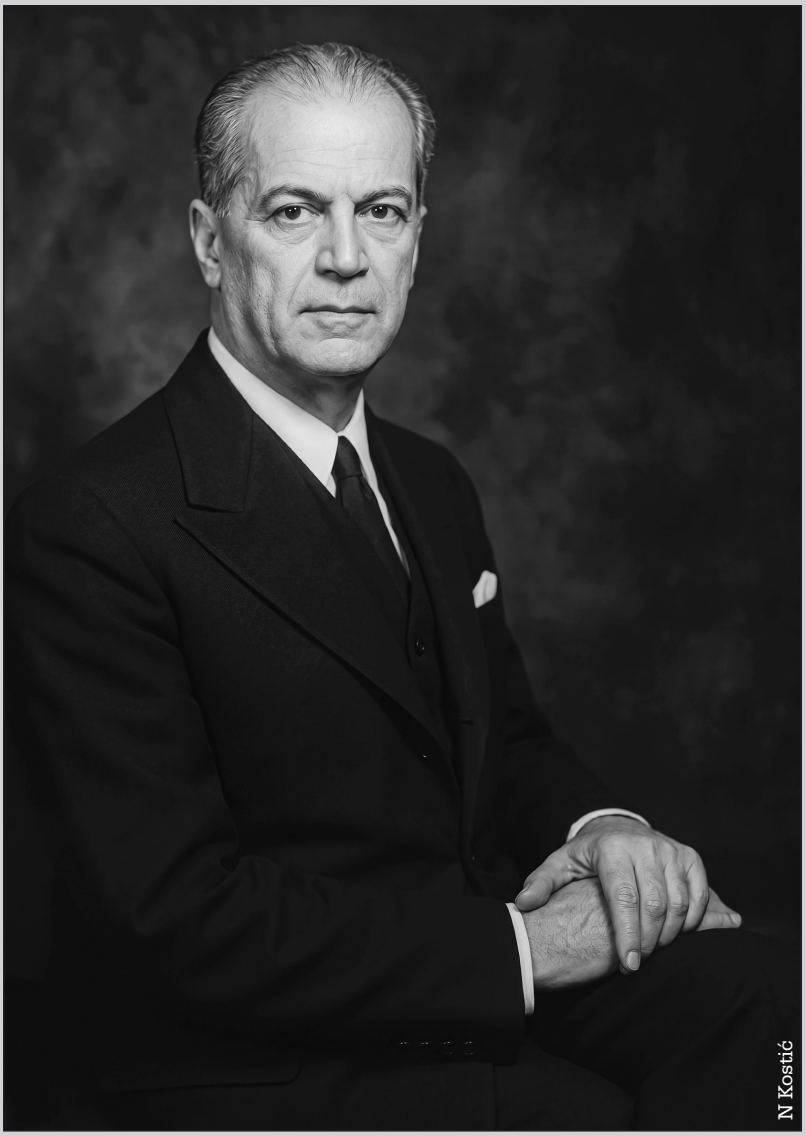

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COVER PHOTO PICTURE

Milivoje Kostić (Sarajevo, BA 24 August 1883 – Belgrade, RS, 21 February 1974) completed his primary and secondary education in Sarajevo and graduated from the Faculty of Medicine in Vienna in 1906. Immediately after completing his studies, he was employed at the National Hospital in Sarajevo, where he worked for two years. He then pursued specializations at renowned surgical clinics in Hamburg, Berlin, and Paris. After World War I (1918), he became head of the Surgical Department and the director of the Hospital in Sarajevo. In 1923, he moved to Belgrade, where he was elected professor at the Faculty of Medicine. He was a corresponding member of the Serbian Academy of Sciences and Arts (SANU) in the Department of Medical Sciences from 10 June 1955 and a full member from 30 January 1958. He served as a member of the Presidency of SANU from 15 April 1959 to 15 April 1960. In 1955, he was elected a full member of the Scientific Society of Bosnia and Herzegovina in the Department of Medical Sciences, and in 1966, he became a full member of the Academy of Sciences and Arts of Bosnia and Herzegovina upon its establishment. At the session of the Faculty of Medicine in Belgrade in 1945, he advocated for the establishment of the Faculty of Medicine in Sarajevo rather than in Kragujevac or Niš. He was awarded the Order of Labor, First Class (1956), the Order of Merit for the People with the Gold Star (1972), and numerous charters and plaques. The photo is printed with the permission of the Serbian Academy of Sciences and Arts Library

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Clinical Guidelines
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Clinical Practice Guidelines for Adult MASLD in Bosnia and Herzegovina: National Recommendations for Diagnosis, Risk Stratification, and Treatment

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Abstract

Metabolic dysfunction–associated steatotic liver disease (MASLD) is the leading cause of chronic liver disease in Bosnia and Herzegovina and a major contributor to liver- and cardiovascular-related morbidity and mortality. These national guidelines provide the first country-specific, evidence-based recommendations for the diagnosis, risk stratification, and management of MASLD, covering the full disease spectrum from steatosis to cirrhosis and its complications. MASLD management is based on a coordinated, stepwise model across primary, secondary, and tertiary healthcare levels, with primary healthcare serving as the central gatekeeper and coordinator of care. Active case-finding of clinically significant fibrosis is emphasized, particularly in high-risk populations, such as individuals with type 2 diabetes mellitus or obesity. Primary healthcare plays a key role in early risk assessment through systematic evaluation of metabolic risk factors and first-line use of simple non-invasive tools, notably the FIB-4 index. A stepwise non-invasive diagnostic algorithm is recommended, with elastography (preferably vibration-controlled transient elastography or 2D shear wave elastography where available) used for further assessment in patients with indeterminate or high-risk results. Lifestyle modification and optimal management of cardiometabolic comorbidities remain the foundations of MASLD treatment. In eligible patients with type 2 diabetes mellitus and/or overweight or obesity, glucagon-like peptide-1 receptor agonists may be considered, while bariatric surgery represents an option for selected individuals with persistent obesity. Advanced MASLD, including cirrhosis, requires multidisciplinary management, surveillance for hepatocellular carcinoma, and evaluation for liver transplantation in cases of decompensation. These guidelines aim to standardize MASLD care in Bosnia and Herzegovina and strengthen the pivotal role of primary healthcare within the national MASLD care pathway.

Key Words: MASLD ▪ Clinical Guidelines ▪ Management ▪ Bosnia and Herzegovina.

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Purpose of the Guidelines

These national guidelines provide healthcare professionals in Bosnia and Herzegovina (BA) with a practical, evidence-based framework for the diagnosis, risk stratification, and management of metabolic dysfunction-associated steatotic liver disease (MASLD). These recommendations represent an adaptation of the EASL-EASD-EASO Clinical Practice Guidelines for MASLD (1) to the healthcare setting of BA. Accordingly, the guideline does not independently re-grade the available evidence but relies primarily on the evidence appraisal and recommendation framework established in the source guideline, while adapting diagnostic and therapeutic pathways to local healthcare resources and availability. In addition to international evidence and consensus statements, the recommendations incorporate expert opinions from leading national specialists and are adapted to the specific context of BA, including the availability of approved pharmacological therapies, diagnostic resources, and the organizational structure of primary, secondary, and tertiary healthcare levels. By integrating current scientific evidence with local regulatory, infrastructural, and cost-effectiveness considerations, the guidelines support rational clinical decision-making and efficient use of healthcare resources. They are intended for all healthcare professionals involved in MASLD care, including family physicians, internists, gastroenterologists, endocrinologists, and other relevant specialists. Particular emphasis is placed on the central gatekeeping and coordinating role of primary healthcare as the foundation for early detection, risk stratification, and longitudinal management of MASLD within the national care pathway.

Definition of MASLD

MASLD formerly known as non-alcoholic fatty liver disease (NAFLD), encompasses a broad spectrum of liver disease associated with metabolic dysfunction. It is defined by the presence of hepatic steatosis in individuals with at least one established cardiometabolic risk factor, in the absence of significant alcohol consumption or other secondary causes of hepatic steatosis (1, 2).

The progressive inflammatory form of the disease, termed metabolic dysfunction-associated steatohepatitis (MASH), is characterized histologically by steatosis, hepatocellular ballooning, and lobular inflammation. Clinical prognosis is primarily determined by fibrosis stage rather than by steatosis alone. The disease is closely linked to systemic metabolic dysfunction and is currently regarded as the hepatic manifestation of metabolic syndrome (1).

Overlap Between MASLD and Alcohol-Related Liver Disease: MetALD

The current nomenclature also includes metabolic dysfunction-associated alcohol-related liver disease (MetALD), defined as the coexistence of metabolic dysfunction with alcohol consumption exceeding the thresholds accepted for MASLD diagnosis but not reaching levels traditionally associated with predominant alcohol-related liver disease. Recognition of MetALD reflects the common overlap between metabolic and alcohol-related liver injury and highlights the need to address both metabolic risk factors and alcohol consumption during patient management (1, 2).

Epidemiological Burden, Prevalence, and Natural History of MASLD

MASLD represents a major and growing public health burden worldwide and is increasingly recognized as a significant health concern in BiH (1, 3). Although comprehensive national registries and population-based epidemiological studies are currently lacking, available regional data and the high prevalence of cardiometabolic risk factors suggest that the burden of MASLD in BiH is substantial.

Based on European population studies, the overall prevalence of MASLD in adults is estimated at approximately 30–35% (3-6). Given the comparable or higher prevalence of obesity, type 2 diabetes mellitus (T2D), arterial hypertension, and dyslipidaemia in BiH, a similar prevalence in

the general population is highly plausible. In high-risk groups, particularly individuals with T2D, MASLD prevalence exceeds 60–70%, consistent with data from other European countries (1, 6).

The natural history of MASLD is heterogeneous. While a substantial proportion of individuals remain asymptomatic with stable hepatic steatosis, approximately 10–30% progress to MASH and clinically significant fibrosis over time (1, 5). In patients with T2D and multiple metabolic risk factors, disease progression is markedly accelerated, with advanced fibrosis or MASH reported in up to 40–60% of cases (1, 6). Progressive fibrosis is the strongest predictor of liver-related morbidity and mortality (7, 8).

In BA, advanced stages of MASLD, including cirrhosis and HCC, are increasingly encountered in secondary and tertiary healthcare settings. MASLD is now recognized as a leading cause of cryptogenic cirrhosis and an increasingly important indication for liver transplantation in Europe, a trend that is likely to be mirrored in BiH as diagnostic awareness and case detection improve. Beyond liver-related outcomes, MASLD is associated with increased all-cause and cause-specific mortality, predominantly driven by cardiovascular disease, which represents the leading cause of death in this patient population. The coexistence of MASLD with cardiovascular disease, chronic kidney disease, and other metabolic complications substantially increases healthcare utilization and long-term costs, underscoring the need for early detection and risk stratification, particularly in primary healthcare settings. In addition to cardiovascular and liver-related mortality, accumulating evidence suggests that extrahepatic malignancies contribute substantially to the overall mortality burden associated with MASLD (1, 5).

Pathogenesis and Comorbidities of MASLD

MASLD is a complex, multisystem disorder and represents the hepatic manifestation of systemic metabolic dysfunction. Rather than an isolated liver condition, MASLD reflects the interplay between insulin resistance, dysregulated lipid metabolism, chronic low-grade inflammation, and progressive fibrogenesis, and is tightly linked to cardiometabolic and endocrine comorbidities (1, 9, 10).

Understanding the pathophysiological mechanisms and associated comorbidities of MASLD is essential for appropriate risk stratification and for designing integrated management strategies. This chapter outlines the key pathogenic mechanisms and the major metabolic comorbidities that determine prognosis and guide clinical decision-making.

Pathophysiological Mechanisms of MASLD

At the core of MASLD pathogenesis lies insulin resistance, which drives a cascade of metabolic disturbances within the liver and peripheral tissues. Insulin resistance promotes increased lipolysis in adipose tissue, enhanced hepatic de novo lipogenesis, and impaired export of triglycerides from hepatocytes. The net result is excessive intrahepatic lipid accumulation and hepatocellular lipotoxicity (9, 10).

Lipotoxic stress induces oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum stress, leading to hepatocyte injury and apoptosis. These processes activate innate immune pathways, including Kupffer cells, and stimulate hepatic stellate cell activation, thereby promoting chronic inflammation and progressive fibrosis. In a subset of patients, this culminates in metabolic dysfunction–associated steatohepatitis (MASH), cirrhosis, and hepatocellular carcinoma (1, 10).

Disease susceptibility and progression are further modified by genetic and hormonal factors. Variants in genes such as *PNPLA3*, *TM6SF2*, and *MBOAT7* are associated with increased hepatic fat accumulation, more severe necroinflammation, and accelerated fibrosis, independent of classical metabolic risk factors (11). Hormonal influences (e.g., postmenopausal status) and environmental factors, including sedentary lifestyle, smoking, and diets rich in refined carbohydrates and saturated fats, additionally contribute to disease development and progression (12).

Collectively, these mechanisms support the concept of MASLD as a systemic metabolic disease

with hepatic involvement, a paradigm that underpins modern approaches to screening, prognostication, and integrated care.

MASLD and Metabolic Comorbidities: Clinical Implications

MASLD is a hepatic manifestation of systemic metabolic dysfunction and is frequently accompanied by cardiometabolic comorbidities that adversely affect both overall and liver-related outcomes. As cardiovascular disease remains the leading cause of death in this population, comprehensive cardiometabolic risk assessment and management are essential components of care (1, 13). Table 1 summarizes the wide range of cardiometabolic and systemic comorbidities associated with MASLD and their clinical implications. In the following sections, we focus on those comorbidities with the strongest evidence for impact on disease progression, prognosis, and therapeutic decision-making.

Type 2 Diabetes Mellitus

Type 2 diabetes (T2D) is the most important clinical risk factor for progression from simple steatosis to MASH, advanced fibrosis, cirrhosis, and hepatocellular carcinoma. Approximately 60–70% of patients with T2D have MASLD, and the presence of diabetes roughly doubles the risk of advanced fibrosis (13). Effective glycaemic control is therefore a cornerstone of management. Antidiabetic agents with proven or potential hepatic benefit, such as GLP-1 receptor agonists and SGLT2 inhibitors, are preferred, while pioglitazone may be considered in carefully selected patients with biopsy-proven MASH (1, 14).

Dyslipidemia

Atherogenic dyslipidemia is highly prevalent in MASLD and represents a major driver of cardiovascular morbidity and mortality. Statins are safe across the entire spectrum of MASLD, including compensated cirrhosis, and are strongly recommended for both primary and secondary cardiovascular prevention. Importantly, statin therapy does not exacerbate liver disease and may reduce both cardiovascular and liver-related outcomes (15).

Arterial Hypertension

Arterial hypertension commonly coexists with MASLD as part of the metabolic syndrome and contributes substantially to cardiovascular risk. Adequate blood pressure control is essential for reducing overall cardiovascular morbidity and mortality in patients with MASLD. Management should follow established hypertension guidelines and be individualized according to the patient's overall cardiometabolic profile (16).

Cardiovascular Disease

Cardiovascular disease represents the leading cause of death in patients with MASLD, surpassing liver-related mortality. MASLD should therefore be considered a cardiovascular risk enhancer, warranting aggressive risk factor modification and early preventive strategies. Several studies suggest that MASLD may represent an independent cardiovascular risk factor, although residual confounding and causal pathways remain under investigation (1, 13).

Obesity

Obesity, particularly visceral adiposity, is a central driver of MASLD pathogenesis, promoting insulin resistance, hepatic inflammation, and fibrogenesis. Sustained weight loss remains the most effective intervention for improving hepatic steatosis, necroinflammation, and fibrosis. Lifestyle modification is first-line therapy, while pharmacological and surgical weight-loss strategies may be appropriate in selected patients with obesity and metabolic complications (1, 17).

Integrated Clinical Perspective

All patients with MASLD should undergo systematic evaluation for metabolic comorbidities, including T2D, dyslipidemia, hypertension, and

Table 1. Key Cardiometabolic Comorbidities Associated with MASLD* and Their Clinical Implications

MASLD*- associated comorbidity	Relationship with MASLD*	Key clinical implications
Type 2 diabetes mellitus	Key risk factor for progression to MASH†, fibrosis, cirrhosis, HCC‡	High-priority group for fibrosis risk stratification and proactive liver assessment
Obesity (visceral adiposity)	Key driver of steatosis, inflammation, fibrogenesis	Sustained weight loss key to liver improvement
Dyslipidemia	Nearly universal; major determinant of CV§ risk	Reduce CV§ risk: statins safe, recommended
Arterial hypertension	Common MetS‖ component in MASLD*	Adequate BP¶ control reduces CV§ morbidity
Metabolic syndrome	Reflects systemic metabolic dysfunction in MASLD*	Predicts liver disease progression and CV§ events
Cardiovascular disease	Leading cause of death in MASLD*	CV§ risk management core to MASLD*
Chronic kidney disease	Frequently coexists due to shared metabolic risk factors	Linked to higher overall and liver-related mortality
Polycystic ovary syndrome	Associated with IR** and increased MASLD* prevalence	Assess liver in PCOS†† women with metabolic risk
Obstructive sleep apnea	IH‡‡ drives inflammation and fibrosis	Associated with more severe steatosis and fibrosis
Hypothyroidism	Alters lipid metabolism and energy homeostasis	May worsen hepatic steatosis and metabolic profile

*Metabolic dysfunction–associated steatotic liver disease; †Metabolic dysfunction–associated steatohepatitis; ‡Hepatocellular carcinoma; §Cardiovascular; ‖Metabolic dysfunction-associated alcohol-related liver disease; ¶Blood pressure; **Insulin resistance; ††PCOS – Polycystic ovarian syndrome; ‡‡Intermittent hypoxia.

obesity. Conversely, individuals with metabolic syndrome or T2D should be proactively assessed for MASLD and for the presence of advanced liver fibrosis using validated non-invasive tools, as outlined in subsequent diagnostic chapters (1, 9).

An integrated, multidisciplinary approach—linking primary care physicians, endocrinologists, cardiologists, and hepatologists—is essential. Optimal control of cardiometabolic comorbidities not only reduces overall mortality but also represents a fundamental component of effective liver disease management.

Management of cardiometabolic comorbidities should be initiated according to standard clinical indications irrespective of fibrosis stage, as cardiovascular disease remains the leading cause of mortality in patients with MASLD. Treatment decisions should be guided by established cardiovascular, metabolic, and endocrine indications (1).

Diagnosis and Differential Diagnosis of MASLD

The diagnosis of MASLD requires objective evidence of hepatic steatosis in the presence of metabolic risk factors, following the exclusion of alternative causes of liver disease, particularly significant alcohol consumption. A structured, stepwise diagnostic approach is recommended to ensure diagnostic accuracy, appropriate risk stratification, and rational use of non-invasive and invasive diagnostic tools (1, 2, 18-20).

Diagnostic Criteria for MASLD

MASLD is diagnosed when all of the following conditions are fulfilled:

- Evidence of hepatic steatosis (by imaging, validated serum-based steatosis scores and composite non-invasive assessment tools, or histology).
- Presence of at least one cardiometabolic risk factor (overweight/obesity, insulin resistance and its clinical manifestations, particularly type 2 diabetes mellitus, dyslipidemia, or arterial hypertension).
- Absence of other primary causes of steatotic or chronic liver disease.

Initial Clinical Evaluation

Medical History and Physical Examination

A comprehensive medical history should focus on:
- Quantification of alcohol intake; alcohol consumption exceeding accepted thresholds should prompt consideration of alcohol-related liver disease or mixed etiologies (1, 2).
- Presence and duration of metabolic comorbidities (type 2 diabetes, obesity, and metabolic syndrome).
- History of medication use, including drugs associated with steatosis or steatohepatitis (e.g. amiodarone, methotrexate, tamoxifen).
- Family history of liver disease, diabetes, or hereditary metabolic disorders. Physical examination should assess signs of insulin resistance, obesity distribution, and features of chronic liver disease.

Laboratory Assessment

Baseline laboratory evaluation should include:
- Liver enzymes: ALT, AST, gamma-glutamyl transferase (GGT), and alkaline phosphatase.
- Platelet count, as a surrogate marker of portal hypertension and advanced fibrosis.
- Metabolic profile: fasting glucose or HbA1c, lipid profile.
- Additional tests as indicated: ferritin, transferrin saturation, autoimmune markers, viral hepatitis serology.

Importantly, normal aminotransferase levels do not exclude MASLD, MASH, or advanced fibrosis, and liver enzyme levels correlate poorly with disease severity (18, 21-23).

Imaging for Detection of Hepatic Steatosis

Ultrasound

Abdominal ultrasound is recommended as the first-line imaging modality for detecting hepatic steatosis due to its wide availability, safety, and cost-effectiveness, particularly in primary and secondary care settings in BiH. Conventional ultrasonography demonstrates sensitivity of approximately 85% and specificity of approximately 94% for moderate-to-severe hepatic steatosis but performs less reliably in mild steatosis and severe obesity (1, 22, 24).

Advanced Imaging

Magnetic resonance imaging–proton density fat fraction (MRI-PDFF) and magnetic resonance elastography (MRE) should be reserved for specialized centers and selected cases where non-invasive tests are inconclusive or when advanced disease is suspected. MRI-PDFF is currently regarded as the most accurate non-invasive modality for quantifying liver fat, while magnetic resonance elastography demonstrates excellent diagnostic performance for advanced fibrosis (AUROC frequently >0.90). Their use is largely limited by availability and cost (25, 26).

Elastography

In patients with indeterminate or high-risk serum fibrosis scores, vibration-controlled transient elastography (VCTE) remains the preferred second-line test because of its extensive validation for the assessment of significant and advanced fibrosis. However, two-dimensional shear wave elastography (2D-SWE) demonstrates comparable diagnostic performance and may be used as an alternative where appropriate expertise and equipment are available (2, 27-29). Elastography also provides prognostic information and supports longitudinal disease monitoring. In patients with MASLD, liver stiffness measurement obtained by VCTE can be used for fibrosis risk stratification. Although cut-off values vary among studies and according to the clinical context, liver stiffness values below approximately 8 kPa generally indicate a low probability of advanced fibrosis, values between 8 and 12 kPa suggest an increased likelihood of significant or advanced fibrosis, whereas values above 12 kPa are associated with a high probability of advanced fibrosis or cirrhosis and warrant specialist evaluation (1, 22).

Non-Invasive Assessment of Liver Fibrosis

Serum-Based Fibrosis Scores

Risk stratification for advanced fibrosis should be performed using validated non-invasive scores, including:

- FIB-4 index.
- NAFLD Fibrosis Score (NFS).
- Enhanced Liver Fibrosis (ELF) test, where available.

These tools are recommended as first-step triage tests to identify patients at low risk of advanced fibrosis and to reduce unnecessary referrals (2, 22, 23, 27, 28).

The diagnostic performance of commonly used non-invasive fibrosis assessment tools varies according to the fibrosis stage being evaluated, selected cut-off values, and study population characteristics. Nevertheless, these tools have been extensively validated and are recommended by major international guidelines as part of a stepwise approach to fibrosis risk stratification. Table 2 summarizes the overall diagnostic performance of the most frequently used non-invasive tests in patients with MASLD (1, 22, 26-33).

Among currently available non-invasive modalities, simple serum-based scores such as FIB-4 and NFS are primarily useful for excluding advanced fibrosis in low-risk populations and represent appropriate first-line tools in primary care. Elastography-based methods, particularly VCTE and magnetic resonance elastography, provide greater diagnostic accuracy for fibrosis staging and are generally used in secondary and tertiary healthcare settings. These complementary characteristics support the stepwise diagnostic algorithm proposed in this guideline, balancing diagnostic accuracy with resource availability and cost-effectiveness (1, 2, 22).

Role of Liver Biopsy in MASLD

Liver biopsy is not routinely indicated for the diagnosis of MASLD but may be considered in selected patients. It should be considered when non-invasive tests provide discordant results, when alternative liver diseases cannot be excluded, or when histological confirmation of MASH may influence eligibility for disease-specific pharmacological therapy (1).

Differential Diagnosis of MASLD

A thorough differential diagnosis is mandatory before establishing MASLD, as hepatic steatosis may occur in several other liver diseases. Conditions that must be excluded include:

- Alcohol-related liver disease (ALD) and metabolic dysfunction-associated alcohol-related liver disease (MetALD)
- Chronic viral hepatitis B and C
- Autoimmune hepatitis
- Cholestatic liver diseases (primary biliary cholangitis, primary sclerosing cholangitis)
- Wilson disease
- Hereditary hemochromatosis
- Drug-induced liver injury
- Other genetic or metabolic liver disorders

Table 2. Diagnostic Performance of Commonly Used Non-invasive Tests for Liver Fibrosis in MASLD*

Test	Primary Clinical Role	Approximate AUROC† for Advanced Fibrosis (F≥3)
FIB-4 Index‡	First-line risk stratification/exclusion of advanced fibrosis	0.76–0.84
NFS§	First-line risk stratification/exclusion of advanced fibrosis	0.72–0.82
VCTE/FibroScan‖	Secondary assessment of fibrosis severity	0.85–0.90
MRE¶	Advanced fibrosis assessment	0.90–0.95
FAST Score**	Identification of patients with at-risk MASH††	0.80–0.85

*Metabolic dysfunction–associated steatotic liver disease; †Area under the receiver operating characteristic curve; ‡Fibrosis-4 index; §Non-alcoholic fatty liver disease fibrosis score; ‖Vibration-controlled transient elastography; ¶Magnetic resonance elastography; **FibroScan-AST score; ††Metabolic dysfunction-associated steatohepatitis. *AUROC values are approximate ranges derived from representative validation studies and may vary according to fibrosis stage, cut-off values, and study population.*

Appropriate serological, biochemical, imaging, and genetic testing should be performed based on clinical suspicion and patient phenotype (1, 18, 21).

Risk Stratification and Follow-Up

Risk stratification is a cornerstone of MASLD management, as the stage of liver fibrosis represents the strongest predictor of liver-related outcomes and overall mortality. Accordingly, a pragmatic, non-invasive, stepwise risk stratification algorithm is recommended, particularly in the primary care setting, to identify patients who require specialist referral and structured follow-up (1, 2, 25).

Risk Stratification in Primary Care

Step 1: Serum-Based Fibrosis Assessment (FIB-4)

The fibrosis-4 (FIB-4) index should be used as the initial screening tool in patients with MASLD (2, 23-26, 29).

- A FIB-4 value <1.3 reliably excludes advanced fibrosis in individuals younger than 65 years, whereas a threshold <2.0 should be used in patients aged ≥65 years.
- FIB-4 values between 1.3 and 2.67 (<65 years) should be considered indeterminate and require further evaluation.
- FIB-4 values >2.67 indicate a high probability of advanced fibrosis and warrant specialist assessment.

Step 2: Elastography

Patients with indeterminate or high-risk FIB-4 values should undergo liver stiffness measurement using validated elastography techniques, such as vibration-controlled transient elastography (VCTE) or two-dimensional shear wave elastography (2D-SWE) (2, 25, 29-34).

Management of Advanced Fibrosis (F3–F4)

Patients with suspected advanced fibrosis based on non-invasive testing (e.g., FIB-4 >2.67 and/or liver stiffness values compatible with F3-F4 fibrosis) and those with confirmed advanced fibrosis or cirrhosis should be referred to specialized hepatology care (1, 35).

Rationale for the Stepwise Approach

This non-invasive, stepwise strategy:

- Enables early identification of patients at highest risk for liver-related complications,
- Reduces unnecessary liver biopsies,
- Optimizes the use of healthcare resources,
- Strengthens the role of primary care in long-term MASLD management.

Prevention and Treatment of MASLD

Prevention and management of MASLD require early identification of individuals at risk and a comprehensive, long-term strategy targeting metabolic dysfunction, lifestyle factors, and liver-specific disease progression (1, 2, 18). Since cardiovascular disease represents the leading cause of mortality in patients with MASLD, preventive and therapeutic approaches must prioritize both hepatic and systemic cardiometabolic outcomes (36, 37). Public health strategies in Bosnia and Herzegovina should emphasize population-level interventions addressing obesity, type 2 diabetes mellitus (T2D), unhealthy dietary patterns, physical inactivity, and smoking, supported by structured patient education and access to nutritional counseling (2, 38). Key strategies for MASLD prevention and treatment are shown in Table 3.

Non-pharmacological Treatment (Lifestyle Intervention)

Lifestyle modification is the cornerstone of MASLD management and should be recommended to all patients, regardless of disease severity or concomitant pharmacological treatment (1, 2, 18).

Weight reduction

- A ≥5% reduction in body weight is associated with a significant decrease in hepatic steatosis.

Table 3. Strategies for the Prevention and Treatment of MASLD*

Category	Specific Measure/Intervention		Key Goals/Effects	Key Message
Core principles	Early identification and long-term strategy		Targeting metabolic dysfunction, liver disease progression, and CV† risk	Multidisciplinary approach
Public health strategies	Population-level interventions		Addressing obesity, T2D‡, unhealthy diet, physical inactivity, and smoking	Education and structured prevention programs
Non-pharmacological treatment	Cornerstone for all patients (Lifestyle intervention)	Weight reduction	≥5%: Reduces hepatic steatosis ≥7–10%; Improves steatohepatitis ≥10%; May induce fibrosis regression	Individualized approach is essential
		Dietary measures	Mediterranean hypocaloric style diet improves metabolic control	Reduce free sugars and ultra-processed foods
		Physical activity	Aerobic and resistance exercise improve liver enzymes, reduce hepatic fat, and boost insulin sensitivity	Regular activity is recommended
		Additional factors	Moderate coffee consumption and smoking cessation	Improve metabolic control
Management of comorbidities	Control of T2D‡ hypertension, dyslipidemia, and obesity		Reduces overall CV† risk and indirectly slows liver disease progression	Statins should be prescribed for CV† indications
MASLD*-directed pharmacotherapy	Patients with fibrosis (≥F2) or high risk of progression		Always complements, does not replace, lifestyle modification.	It is used when lifestyle interventions fail
Drugs for comorbidities (not MASLD*-specific)	GLP-1 Receptor Agonists§ (e.g., semaglutide)		Weight loss; steatosis/MASH‖ improvement	Not approved as MASLD*-specific therapy, used for T2D‡
	Pioglitazone		Improves MASH‖ histology, especially in T2D‡. Only for biopsy-proven patients	Limited by side effects (weight gain, edema)
	SGLT2¶ Inhibitors		May reduce hepatic fat. Safe in T2D‡	Not approved as specific MASLD*/MASH‖ therapy
	Metformin		Improves insulin sensitivity	No benefit in liver histology
Agents available for MASLD* in BiH	Ursodeoxycholic acid		Improves liver enzymes and inflammatory markers	Useful in early stages (F0-F2) with elevated enzymes
	Vitamin D		Correction of deficiency may improve metabolic and inflammatory profiles	Safe, inexpensive supportive therapy
	Vitamin E (α-tocopherol)		Histological benefit in non-diabetic, non-cirrhotic biopsy-proven MASH‖	Not for routine use due to long-term safety concerns
Emerging and non-available therapies	Resmetirom (THR-β** agonist)		Phase III trials show improvement in steatohepatitis and fibrosis	Currently not available on the market in BA
	Semaglutide 2.4 mg			
	Bile acid-targeting agents (e.g., OCA††)		Antifibrotic effects without proven clinical benefit	Not part of routine MASLD* management
Interventional and surgical approaches	Endoscopic bariatric Therapy		Weight loss (~10–12% at 6 months)	In obese patients refractory to lifestyle modifications
	Bariatric Surgery		Sustained weight loss, improvement of comorbidities and liver histology	For selected obese patients with compensated cirrhosis
	Liver transplantation		Treatment for end-stage cirrhosis and hepatocellular carcinoma	Post-transplant management focuses on metabolic risk control

*Metabolic dysfunction-associated steatotic liver disease; †Cardiovascular; ‡Type 2 Diabetes; §Glucagon-like peptide-1 receptor agonists; ‖Metabolic dysfunction-associated steatohepatitis; ¶Sodium-glucose co-transporter-2 inhibitors; **Thyroid hormone receptor-β ††Obeticholic Acid.

– ≥7–10% weight loss improves steatohepatitis (MASH) and necroinflammatory activity.
– ≥10% weight loss may induce fibrosis regression in selected patients (39-42).

Dietary measures

– Hypocaloric diet individualized according to metabolic needs.
– Preference for Mediterranean-style dietary patterns.
– Reduction of free sugars, fructose-containing beverages, and ultra-processed foods (42-44).

Physical activity

– Regular aerobic and resistance exercise improves liver enzymes, reduces hepatic fat content, and enhances insulin sensitivity, even in the absence of significant weight loss. At least 150–300 minutes of moderate-intensity aerobic activity per week, combined with resistance training 2–3 times weekly, is recommended (45-47).

Additional lifestyle factors

– Moderate coffee consumption is associated with hepatoprotective effects, including reduced fibrosis progression. Moderate coffee consumption generally refers to approximately 2–3 cups daily (48, 49).
– Smoking cessation is strongly recommended due to its adverse metabolic and cardiovascular effects (50, 51).

Integrated Management of Metabolic Comorbidities

Optimal control of metabolic comorbidities is a fundamental component of MASLD management and should be pursued in all patients, regardless of fibrosis stage, as cardiovascular disease is the leading cause of mortality in these patients. However, management of cardiometabolic risk factors should be conceptually distinguished from MASLD-specific pharmacotherapy (1, 18).

– Type 2 diabetes mellitus, hypertension, dyslipidaemia, and obesity should be managed according to established clinical practice guidelines.
– Pharmacological treatment of metabolic comorbidities should not be withheld because of a diagnosis of MASLD and should follow standard cardiovascular, metabolic, and endocrine indications.
– Although these interventions are not considered MASLD-specific therapies, effective cardiometabolic risk reduction may slow liver disease progression and reduces cardiovascular and overall mortality (52).

Pharmacological Therapy for MASLD in Bosnia and Herzegovina

Pharmacological therapy should be considered only in patients with confirmed MASLD and significant fibrosis (≥F2) or in those at high risk of disease progression (age >50 years, T2D, metabolic syndrome, persistently elevated ALT), particularly when structured lifestyle interventions fail (1, 2, 18). Pharmacological treatment must always complement, not replace, lifestyle modification and optimal management of metabolic comorbidities.

Drugs Used for Comorbidities (Not MASLD-Specific Therapy)

Several medications commonly prescribed in patients with MASLD provide metabolic and indirect hepatic benefits. However, they are not currently considered MASLD-specific disease-modifying therapies and should be prescribed according to their approved indications:

– ***Statins***
Statins are safe across the MASLD spectrum, including in patients with compensated cirrhosis, and should not be withheld when indicated for cardiovascular risk reduction. They do not improve steatosis, steatohepatitis, or fibrosis sufficiently to justify a MASLD-specific indication. Although observational studies suggest a potential reduction in progression to advanced fibrosis, cirrhosis, and liver-related

complications, current evidence remains insufficient to recommend statins specifically for the treatment of MASLD (53-55).

- ***Metformin***
  Metformin improves insulin sensitivity and glycaemic control and should be used in patients with type 2 diabetes mellitus according to standard diabetes care. However, it has not demonstrated meaningful histological benefit in MASLD and should not be used specifically for the treatment of MASLD (56, 57).
- ***GLP-1 receptor agonists (e.g., semaglutide, liraglutide)***
  GLP-1 receptor agonists used for the treatment of type 2 diabetes mellitus (e.g., semaglutide up to 1.0 mg, liraglutide) promote weight loss and improve glycaemic and cardiometabolic control. Although clinical trials have shown reductions in hepatic steatosis and increased rates of MASH resolution, consistent antifibrotic effects have not been established. Accordingly, they are not currently recommended as MASLD-specific pharmacotherapy. Higher-dose semaglutide (2.4 mg) used for obesity treatment is discussed separately (58-60).
- ***Sodium-glucose co-transporter-2 inhibitors (SGLT2 inhibitors)***
  SGLT2 inhibitors are safe in patients with MASLD and type 2 diabetes mellitus and may reduce hepatic fat content while providing cardiovascular and renal benefits. They should be prescribed according to diabetes indications but are not approved as MASLD/MASH-specific therapy (61, 62).
- ***Pioglitazone***
  Pioglitazone has demonstrated histological benefits in patients with MASH, particularly those with type 2 diabetes mellitus. However, its use is limited by adverse effects, including weight gain, fluid retention, and increased risk of heart failure. Pioglitazone may be considered in carefully selected patients with biopsy-confirmed MASH but is not recommended for routine treatment of MASLD (63).

Pharmacological Agents Available for MASLD Management in BA

At present, pharmacological options available for use in MASLD management in BA are limited. Three agents are available and may be used in selected patients as part of an integrated, individualized treatment strategy: ursodeoxycholic acid (UDCA), vitamin D, and vitamin E. Importantly, none of these agents can be considered disease-modifying therapy for MASLD, and their use should always complement lifestyle intervention and optimal management of metabolic comorbidities.

Ursodeoxycholic Acid (UDCA)

Ursodeoxycholic acid (UDCA) is a naturally occurring, hydrophilic bile acid with well-established anti-inflammatory, immunomodulatory, antioxidant, and anti-apoptotic properties. The biological effects of UDCA may be more relevant during earlier stages of MASLD, when hepatocellular injury and inflammatory activity predominate, although convincing evidence of disease modification remains lacking (63-65). Multiple randomized controlled trials and meta-analyses have consistently demonstrated that standard-dose UDCA (10–15 mg/kg/day) leads to significant reductions in serum aminotransferases (ALT and AST) within 1–6 months, while maintaining an excellent safety and tolerability profile (65-70). In addition, UDCA therapy is associated with a reduction in gamma-glutamyl transferase (GGT) levels in patients with MASLD (66, 67), a finding of clinical relevance given that GGT is an independent predictor of liver-related, cardiovascular, and all-cause mortality (71, 72).

Beyond liver enzyme normalization, UDCA may exert favorable effects on metabolic and cardiovascular risk factors, including total and LDL cholesterol and glycemic parameters—benefits not consistently achieved with lifestyle intervention alone. These pleiotropic effects may improve the overall cardiometabolic risk profile and facilitate adherence during the early phase of lifestyle

modification, thereby reinforcing the rationale for UDCA use in carefully selected patients with active biochemical disease (68, 69, 73, 74). Despite these consistent biochemical and metabolic improvements, evidence supporting a clinically meaningful antifibrotic effect of UDCA remains inconsistent and largely inconclusive (67, 68, 70). Consequently, the therapeutic benefit of UDCA appears to be limited to early disease stages, with minimal or no efficacy in patients with advanced fibrosis or established MASH, where structural liver damage predominates.

Clinical position: Current evidence does not support UDCA as a disease-modifying therapy for MASLD or MASH, as consistent histological improvement and antifibrotic effects have not been demonstrated. However, owing to its favourable safety profile and consistent biochemical effects, UDCA may be considered as an adjunct to lifestyle-based management in selected patients, particularly when improvement of liver biochemical parameters is a therapeutic goal (F0-F2).

Vitamin E

Vitamin E (α-tocopherol, 800 IU/day) has demonstrated histological benefit in carefully selected patients with biopsy-proven steatohepatitis who do not have diabetes or cirrhosis (75). Despite these findings, its use in routine clinical practice remains limited due to unresolved concerns regarding long-term safety, including potential associations with prostate cancer, hemorrhagic stroke, and increased all-cause mortality (76). Consequently, vitamin E should not be recommended for routine treatment of MASLD, but may be considered on an individual basis in highly selected, non-diabetic, non-cirrhotic patients after careful assessment of potential benefits and risks. In this context, combination therapy with UDCA and antioxidant supplementation has been shown to be generally well tolerated and associated with sustained improvements in liver biochemistry; however, evidence from comparative studies indicates that while vitamin-based antioxidant therapy may improve fibrosis, it does not significantly reduce GGT levels or inflammatory activity, and overall data remain insufficient to support routine use, restricting such combination approaches to selected patients under specialist supervision (77-79).

Vitamin D

Vitamin D deficiency is highly prevalent among patients with MASLD and is associated with insulin resistance, low-grade inflammation, and fibrosis progression. Vitamin D supplementation is safe, inexpensive, and widely accessible in Bosnia and Herzegovina (80). Correction of vitamin D deficiency should be considered part of general metabolic health management rather than MASLD-specific treatment. Although vitamin D cannot be regarded as MASLD-specific therapy, correction of documented deficiency may improve metabolic and inflammatory profiles and should be considered supportive treatment, particularly in patients with coexisting metabolic dysfunction (81).

Emerging, Experimental, and Currently Non-Available Therapies

Several emerging pharmacological therapies for MASLD/MASH show promising mechanistic and histological effects but remain investigational or unavailable for routine clinical use.

– Resmetirom, a selective thyroid hormone receptor-β (THR-β) agonist, has demonstrated significant histological efficacy in patients with non-cirrhotic MASLD/MASH and moderate to advanced fibrosis (predominantly stages F2–F3) in phase III registration trials conducted over a 52-week treatment period. Compared with placebo, resmetirom achieved higher rates of both steatohepatitis resolution and fibrosis improvement, accompanied by consistent reductions in liver enzymes and atherogenic lipid parameters. Adverse events were predominantly mild to moderate and mainly gastrointestinal in nature. On the basis of these findings, derived from a manufacturer-sponsored phase III registration trial, resmetirom received accelerated

(conditional) regulatory approval by the U.S. Food and Drug Administration (FDA) in March 2024, with a weight-based dosing regimen of 80–100 mg/day (82). Current evidence suggests that the most appropriate candidates for treatment are carefully selected non-cirrhotic patients with MASH and fibrosis stages *F2–F3*. Nevertheless, despite these encouraging results, the drug is not currently available on the local market, and important uncertainties remain regarding its long-term impact on hard clinical outcomes, optimal treatment duration, durability of histological response, and reliable predictors of treatment benefit. Accordingly, the definitive clinical positioning of resmetirom in MASLD management has yet to be fully established, and routine incorporation into national treatment pathways cannot currently be recommended (83-86).

- Bile acid–targeting strategies (FXR-, TGR5-, and FGF19-mediated pathways) represent a biologically plausible therapeutic direction, as bile acids act as central regulators of lipid and glucose metabolism, inflammation, fibrogenesis, and the gut–liver axis. Modulation of bile acid signaling and microbiota composition may provide indirect metabolic benefits, particularly when integrated into multimodal treatment approaches (87-90).
- Among bile acid-based agents, obeticholic acid (OCA) demonstrated antifibrotic and biochemical effects in clinical trials. However, concerns regarding safety, particularly pruritus and adverse lipid profile changes, together with the absence of demonstrated clinical outcome benefits, have limited its regulatory acceptance and routine clinical use. Consequently, OCA is not currently considered part of standard MASLD management and is mentioned here only in the context of bile acid-targeted therapeutic development (1, 91, 92). Tauroursodeoxycholic acid and other experimental bile acid derivatives (e.g. norUDCA, cilofexor, tropifexor, INT-767) remain in early or mid-stage development, with insufficient evidence to support routine clinical use (93-98).
- Recent phase III data from the ESSENCE trial have shown that once-weekly semaglutide 2.4 mg produces robust improvements in liver histology in adults with MASH and moderate to advanced fibrosis, demonstrating both higher rates of MASH resolution and clinically meaningful fibrosis improvement compared with placebo at 72 weeks. These results supported accelerated (conditional) approval by the FDA of Wegovy® (semaglutide 2.4 mg) for the treatment of non-cirrhotic MASH with fibrosis stages F2–F3 in adults, in combination with lifestyle interventions, representing the first GLP-1 receptor agonist to receive a liver-specific indication in this population (99). Current evidence suggests that the most appropriate candidates for treatment are carefully selected adults with non-cirrhotic MASH and fibrosis stages F2–F3, particularly in the presence of overweight, obesity, or other cardiometabolic comorbidities. Nevertheless, confirmatory long-term outcome data and broader regulatory approvals (EMA: CHMP positive opinion; European Commission decision pending) are still awaited, and its definitive role within national treatment pathways remains to be established (100-102). Dual and triple incretin receptor agonists, including agents such as tirzepatide and survodutide, remain investigational in MASLD/MASH and cannot currently be recommended outside clinical trials, as no liver-specific indication has yet been formally approved (103-105).

Interventional and Surgical Approaches

Endoscopic Therapy

Endoscopic bariatric interventions may be considered in selected patients with obesity who do not achieve sufficient weight loss with lifestyle modification alone. Among these, intragastric balloon placement has been associated with short-term weight reduction and improvements in metabolic parameters, including hepatic steatosis in some studies. However, the available evidence

in MASLD is limited, and the durability of these benefits after balloon removal remains uncertain. These procedures should be avoided in patients with esophageal or gastric varices or clinically significant portal hypertension. Available evidence from small studies suggests an average weight reduction of approximately 10–12% at 6 months following treatment (106, 107). In carefully selected patients with obesity and MASLD-associated compensated cirrhosis, bariatric surgery may be considered following thorough multidisciplinary assessment. Indications generally include a body mass index (BMI) ≥40 kg/m², ≥35–40 kg/m² in the presence of obesity-related comorbidities, or ≥30–35 kg/m² in patients with poorly controlled type 2 diabetes and/or arterial hypertension, provided there are no features of decompensated liver disease. Emerging evidence suggests that bariatric surgery may result in fibrosis regression and even partial reversal of compensated cirrhosis in selected patients, although careful patient selection remains essential (107, 108).

Liver Transplantation

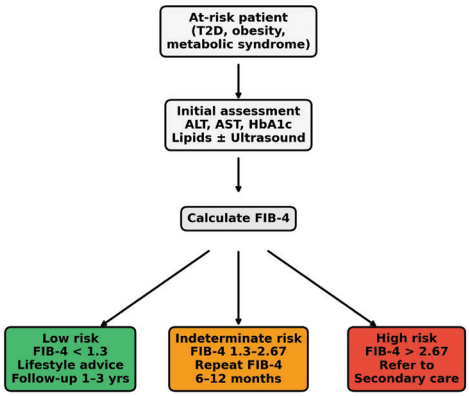
MASLD-related cirrhosis and hepatocellular carcinoma (HCC) are rapidly emerging as leading indications for liver transplantation worldwide. In appropriately selected patients, liver transplantation provides a significant survival benefit, with post-transplant outcomes comparable to those of non-MASLD recipients (109, 110). Although recurrence of metabolic liver disease after transplantation is common, disease progression is typically slow and rarely compromises graft survival. Timely referral to a transplant center is essential, particularly for patients with decompensated cirrhosis or those developing HCC within accepted transplant criteria. Given the high prevalence of metabolic comorbidities, sarcopenia, and frailty in MASLD/MASH, routine assessment during transplant evaluation is recommended, as early identification and targeted interventions may improve peri- and post-transplant outcomes (111). Surveillance for HCC plays a critical role in maintaining transplant eligibility. In patients with MASLD-related cirrhosis, abdominal ultrasound combined with alpha-fetoprotein (AFP) measurement every 6 months is recommended (104). Cross-sectional imaging (CT or MRI) should be reserved for further characterization of lesions detected on ultrasound. Treatment strategy and liver transplant eligibility are determined according to established staging systems and transplant criteria, including the Barcelona Clinic Liver Cancer (BCLC) classification and Milan criteria (112).

Role of Health Care System Levels in the Management of MASLD

Effective management of MASLD requires an integrated, stepwise approach across primary, secondary, and tertiary levels of healthcare. Given the high prevalence of MASLD, its close association with cardiometabolic disorders, and its frequently asymptomatic course, primary healthcare plays a central gatekeeping and coordinating role within the national MASLD care pathway (1, 2).

Primary healthcare is pivotal for early identification of individuals at risk, initiation of first-line non-invasive fibrosis risk stratification using simple tools such as the FIB-4 index, implementation of lifestyle and behavioral interventions, and long-term follow-up of patients at low risk of advanced fibrosis (1, 2, 22). In addition, primary care ensures optimal management of metabolic comorbidities and coordinates timely referral to specialist care for patients with indeterminate or high-risk non-invasive test results (1, 2, 36). The central role of primary healthcare as the first point of contact for MASLD detection and risk stratification is summarized in Figure 1.

Secondary healthcare provides diagnostic refinement and specialist management for patients referred from primary care. Its core responsibilities include confirmation of disease stage using advanced non-invasive methods, particularly vibration-controlled transient elastography, identification of patients with significant or progressive fibrosis, and supervision of pharmacological therapies where indicated, in close collaboration with primary healthcare (1, 2, 22, 31).



At-risk individuals (obesity, type 2 diabetes, or metabolic syndrome) are evaluated in primary care using routine laboratory tests, with or without liver ultrasound, followed by first-line fibrosis risk stratification using the fibrosis-4 (FIB-4) index. Patients at low risk (FIB-4 <1.3; green) are managed with lifestyle counselling and cardiometabolic risk control, with periodic reassessment, whereas those with indeterminate risk (FIB-4 1.3–2.67; amber) require closer follow-up. Patients at high risk (FIB-4 >2.67; red) are referred for specialist evaluation.

Figure 1. Primary healthcare algorithm for MASLD management.

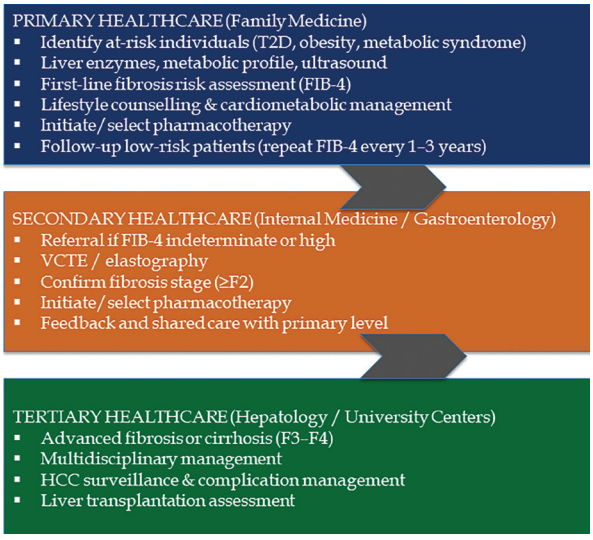


Figure 2. Organization of MASLD care across primary, secondary, and tertiary healthcare levels in BA.

Tertiary healthcare is reserved for patients with advanced fibrosis, cirrhosis, or MASLD-related complications and is responsible for comprehensive multidisciplinary management, surveillance for hepatocellular carcinoma, use of advanced diagnostic modalities when required, and assessment for liver transplantation where appropriate (1, 8, 35). Strengthening primary healthcare as the foundation of MASLD management is essential to improve early detection of advanced fibrosis, optimize healthcare resource utilization, and reduce liver-related and cardiovascular morbidity and mortality in BiH (3, 4, 36). The organization and coordination of MASLD management across primary, secondary, and tertiary healthcare levels in BiH are summarized in Figure 2.

Referral from primary to secondary care is recommended in patients with FIB-4 values above the established low-risk threshold, indeterminate non-invasive test results, persistently elevated liver enzymes, or suspicion of advanced fibrosis. Referral to tertiary care is recommended in patients with confirmed advanced fibrosis, cirrhosis, portal hypertension, hepatocellular carcinoma, or when consideration of advanced pharmacological, bariatric, or transplant-related interventions is required.

The role of Public Health and Prevention in the Management of MASLD

1. Education and support in primary health care:
   - Developing patient education programs to increase awareness of MASLD, risk factors and the importance of early disease recognition.
   - Incorporating strategies to encourage lifestyle changes through educational workshops, support groups and self-help materials.
2. Formalized care pathways:
   - Defining clear criteria and thresholds for the referral process between primary, secondary and tertiary health care.
   - Creating multidisciplinary teams that will collaborate at different levels and ensure an integrated approach to patients with MASLD. Primary care should serve not only as the entry point for diagnosis and risk stratification but also as the central setting for structured lifestyle intervention. Educational programmes, dietary counselling, physical activity promotion, and

behavioural support strategies should be integrated whenever feasible to facilitate long-term adherence to lifestyle modification. Given the high prevalence of obesity, type 2 diabetes, hypertension, and sedentary lifestyle patterns in Bosnia and Herzegovina, population-level preventive strategies are essential. Public health initiatives should focus on healthy nutrition, physical activity promotion, obesity prevention, diabetes screening, and awareness campaigns regarding MASLD and cardiometabolic health.

Key Recommendations

1. **Lifestyle intervention remains the foundation of MASLD management**
   Sustained weight reduction of ≥7–10% achieved through dietary modification and regular physical activity is the cornerstone of MASLD treatment and is associated with improvements in hepatic steatosis, necroinflammation, and, in some patients, fibrosis.
2. **Pharmacological therapy should be reserved for patients at increased risk of progression**
   Drug therapy may be considered in patients with significant fibrosis (F ≥2) or high-risk clinical profiles (age >50 years, type 2 diabetes, metabolic syndrome, persistently elevated aminotransferases, or evidence of active inflammation), particularly when lifestyle interventions alone are insufficient.
3. **Optimal management of cardiometabolic comorbidities is mandatory**
   Strict glycaemic control in type 2 diabetes and aggressive lipid-lowering therapy, including statins, are integral components of MASLD care and are essential for reducing long-term cardiovascular morbidity and mortality.
4. **Statins are safe in MASLD and compensated cirrhosis**
   Although not MASLD-specific therapies, statins should not be withheld when clinically indicated for dyslipidaemia or cardiovascular risk prevention, including in patients with compensated cirrhosis.
5. **Selected agents may be used in carefully defined patient subgroups**
   Pioglitazone may be considered in patients with biopsy-proven MASH, while vitamin E can be used in non-diabetic patients without cirrhosis; both require individualized benefit–risk assessment due to potential adverse effects.
6. **UDCA has a favourable safety profile but limited histological efficacy**
   Ursodeoxycholic acid improves liver enzymes and metabolic parameters and appears most useful in early disease stages (F0–F2), although current evidence does not support a consistent histological benefit.
7. **Metformin, GLP-1 receptor agonists, and SGLT2 inhibitors should be used for metabolic indications**
   These agents are not approved as MASLD-specific therapies but may confer indirect hepatic benefits through weight loss, improved insulin sensitivity, and cardiometabolic risk reduction when prescribed for diabetes or obesity.
8. **Bile acids are key regulators in MASLD pathophysiology**
   Alterations in bile acid composition and signaling via FXR-, TGR5-, and FGF19-mediated pathways play a central role in lipid metabolism, glucose homeostasis, inflammation, and fibrosis progression, and correlate with disease severity.
9. **Bile acid–targeting therapies represent an important future direction**
   Pharmacological modulation of bile acid signaling (e.g., FXR agonists, TGR5 agonists, FGF19 analogues, nor-UDCA) shows promise for improving steatosis, inflammation, and fibrosis, but current evidence remains insufficient for routine clinical use.
10. **Advanced and obesity-directed interventions should be individualized and centralized**
    Resmetirom may be considered in non-cirrhotic patients with F ≥2 fibrosis, while endoscopic

bariatric procedures, bariatric surgery, and liver transplantation are reserved for selected patients and should be managed within secondary and tertiary healthcare settings through a multidisciplinary approach.

Evidence Gaps and Research Priorities

Despite the growing burden of MASLD, significant evidence gaps remain in BiH. Currently, no population-based epidemiological studies are available to accurately define the prevalence, incidence, fibrosis burden, or long-term outcomes of MASLD in the country. Consequently, most recommendations presented in this guideline are based on international evidence adapted to the local healthcare setting. Future research efforts should prioritize the establishment of national epidemiological data, including studies assessing disease prevalence in the general population and in high-risk groups such as individuals with obesity, type 2 diabetes, and metabolic syndrome. Additional priorities include evaluation of the diagnostic performance and cost-effectiveness of non-invasive fibrosis assessment strategies in routine clinical practice, assessment of referral pathways across different levels of healthcare, and characterization of liver-related and cardiovascular outcomes among patients with MASLD.

In addition to epidemiological and clinical research, implementation research should be encouraged to evaluate the effectiveness of integrated care pathways across primary, secondary, and tertiary healthcare, the feasibility of structured lifestyle intervention programmes, and the impact of public health strategies aimed at improving awareness, early detection, and prevention of MASLD. Such studies would support optimization of healthcare delivery and facilitate future refinement of national recommendations. The development of national registries and multicentre collaborative studies would provide valuable information regarding disease burden, healthcare resource utilization, and treatment outcomes. Such data are essential for future updates of these guidelines and for informing healthcare policy, resource allocation, and public health strategies aimed at reducing the growing impact of MASLD in BA.

Conclusion

MASLD is one of the most prevalent chronic liver diseases and represents a major public health challenge in Bosnia and Herzegovina. MASLD should not be regarded as an isolated liver condition but rather as the hepatic manifestation of systemic metabolic dysfunction, closely linked to type 2 diabetes, obesity, dyslipidemia, and cardiovascular disease, which together determine overall morbidity and mortality.

The stage of liver fibrosis is the strongest predictor of liver-related outcomes in MASLD. Consequently, early identification of patients at risk, appropriate risk stratification, and timely referral of individuals with advanced fibrosis are central elements of effective disease management. The use of validated non-invasive tests—particularly the FIB-4 index as a first-line tool in primary care, followed by selective application of elastography—enables accurate, safe, and resource-efficient assessment while minimizing the need for invasive diagnostic procedures.

Primary care plays a pivotal role in the management of MASLD, including early detection, long-term follow-up of low-risk patients, and optimization of cardiometabolic comorbidities. Clearly defined referral pathways allow timely specialist involvement for patients with suspected advanced fibrosis or cirrhosis, ensuring appropriate surveillance for hepatocellular carcinoma and management of liver-related complications.

Currently, no pharmacological therapy is approved exclusively for MASLD; therefore, lifestyle modification, sustained weight reduction, and optimal treatment of associated metabolic disorders remain the cornerstone of therapy. An integrated, multidisciplinary approach involving family physicians, endocrinologists, cardiologists, and hepatologists is essential to improve clinical outcomes and reduce the long-term burden of MASLD on the healthcare system. The implementation of this guideline aims to standardize clinical practice

across BiH, promote early diagnosis, support rational use of healthcare resources, and ultimately reduce MASLD-related complications while improving quality of life and long-term outcomes for affected patients.

What Is Already Known on This Topic

Metabolic dysfunction–associated steatotic liver disease (MASLD) is the most common chronic liver disease globally and a major driver of liver- and cardiovascular-related morbidity and mortality. Disease severity and long-term outcomes are primarily determined by the stage of liver fibrosis. International guidelines emphasize lifestyle modification, management of cardiometabolic comorbidities, non-invasive fibrosis risk stratification, and surveillance for hepatocellular carcinoma in advanced disease. However, translating these recommendations into routine clinical practice remains challenging, particularly in healthcare systems with limited resources and fragmented care pathways. Clear delineation of roles across primary, secondary, and tertiary healthcare levels, as well as adaptation of international recommendations to local healthcare structures, is often lacking.

What This Study Adds

These national clinical practice guidelines provide the first country-specific, consensus-based recommendations for the diagnosis, risk stratification, and management of MASLD in Bosnia and Herzegovina. The document adapts international evidence to local healthcare structures and resource availability, emphasizing a stepwise, non-invasive diagnostic approach and the central coordinating role of primary healthcare. By defining clear referral pathways and integrating lifestyle, pharmacological, interventional, and transplant-related strategies, the guidelines aim to standardize MASLD care, improve early detection of advanced fibrosis, and support practical implementation of evidence-based management across all levels of care.

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References

1. European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines on the management of metabolic dysfunction-associated steatotic liver disease (MASLD). J Hepatol. 2024;81(3):492-542. doi: 10.1016/j.jhep.2024.04.031. PMID: 38851997.
2. Rinella ME, Neuschwander-Tetri BA, Siddiqui MS, Abdelmalek MF, Caldwell S, Barb D, et al. AASLD Practice Guidance on the clinical assessment and management of nonalcoholic fatty liver disease. Hepatology. 2023;77(5):1797-835. doi: 10.1097/HEP.0000000000000323. PMID: 36727674.
3. Younossi ZM, Golabi P, Paik JM, Henry A, Van Dongen C, Henry L. The global epidemiology of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH): a systematic review. Hepatology. 2023;77(4):1335-47. doi: 10.1097/HEP.0000000000000004. PMID: 36626630.
4. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64(1):73-84. doi: 10.1002/hep.28431. PMID: 26707365.
5. Estes C, Anstee QM, Arias-Loste MT, Bantel H, Bellentani S, Caballeria J, et al. Modeling NAFLD disease burden in China, France, Germany, Italy, Japan, Spain, United Kingdom, and United States for the period 2016-2030. J Hepatol. 2018;69(4):896-904. doi: 10.1016/j.jhep.2018.05.036. PMID: 29886156.
6. Mantovani A, Zaza G, Byrne CD, Lonardo A, Zoppini G, Bonora E, et al. Nonalcoholic fatty liver disease increases risk of incident chronic kidney disease: A systematic review and meta-analysis. Metabolism. 2018;79:64-76. doi: 10.1016/j.metabol.2017.11.003. PMID: 29137912.
7. Dulai PS, Singh S, Patel J, Soni M, Prokop LJ, Younossi Z, et al. Increased risk of mortality by fibrosis stage in nonalcoholic fatty liver disease: Systematic review and meta-analysis. Hepatology. 2017;65(5):1557-65. doi: 10.1002/hep.29085. PMID: 28130788.
8. Ekstedt M, Hagström H, Nasr P, Fredrikson M, Stål P, Kechagias S, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. Hepatology. 2015;61(5):1547-54. doi: 10.1002/hep.27368. PMID: 25125077.
9. Eslam M, Newsome PN, Sarin SK, Anstee QM, Targher G, Romero-Gomez M, et al. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. J Hepatol. 2020;73(1):202-9. doi: 10.1016/j.jhep.2020.03.039. PMID: 32278004.
10. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nat Med. 2018;24(7):908-22. doi: 10.1038/s41591-018-0104-9. PMID: 29967350.
11. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2008;40(12):1461-5. doi: 10.1038/ng.257. PMID: 18820647.
12. Lonardo A, Nascimbeni F, Ballestri S, Fairweather D, Win S, Than TA, et al. Sex Differences in Nonalcoholic

Fatty Liver Disease: State of the Art and Identification of Research Gaps. Hepatology. 2019;70(4):1457-69. doi: 10.1002/hep.30626. PMID: 30924946.

13. Younossi ZM, Golabi P, de Avila L, Paik JM, Srishord M, Fukui N, et al. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. J Hepatol. 2019;71(4):793-801. doi: 10.1016/j.jhep.2019.06.021. PMID: 31279902.
14. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet. 2016;387(10019):679-90. doi: 10.1016/S0140-6736(15)00803-X. PMID: 26608256.
15. da Silva Pereira ENG, Franco RLC, Santos RDCD, Daliry A. Statins and non-alcoholic fatty liver disease: A concise review. Biomed Pharmacother. 2025;183:117805. doi: 10.1016/j.biopha.2024.117805. PMID: 39755024.
16. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. Eur Heart J. 2018;39(33):3021-104. doi: 10.1093/eurheartj/ehy339. PMID: 30165516.
17. Lassailly G, Caiazzo R, Buob D, Pigeyre M, Verkindt H, Labreuche J, et al. Bariatric Surgery Reduces Features of Nonalcoholic Steatohepatitis in Morbidly Obese Patients. Gastroenterology. 2015;149(2):379-88; quiz e15-6. doi: 10.1053/j.gastro.2015.04.014. PMID: 25917783.
18. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology. 2018;67(1):328-57. doi: 10.1002/hep.29367. PMID: 28714183.
19. Paul S, Davis AM. Diagnosis and Management of Nonalcoholic Fatty Liver Disease. JAMA. 2018;320(23):2474-5. doi: 10.1001/jama.2018.17365. PMID: 30476962.
20. Başaranoğlu M, Örmeci N. Nonalcoholic fatty liver disease: diagnosis, pathogenesis, and management. Turk J Gastroenterol. 2014;25(2):127-32. doi: 10.5152/tjg.2014.7675. PMID: 25003670.
21. Younossi ZM, Golabi P, Price JK, Owrangi S, Gundu-Rao N, Satchi R, et al. The Global Epidemiology of Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis Among Patients With Type 2 Diabetes. Clin Gastroenterol Hepatol. 2024;22(10):1999-2010.e8. doi: 10.1016/j.cgh.2024.03.006. PMID: 38521116.
22. European Association for the Study of the Liver. EASL Clinical Practice Guidelines on non-invasive tests for evaluation of liver disease severity and prognosis - 2021 update. J Hepatol. 2021;75(3):659-89. doi: 10.1016/j.jhep.2021.05.025. PMID: 34166721.
23. van Kleef LA, Sonneveld MJ, de Man RA, de Knegt RJ. Poor performance of FIB-4 in elderly individuals at risk for chronic liver disease - implications for the clinical utility of the EASL NIT guideline. J Hepatol. 2022;76(1):245-6. doi: 10.1016/j.jhep.2021.08.017. PMID: 34461208.
24. Hernaez R, Lazo M, Bonekamp S, Kamel I, Brancati FL, Guallar E, et al. Diagnostic accuracy and reliability of ultrasonography for the detection of fatty liver: a meta-analysis. Hepatology. 2011;54(3):1082-90. doi: 10.1002/hep.24452. PMID: 21618575.
25. Caussy C, Johansson L. Magnetic resonance-based biomarkers in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Endocrinol Diabetes Metab. 2020;3(4):e00134. doi: 10.1002/edm2.134. PMID: 33102797.
26. Singh S, Venkatesh SK, Loomba R, Wang Z, Sirlin C, Chen J, et al. Magnetic resonance elastography for staging liver fibrosis in non-alcoholic fatty liver disease: a diagnostic accuracy systematic review and individual participant data pooled analysis. Eur Radiol. 2016;26(5):1431-40. doi: 10.1007/s00330-015-3949-z. PMID: 26314479.
27. Newsome PN, Sasso M, Deeks JJ, Paredes A, Boursier J, Chan WK, et al. FibroScan-AST (FAST) score for the non-invasive identification of patients with non-alcoholic steatohepatitis with significant activity and fibrosis: a prospective derivation and global validation study. Lancet Gastroenterol Hepatol. 2020;5(4):362-73. doi: 10.1016/S2468-1253(19)30383-8. PMID: 32027858.
28. Ravaioli F, Dajti E, Mantovani A, Newsome PN, Targher G, Colecchia A. Diagnostic accuracy of FibroScan-AST (FAST) score for the non-invasive identification of patients with fibrotic non-alcoholic steatohepatitis: a systematic review and meta-analysis. Gut. 2023;72(7):1399-409. doi: 10.1136/gutjnl-2022-328689. PMID: 36599683.
29. Natarajan Y, Loomba R. Magnetic Resonance Elastography for the Clinical Risk Assessment of Fibrosis, Cirrhosis, and Portal Hypertension in Patients With NAFLD. J Clin Exp Hepatol. 2022;12(1):174-9. doi: 10.1016/j.jceh.2021.08.002. PMID: 35068797.
30. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology. 2007;45(4):846-54. doi: 10.1002/hep.21496. PMID: 17393509.
31. McPherson S, Stewart SF, Henderson E, Burt AD, Day CP. Simple non-invasive fibrosis scoring systems can reliably exclude advanced fibrosis in patients with non-alcoholic fatty liver disease. Gut. 2010;59(9):1265-9. doi: 10.1136/gut.2010.216077. PMID: 20801772.
32. McPherson S, Anstee QM, Henderson E, Day CP, Burt AD. Are simple noninvasive scoring systems for fibrosis reliable in patients with NAFLD and normal ALT levels? Eur J Gastroenterol Hepatol. 2013;25(6):652-8. doi: 10.1097/MEG.0b013e32835d72cf. PMID: 23325287.
33. Demir M, Lang S, Nierhoff D, Drebber U, Hardt A, Wedemeyer I, et al. Stepwise combination of

simple noninvasive fibrosis scoring systems increases diagnostic accuracy in nonalcoholic fatty liver disease. J Clin Gastroenterol. 2013;47(8):719-26. doi: 10.1097/MCG.0b013e3182819a89. PMID: 23442837.

34. Alkhouri N, Sedki E, Alisi A, Lopez R, Pinzani M, Feldstein AE, et al. Combined pediatric NAFLD fibrosis index and transient elastography to predict clinically significant fibrosis in children with fatty liver disease. Liver Int. 2013;33(1):79-85. doi: 10.1111/liv.12024. PMID: 23146095.
35. European Association for the Study of the Liver. EASL Clinical Practice Guidelines on the management of hepatocellular carcinoma. J Hepatol. 2025;82(2):315-74. doi: 10.1016/j.jhep.2024.08.028. PMID: 39690085.
36. Linero PL, Castilla-Guerra L. Management of Cardiovascular Risk in the Non-alcoholic Fatty Liver Disease Setting. Eur Cardiol. 2024;19:e02. doi: 10.15420/ecr.2023.19. PMCID: PMC11131151.
37. Duell PB, Welty FK, Miller M, Chait A, Hammond G, Ahmad Z, et al. Nonalcoholic Fatty Liver Disease and Cardiovascular Risk: A Scientific Statement From the American Heart Association. Arterioscler Thromb Vasc Biol. 2022;42(6):e168-85. doi: 10.1161/ATV.0000000000000153. PMID: 35418240.
38. Saavedra R, Ramirez B, Jay B. Strategies to Manage Obesity: Lifestyle. Methodist Debakey Cardiovasc J. 2025;21(2):53-61. doi: 10.14797/mdcvj.1510. PMID: 39990756.
39. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, Torres-Gonzalez A, Gra-Oramas B, Gonzalez-Fabian L, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. Gastroenterology. 2015;149(2):367-78.e5; quiz e14-5. doi: 10.1053/j.gastro.2015.04.005. PMID: 25865049.
40. Romero-Gómez M, Zelber-Sagi S, Trenell M. Treatment of NAFLD with diet, physical activity and exercise. J Hepatol. 2017;67(4):829-46. doi: 10.1016/j.jhep.2017.05.016. PMID: 28545937.
41. Stavropoulos K, Imprialos K, Pittaras A, Faselis C, Narayan P, Kokkinos P. Lifestyle Modifications in Non-Alcoholic Fatty Liver Disease and Non- Alcoholic Steatohepatitis. Curr Vasc Pharmacol. 2018;16(3):239-45. doi: 10.2174/1570161115666170621080835. PMID: 28637408.
42. Lassailly G, Caiazzo R, Ntandja-Wandji LC, Gnemmi V, Baud G, Verkindt H, et al. Bariatric Surgery Provides Long-term Resolution of Nonalcoholic Steatohepatitis and Regression of Fibrosis. Gastroenterology. 2020;159(4):1290-301.e5. doi: 10.1053/j.gastro.2020.06.006. PMID: 32553765.
43. Estruch R, Ros E, Salas-Salvadó J, Covas MI, Corella D, Arós F, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. N Engl J Med. 2018;378(25):e34. doi: 10.1056/NEJMoa1800389. PMID: 29897866.
44. Nseir W, Hellou E, Assy N. Role of diet and lifestyle changes in nonalcoholic fatty liver disease. World J Gastroenterol. 2014;20(28):9338-44. doi: 10.3748/wjg.v20.i28.9338. PMID: 25071328.
45. Keating SE, Hackett DA, George J, Johnson NA. Exercise and non-alcoholic fatty liver disease: a systematic review and meta-analysis. J Hepatol. 2012;57(1):157-66. doi: 10.1016/j.jhep.2012.02.023. PMID: 22414768.
46. Hallsworth K, Fattakhova G, Hollingsworth KG, Thoma C, Moore S, Taylor R, et al. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. Gut. 2011;60(9):1278-83. doi: 10.1136/gut.2011.242073. PMID: 21708823.
47. Johnson NA, Sachinwalla T, Walton DW, Smith K, Armstrong A, Thompson MW, et al. Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. Hepatology. 2009;50(4):1105-12. doi: 10.1002/hep.23129. PMID: 19637289.
48. Poole R, Kennedy OJ, Roderick P, Fallowfield JA, Hayes PC, Parkes J. Coffee consumption and health: umbrella review of meta-analyses of multiple health outcomes. BMJ. 2017;359:j5024. doi: 10.1136/bmj.j5024. PMID: 29167102.
49. Kennedy OJ, Roderick P, Poole R, Parkes J. Coffee, caffeine and non-alcoholic fatty liver disease? Therap Adv Gastroenterol. 2016;9(3):417-8. doi: 10.1177/1756283X16636765. PMID: 27134669.
50. Jung HS, Chang Y, Kwon MJ, Sung E, Yun KE, Cho YK, et al. Smoking and the Risk of Non-Alcoholic Fatty Liver Disease: A Cohort Study. Am J Gastroenterol. 2019;114(3):453-63. doi: 10.1038/s41395-018-0283-5. PMID: 30353055.
51. Kim NH, Jung YS, Hong HP, Park JH, Kim HJ, Park DI, et al. Association between cotinine-verified smoking status and risk of nonalcoholic fatty liver disease. Liver Int. 2018;38(8):1487-94. doi: 10.1111/liv.13701. PMID: 29359396.
52. Kasper P, Martin A, Lang S, Kütting F, Goeser T, Demir M, et al. NAFLD and cardiovascular diseases: a clinical review. Clin Res Cardiol. 2021;110(7):921-37. doi: 10.1007/s00392-020-01709-7. PMID: 32696080.
53. Athyros VG, Tziomalos K, Gossios TD, Griva T, Anagnostis P, Kargiotis K, et al. Safety and efficacy of long-term statin treatment for cardiovascular events in patients with coronary heart disease and abnormal liver tests in the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) Study: a post-hoc analysis. Lancet. 2010;376(9756):1916-22. doi: 10.1016/S0140-6736(10)61272-X. PMID: 21109302.
54. Alkasabrah O, Joshi DK, Hafeez A, Agrawal SP, Frishman WH, Aronow WS. Statins and the Cardio-Hepatic Axis: Balancing Cardiovascular Protection with Liver Disease Safety. Cardiol Rev. 2025 Nov 8. doi: 10.1097/CRD.0000000000001122. PMID: 41204625.
55. Abdulrazzak E, Fakhoury B, Jaan A, Ebrahim M, Alabdul Razzak I, Shehadah A, et al. Statin Therapy and Portal

Pressure Reduction in Cirrhosis: A Systematic Review and Meta-Analysis. Liver Int. 2025;45(10):e70326. doi: 10.1111/liv.70326. PMID: 40891266.

56. Haukeland JW, Konopski Z, Eggesbø HB, von Volkmann HL, Raschpichler G, Bjøro K, et al. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. Scand J Gastroenterol. 2009;44(7):853-60. doi: 10.1080/00365520902845268. PMID: 19811343.
57. Rouabhia S, Milic N, Abenavoli L. Metformin in the treatment of non-alcoholic fatty liver disease: safety, efficacy and mechanism. Expert Rev Gastroenterol Hepatol. 2014;8(4):343-9. doi: 10.1586/17474124.2014.894880. PMID: 24580044.
58. Newsome PN, Buchholtz K, Cusi K, Linder M, Okanoue T, Ratziu V, et al. A Placebo-Controlled Trial of Subcutaneous Semaglutide in Nonalcoholic Steatohepatitis. N Engl J Med. 2021;384(12):1113-24. doi: 10.1056/NEJMoa2028395. PMID: 33185364.
59. Armstrong MJ, Gaunt P, Aithal GP, Barton D, Hull D, Parker R, et al. Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. Lancet. 2016;387(10019):679-90. doi: 10.1016/S0140-6736(15)00803-X. PMID: 26608256.
60. Borodavkin P, Sheridan W, Coelho C, Oštarijaš E, Zaïr ZM, Miras AD, et al. Effects of glucagon-like peptide-1 receptor agonists on histopathological and secondary biomarkers of non-alcoholic steatohepatitis: A systematic review and meta-analysis. Diabetes Obes Metab. 2022;24(2):337-42. doi: 10.1111/dom.14565. PMID: 34605124.
61. Kuchay MS, Krishan S, Mishra SK, Farooqui KJ, Singh MK, Wasir JS, et al. Effect of Empagliflozin on Liver Fat in Patients With Type 2 Diabetes and Nonalcoholic Fatty Liver Disease: A Randomized Controlled Trial (E-LIFT Trial). Diabetes Care. 2018;41(8):1801-8. doi: 10.2337/dc18-0165. PMID: 29895557.
62. Sattar N, Fitchett D, Hantel S, George JT, Zinman B. Empagliflozin is associated with improvements in liver enzymes potentially consistent with reductions in liver fat: results from randomised trials including the EMPA-REG OUTCOME® trial. Diabetologia. 2018;61(10):2155-63. doi: 10.1007/s00125-018-4702-3. PMID: 30066148.
63. Hofmann AF, Hagey LR. Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci. 2008;65:2461-83. doi: 10.1007/s00018-008-7568-6. PMID: 18488143.
64. Amaral JD, Viana RJ, Ramalho RM, Steer CJ, Rodrigues CM. Bile acids: regulation of apoptosis by ursodeoxycholic acid. J Lipid Res. 2009;50:1721-34. doi: 10.1194/jlr.R900011-JLR200. PMID: 19417220.
65. Zerem E, Kunosic S, Kurtcehajic A, Zerem D, Zerem O. Bile acids in metabolic dysfunction-associated steatotic liver disease. World J Hepatol. 2025;17(8):108606. doi: 10.4254/wjh.v17.i8.108606. PMID: 40901599.
66. Simental-Mendía M, Sánchez-García A, Simental-Mendía LE. Effect of ursodeoxycholic acid on liver markers: A systematic review and meta-analysis of randomized placebo-controlled clinical trials. Br J Clin Pharmacol. 2020;86:1476-88. doi: 10.1111/bcp.14311. PMID: 32285958.
67. Lin X, Mai M, He T, Huang H, Zhang P, Xia E, et al. Efficiency of ursodeoxycholic acid for the treatment of nonalcoholic steatohepatitis: A systematic review and meta-analysis. Expert Rev Gastroenterol Hepatol. 2022;16:537-45. doi: 10.1080/17474124.2022.2083605. PMID: 35617696.
68. Ratziu V, de Ledinghen V, Oberti F, Mathurin P, Wartelle-Bladou C, Renou C, et al. A randomized controlled trial of high-dose ursodesoxycholic acid for nonalcoholic steatohepatitis. J Hepatol. 2011;54:1011-9. doi: 10.1016/j.jhep.2010.08.030. PMID: 21145828.
69. Zhang W, Tang Y, Huang J, Hu H. Efficacy of ursodeoxycholic acid in nonalcoholic fatty liver disease: An updated meta-analysis of randomized controlled trials. Asia Pac J Clin Nutr. 2020;29(4):696-705. doi: 10.6133/apjcn.202012_29(4).0004. PMID: 33377363.
70. Leuschner UF, Lindenthal B, Herrmann G, Arnold JC, Rössle M, Cordes HJ, et al. High-dose ursodeoxycholic acid therapy for nonalcoholic steatohepatitis: a double-blind, randomized, placebo-controlled trial. Hepatology. 2010;52(2):472-9. doi: 10.1002/hep.23727. PMID: 20683947.
71. Neuman MG, Malnick S, Chertin L. Gamma glutamyl transferase - an underestimated marker for cardiovascular disease and the metabolic syndrome. J Pharm Pharm Sci. 2020;23(1):65-74. doi: 10.18433/jpps30923. PMID: 32310756.
72. Zhao Y, Xin X, Luo XP. The relationship between the ratio of gamma-glutamyltransferase to high-density lipoprotein cholesterol and the risk of diabetes mellitus using publicly available data: a secondary analysis based on a longitudinal study in Japan. Lipids Health Dis. 2023;22(1):7. doi: 10.1186/s12944-023-01772-9. PMID: 36650520.
73. Nadinskaia M, Maevskaya M, Ivashkin V, Kodzoeva K, Pirogova I, Chesnokov E, et al. Ursodeoxycholic acid as a means of preventing atherosclerosis, steatosis and liver fibrosis in patients with nonalcoholic fatty liver disease. World J Gastroenterol. 2021;27(10):959-75. doi: 10.3748/wjg.v27.i10.959. PMID: 33776366.
74. Ko WK, Lee SH, Kim SJ, Jo MJ, Kumar H, Han IB, et al. Anti-inflammatory effects of ursodeoxycholic acid by lipopolysaccharide-stimulated inflammatory responses in RAW 264.7 macrophages. PLoS One. 2017;12(6):e0180673. doi: 10.1371/journal.pone.0180673. PMID: 28665991.
75. Sanyal AJ, Chalasani N, Kowdley KV, McCullough A, Diehl AM, Bass NM, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. N Engl J Med. 2010;362(18):1675-85. doi: 10.1056/NEJMoa0907929. PMID: 20427778.

76. Juraschek SP, Col H, Ferro K, Turkson-Ocran RN, Cluett JL, Davis RB, et al. DASH-Patterned Groceries and Effects on Blood Pressure: The GoFresh Randomized Clinical Trial. JAMA. 2026;335(1):36-48. doi: 10.1001/jama.2025.21112. PMID: 41206973.
77. Pietu F, Guillaud O, Walter T, Vallin M, Hervieu V, Scoazec JY, et al. Ursodeoxycholic acid with vitamin E in patients with nonalcoholic steatohepatitis: long-term results. Clin Res Hepatol Gastroenterol. 2012;36(2):146-55. doi: 10.1016/j.clinre.2011.10.011. PMID: 22154224.
78. Dufour JF, Oneta CM, Gonvers JJ, Bihl F, Cerny A, Cereda JM, et al. Randomized placebo-controlled trial of ursodeoxycholic acid with vitamin e in nonalcoholic steatohepatitis. Clin Gastroenterol Hepatol. 2006;4(12):1537-43. doi: 10.1016/j.cgh.2006.09.025. PMID: 17162245.
79. Ersöz G, Günşar F, Karasu Z, Akay S, Batur Y, Akarca US. Management of fatty liver disease with vitamin E and C compared to ursodeoxycholic acid treatment. Turk J Gastroenterol. 2005;16(3):124-8. PMID: 16245220.
80. Barchetta I, Angelico F, Del Ben M, Baroni MG, Pozzilli P, Morini S, et al. Strong association between nonalcoholic fatty liver disease (NAFLD) and low 25(OH) vitamin D levels in an adult population with normal serum liver enzymes. BMC Med. 2011;9:85. doi: 10.1186/1741-7015-9-85. PMID: 21749681.
81. Kitson MT, Roberts SK. D-livering the message: the importance of vitamin D status in chronic liver disease. J Hepatol. 2012;57(4):897-909. doi: 10.1016/j.jhep.2012.04.033. PMID: 22634121.
82. Harrison SA, Bedossa P, Guy CD, Schattenberg JM, Loomba R, Taub R, et al. A Phase 3, Randomized, Controlled Trial of Resmetirom in NASH with Liver Fibrosis. N Engl J Med. 2024;390(6):497-509. doi: 10.1056/NEJMoa2309000. PMID: 38324483.
83. Hashim HT, Alhatemi AQM, Riaz S, Al-Ghuraibawi MA, Alabide AS, Saeed H, et al. Unveiling Resmetirom: A systematic review and meta-analysis on its impact on liver function and safety in non-alcoholic steatohepatitis treatment. JGH Open. 2024;8(10):e70025. doi: 10.1002/jgh3.70025. PMID: 39359614.
84. Raja A, Subhash Sagar R, Saeed S, Zia Ul Haq A, Khan O, Dileep Bhimani P, et al. Safety and efficacy of resmetirom in the treatment of patients with non-alcoholic steatohepatitis and liver fibrosis: a systematic review and meta-analysis. Ann Med Surg (Lond). 2024;86(7):4130-8. doi: 10.1097/MS9.0000000000002195. PMID: 38989228.
85. Knezović E, Hefer M, Blažanović S, Petrović A, Tomičić V, Srb N, et al. Drug Pipeline for MASLD: What Can Be Learned from the Successful Story of Resmetirom. Curr Issues Mol Biol. 2025;47(3):154. doi: 10.3390/cimb47030154. PMID: 40136408.
86. Au K, Zheng MH, Lee WJ, Ghanem OM, Mahawar K, Shabbir A, et al. Resmetirom and Metabolic Dysfunction-Associated Steatohepatitis: Perspectives on Multidisciplinary Management from Global Healthcare Professionals. Curr Obes Rep. 2024;13(4):818-30. doi: 10.1007/s13679-024-00582-z. PMID: 39110384.
87. Wahlström A, Sayin SI, Marschall HU, Bäckhed F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. Cell Metab. 2016;24:41-50. doi: 10.1016/j.cmet.2016.05.005. PMID: 27320064.
88. Beuers U, Trauner M, Jansen P, Poupon R. New paradigms in the treatment of hepatic cholestasis: from UDCA to FXR, PXR and beyond. J Hepatol. 2015;62: S25-S37. doi: 10.1016/j.jhep.2015.02.023. PMID: 25920087.
89. Wang J, Gao J, Zhang Q, Lu J, Yang Y, Cai X, et al. Ileal FXR Knockdown Ameliorates MASLD Progression in Rats via Modulating Bile Acid Metabolism Mediated by Gut Microbiota. J Gastroenterol Hepatol. 2025;40(8):2091-103. doi: 10.1111/jgh.17017. PMID: 40411313.
90. Rodrigues CM, Steer CJ. The therapeutic effects of ursodeoxycholic acid as an anti-apoptotic agent. Expert Opin Investig Drugs. 2001;10:1243-53. doi: 10.1517/13543784.10.7.1243. PMID: 11772248.
91. Sanyal AJ, Ratziu V, Loomba R, Anstee QM, Kowdley KV, Rinella ME, et al. Results from a new efficacy and safety analysis of the REGENERATE trial of obeticholic acid for treatment of pre-cirrhotic fibrosis due to non-alcoholic steatohepatitis. J Hepatol. 2023;79:1110-20. doi: 10.1016/j.jhep.2023.07.014. PMID: 37517454.
92. Rinella ME, Dufour JF, Anstee QM, Goodman Z, Younossi Z, Harrison SA, et al. Non-invasive evaluation of response to obeticholic acid in patients with NASH: Results from the REGENERATE study. J Hepatol. 2022;76:536-48. doi: 10.1016/j.jhep.2021.10.029. PMID: 34793868.
93. Wang W, Zhao J, Gui W, Sun D, Dai H, Xiao L, et al. Tauroursodeoxycholic acid inhibits intestinal inflammation and barrier disruption in mice with non-alcoholic fatty liver disease. Br J Pharmacol. 2018;175:469-84. doi: 10.1111/bph.14095. PMID: 29139555.
94. Lu Q, Jiang Z, Wang Q, Hu H, Zhao G. The effect of Tauroursodeoxycholic acid (TUDCA) and gut microbiota on murine gallbladder stone formation. Ann Hepatol. 2021;23:100289. doi: 10.1016/j.aohep.2020.100289. PMID: 33217585.
95. Patel K, Harrison SA, Elkhashab M, Trotter JF, Herring R, Rojter SE, et al. Cilofexor, a Nonsteroidal FXR Agonist, in Patients With Noncirrhotic NASH: A Phase 2 Randomized Controlled Trial. Hepatology. 2020;72:58-71. doi: 10.1002/hep.31205. PMID: 32115759.
96. Alkhouri N, Herring R, Kabler H, Kayali Z, Hassanein T, Kohli A, et al. Safety and efficacy of combination therapy with semaglutide, cilofexor and firsocostat in patients with non-alcoholic steatohepatitis: A randomised, open-label phase II trial. J Hepatol. 2022;77:607-18. doi: 10.1016/j.jhep.2022.04.003. PMID: 35439567.
97. Traussnigg S, Schattenberg JM, Demir M, Wiegand J, Geier A, Teuber G, et al. Norursodeoxycholic acid versus placebo in the treatment of non-alcoholic fatty liver disease: a double-blind, randomised, placebo-controlled,

phase 2 dose-finding trial. Lancet Gastroenterol Hepatol. 2019;4:781-93. doi: 10.1016/S2468-1253(19)30184-0. PMID: 31345778.

98. Carlsson B, Lindén D, Brolén G, Liljeblad M, Bjursell M, Romeo S, et al. Review article: the emerging role of genetics in precision medicine for patients with non-alcoholic steatohepatitis. Aliment Pharmacol Ther. 2020;51:1305-20. doi: 10.1111/apt.15738. PMID: 32383295.
99. Sanyal AJ, Newsome PN, Kliers I, Østergaard LH, Long MT, Kjær MS, et al. Phase 3 Trial of Semaglutide in Metabolic Dysfunction-Associated Steatohepatitis. N Engl J Med. 2025;392(21):2089-99. doi: 10.1056/NEJMoa2413258. PMID: 40305708.
100. Alkhouri N. Recent Data from the ESSENCE Trial on Semaglutide in Metabolic Dysfunction-Associated Steatohepatitis. Gastroenterol Hepatol (N Y). 2025;21(2):133-5. PMID: 40115605.
101. Luo S, Liu H, Jin W. Semaglutide in Metabolic Dysfunction-Associated Steatohepatitis. N Engl J Med. 2025;393(8):826. doi: 10.1056/NEJMc2509454. PMID: 40834308.
102. Kim Y, Zeng N, Winer-Jones JP, Bonafede M, Lobo F, O'Donnell J, et al. Retrospective Study of Real-World Treatment Patterns of Subcutaneous Semaglutide Use Among Patients with Metabolic Dysfunction-Associated Steatohepatitis in the United States. Clinicoecon Outcomes Res. 2025; 17:743-54. doi: 10.2147/CEOR.S546841. PMID: 41147033.
103. Fiorucci S, Urbani G. Tirzepatide for metabolic dysfunction-associated steatohepatitis: results from phase II clinical trials and perspectives. Expert Opin Investig Drugs. 2025;34(9):655-63. doi: 10.1080/13543784.2025.2546812. PMID: 40782123.
104. Arai T, Atsukawa M, Nagao C, Yamada Z, Rokugo T, Suzuki K, et al. Effect of Tirzepatide Treatment on Hepatic Biomarkers in Patients With Metabolic Dysfunction-Associated Steatotic Liver Disease and Type 2 Diabetes Mellitus. Hepatol Res. 2025;55(10):1346-52. doi: 10.1111/hepr.14241. PMID: 40616360.
105. Li Y, Sun W, Liu H, Ruan XZ. Tirzepatide, a dual GIP/GLP-1 receptor agonist, alleviates metabolic dysfunction-associated steatotic liver disease by reducing the expression of CD36 and OBP2A. Genes Dis. 2025;12(6):101761. doi: 10.1016/j.gendis.2025.101761. PMID: 40837406.
106. Reja D, Zhang C, Sarkar A. Endoscopic bariatrics: current therapies and future directions. Transl Gastroenterol Hepatol. 2022;7:21. doi: 10.21037/tgh.2020.03.09. PMID: 35548475.
107. Moutzoukis MK, Argyriou K, Manolakis A, Kapsoritakis A, Christodoulou D. Endoscopic bariatrics: Current status and emerging technologies. World J Gastrointest Endosc 2025;17(10):109031. doi: 10.4253/wjge.v17.i10.109031. PMID: 41181529.
108. Mingrone G, Rajagopalan H. Bariatrics and endoscopic therapies for the treatment of metabolic disease: Past, present, and future. Diabetes Res Clin Pract. 2024;211:111651. doi: 10.1016/j.diabres.2024.111651. PMID: 38580037.
109. Younossi ZM, Stepanova M, Ong J, Trimble G, AlQahtani S, Younossi I, et al. Nonalcoholic Steatohepatitis Is the Most Rapidly Increasing Indication for Liver Transplantation in the United States. Clin Gastroenterol Hepatol. 2021; 19(3):580-9.e5. doi: 10.1016/j.cgh.2020.05.064. PMID: 32531342.
110. Lim TY, Steggerda JA, Trivedi H, Luu M, Vipani A, Adjei MA, et al. Outcomes from Referral to Transplant for Patients with MASLD: A California Liver Network Study. J Clin Med. 2025;14(21):7841. doi: 10.3390/jcm14217841. PMID: 41227237.
111. Tzartzeva K, Obi J, Rich NE, Parikh ND, Marrero JA, Yopp A, et al. Surveillance Imaging and Alpha Fetoprotein for Early Detection of Hepatocellular Carcinoma in Patients With Cirrhosis: A Meta-analysis. Gastroenterology. 2018;154(6):1706-18.e1. doi: 10.1053/j.gastro.2018.01.064. PMID: 29425931.
112. Reig M, Forner A, Rimola J, Ferrer-Fàbrega J, Burrel M, Garcia-Criado Á, et al. BCLC strategy for prognosis prediction and treatment recommendation: The 2022 update. J Hepatol. 2022;76(3):681-93. doi: 10.1016/j.jhep.2021.11.018. PMID: 34801630.

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Chronic Neutropenia in Children: A Single-Center Experience

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Abstract

Objective. To analyze the characteristics, etiology, diagnostic evaluation, management, follow-up, and outcomes of children with chronic neutropenia treated at a tertiary care pediatric hospital in Croatia. **Materials and Methods.** We retrospectively reviewed the demographic, clinical, and laboratory data of 48 children (23 males and 25 females; median age 7.75 months [IQR 5.13–11.75]) diagnosed with chronic neutropenia between 2013 and 2021. **Results.** The median absolute neutrophil count (ANC) at presentation was 487/μL (IQR 198.5–837.5), and 52% of the patients had severe neutropenia. Autoimmune neutropenia (AIN) was diagnosed in 21 cases, chronic idiopathic neutropenia (CIN) in 26 cases, and neutropenia in the context of a genetic syndrome in one case. Antineutrophil antibodies were detected in 47% of the children tested. During follow-up, 23% received granulocyte-colony-stimulating factor (G-CSF), and 21% received antibiotics. The median follow-up duration was 21 months (IQR 12–32.75), during which 83% achieved spontaneous remission, with a median time to remission of 13.5 months. Lower ANC at diagnosis was associated with more frequent rehospitalizations, longer time to remission, and longer follow-up. No significant differences were found between AIN and CIN in terms of age, ANC at diagnosis, time to referral, or time to remission, although AIN cases were followed for a longer period. **Conclusion.** Pediatric chronic isolated neutropenia, including AIN and CIN, generally follows a mild clinical course with a low incidence of severe infection. Most children achieve spontaneous remission within one year. Comprehensive genetic testing is essential in children with suspected congenital neutropenia and those with features suggestive of an underlying genetic syndrome. Adherence to European guidelines supports standardized diagnosis, follow-up, and management, thereby improving patient care.

Key Words: Neutropenia ▪ Chronic ▪ Children ▪ Etiology ▪ Prognosis.

Introduction

Neutropenia is defined as a reduction in the number of neutrophils circulating in the blood, with an absolute neutrophil count (ANC) below the lower limit of the normal range for age and ethnic origin. The widely accepted cutoff level for neutropenia in Caucasians is 1.0×10^9/L (1000/μL) up to the age of 1 year and 1.5×10^9/L (1500/μL) from the age of 1 year to adulthood (1, 2). Neutropenia can be classified according to severity (based on ANC), duration, and pathogenesis. Based on severity, neutropenia in individuals older than 1 year is classified as mild (ANC 1000-1500/μL), moderate (ANC 500-1000/μL), and severe (ANC <500/μL). Agranulocytosis denotes a profound degree of neutropenia, defined as an ANC <200/μL. Neutropenia is classified as acute (lasting <3 months) or chronic (>3 months). In a pathogenesis-based framework, neutropenias are categorized as congenital, acquired, and likely acquired, with respective subcategories (1, 3, 4). Neutropenia is a common reason for referral to pediatric hematologists. Until recently, limited data were available on the management and outcomes of children with prolonged isolated neutropenia outside the setting of febrile neutropenia in children with cancer.

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This study aimed to examine the etiology, clinical course, interventions, and outcomes of children with chronic neutropenia admitted to a tertiary care pediatric hospital in Croatia.

Materials and Methods

Study Population

Fifty-seven children with neutropenia who were consecutively admitted to the day hospital or inpatient unit at the Division of Hematology and Oncology, Department of Pediatrics, Clinical Hospital Center Rijeka, Croatia, between January 1, 2013, and December 31, 2021, were enrolled. Patients with chemotherapy-induced febrile neutropenia were excluded from the study. Of the 57 subjects, children with acute neutropenia (ANC recovered to ≥1500/μL within three months) were further excluded. Additionally, two patients with chronic neutropenia were lost to follow-up. After these exclusions, data from 48 children (23 males and 25 females), all of Caucasian origin, were included in the final analysis. The median age at diagnosis was 7.75 months (IQR 5.125–11.75). Thirteen (27%) patients were younger than 6 months, 30 (63%) were between 6 months and 5 years of age, and 5 (10%) were older than 5 years of age. These age categories were defined based on recognized differences in the etiology and clinical course of childhood neutropenia according to the age at onset, rather than on developmental pediatric classifications.

Data Collection

Data were obtained from both paper-based and electronic hospital records. The following variables were collected: (a) Clinical data: sex, ethnic origin, age at presentation, findings from full clinical examination including anthropometry, evidence of recent infection (type and site of infection), history of drug intake and vaccination, comorbidities, family history suggestive of neutropenia and autoimmune disorders, treatment, need for repeated hospitalizations, and duration of follow-up; (b) Laboratory investigations: complete blood count with differential (at diagnosis and throughout follow-up until recovery or for a minimum of 3 months), antineutrophil antibodies, immunoglobulins (IgG, IgM, and IgA), antiglobulin test, viral screening for Epstein-Barr virus (EBV) and cytomegalovirus (CMV), antinuclear antibodies (ANA), vitamin B12, folic acid, vitamin D, bone marrow aspirate with cytological examination, genetic testing for *ELANE* and *HAX-1*, microbiological investigations (pharyngeal swab, urine culture, blood culture) and other investigations in case of findings suggestive of associated diseases (e.g., human herpesvirus 6 [HHV-6] for roseola, peripheral blood flow cytometry for suspected immunodeficiency, chest X-ray for the diagnosis of lower respiratory tract infection, additional bone marrow evaluation – immunophenotype and cytogenetics – in case of signs suspected for malignancy). Antineutrophil antibody detection was performed using the monoclonal antibody immobilization of granulocyte antigen (MAIGA) assay. Targeted genetic testing of the *ELANE* and *HAX1* genes was performed using a next-generation sequencing (NGS) panel. Neutropenia was classified as mild, moderate, or severe based on the ANC, as previously described.

Ethics Statement

The study was approved by the Ethics Committee of the Clinical Hospital Center, Rijeka, Croatia (No. 2170-29-02/1-22-2; May 26, 2022) and was conducted in accordance with the principles of the Declaration of Helsinki.

Statistical Analysis

Frequencies and percentages were used for descriptive statistics. As most variables deviated from the normal distribution, nonparametric tests were applied. The median and interquartile range (IQR) were used as measures of the central tendency. Spearman's correlation coefficient was employed to examine the relationship between two continuous variables, Pearson's chi-square test between two nominal variables, and point-biserial correlation between binomial and continuous variables. The Mann–Whitney U test was used to test the differences between the two

groups. Data were processed using the IBM® SPSS® Statistics Version 25 software package.

Results

Patient Referral and Clinical Presentation

The median time from the first documented neutropenia to referral to a hematologist was 1 month (IQR 0.23–2.50). Referral time was not influenced by neutropenia severity. The median number of blood counts performed prior to the clinic visit was 3 (IQR 2-4). Neutropenia associated with fever was the reason for referral in 25 (52%) participants, most commonly due to fever of unknown origin (N=13, 5%), followed by unspecified respiratory tract infections (N=6, 24%), pneumonia (N=3, 12%), otitis (N=2, 8%), and urinary tract infection (N=1, 4%). In 23 (48%) participants, neutropenia was an incidental finding during routine checkups (N=18), assessment of atopic dermatitis (N=2), evaluation of nonspecific rash (N=2), or poor weight gain (N=1).

Five out of 48 children had comorbidities in their medical history: two had atopic dermatitis, while allergic rhinitis, hypogammaglobulinemia, and failure to thrive were present in one child each. Eighteen (38%) children received antibiotics prior to referral, and none had received any other drugs known to cause neutropenia or any vaccinations. Family history of neutropenia and autoimmune disorders was positive in three children, all reported in mothers: one case of neutropenia, autoimmune thyroiditis, and celiac disease; one case of autoimmune thyroiditis; and one case of rheumatoid arthritis.

On clinical examination, two children had atopic dermatitis, and a one-month-old infant showed poor growth and hypotonia. In all other cases, the clinical findings were normal, except for the signs of infection. The median ANC at diagnosis was 487/μL (IQR 198.5-837.5). According to the severity of neutropenia, 10 (21%) patients had mild neutropenia, 13 (27%) had moderate neutropenia, and 25 (52%) had severe neutropenia. The main characteristics of the children with chronic isolated neutropenia are shown in Table 1.

Table 1. Main Characteristics of Children With Isolated Chronic Neutropenia. Data Is Shown as Either Number of Cases and Percentage or Median and Inter-Quartile Range

Baseline characteristics	
Gender	
Female	25 (52%)
Male	23 (48%)
Median age (IQR) at onset of neutropenia (months)	7.75 (5.125–11.75)
Age group	
≤ 6 months	13 (27%)
6 months – 5 years	30 (63%)
> 5 years	5 (10%)
Reason for CBC at diagnosis	
Fever/infection	25 (52%)
Incidental finding	23 (48%)
Prior antibiotics	
Yes	18 (38%)
No	30 (62%)
Outcome characteristics	
Median ANC (IQR) at diagnosis (μL)	487 (198.5-837.5)
Severity of neutropenia	
Mild	10 (21%)
Moderate	13 (27%)
Severe	25 (52%)
Indirect antineutrophil antibodies	
Positive	21 (47%)
Negative	24 (53%)
Not performed	3
Rehospitalizations	
Yes	15 (31%)
No	33 (69%)
Treatment during neutropenia	
G-CSF	11 (23%)
Antibiotics for infection	10 (21%)
None	27 (56%)
Etiology-based group	
Chronic idiopathic neutropenia	26 (54%)
Autoimmune neutropenia	21 (44%)
Other/complex genetic syndrome	1 (2%)
Median time (IQR) to remission (months)	13.5 (9.00-18.00)
Median follow-up time (IQR) (months)	21 (12.00-32.75)

CBC=Complete blood count; G-CSF=Granulocyte-colony stimulating factor; IQR=Interquartile range.

Autoimmune neutropenia (AIN) was diagnosed upon detection of positive indirect antineutrophil antibodies, while chronic idiopathic neutropenia (CIN) was diagnosed by exclusion, following normal results of all investigations. In the patient with AIN and vitamin B12 deficiency, supplementation was administered without a significant response. In one patient, neutropenia occurred in the context of a genetic syndrome associated with a *MAGEL2* variant, accompanied by failure to thrive and hypotonia. The patient did not experience spontaneous recovery during the follow-up period and was subsequently managed by another subspecialist. Another patient, initially diagnosed with neonatal alloimmune neutropenia (NAN) and showing spontaneous ANC recovery after six weeks, subsequently developed mild neutropenia at 7 months of age. This persisted until 24 months, leading to a revised diagnosis of CIN.

Laboratory Investigations

The MAIGA test for the detection of antineutrophil antibodies was performed in 45 (94%) children. Positive indirect antineutrophil antibodies were found in 21 (47%) cases. The antiglobulin test was performed in 29 (59%) children, and all the results were negative. Ig levels were measured in 43 (90%) children; IgG was below the age-adjusted reference range in one case, and IgM in two cases. In these children, flow cytometric analysis of peripheral blood lymphocyte subsets revealed no abnormalities. Among the children tested, CMV IgM was positive in 1 of 28, and EBV viral capsid antigen (VCA) IgM was positive in 1 of 25. HHV-6 serology was performed in two children with roseola and neutropenia, and both had positive IgM and IgG. ANA testing was performed in six cases, with one positive result. Vitamin B12 was measured in 17 children and was below the lower limit in one previously described case, while folic acid levels were normal in all 16 children tested. Vitamin D levels were determined in 12 children, and were low in two. However, neutropenia did not resolve after the infection cleared or following vitamin B12 and D supplementation; therefore, these conditions were not considered etiological factors for chronic neutropenia.

Genetic and Bone Marrow Findings

Genetic testing for *ELANE* and *HAX-1* was performed in 7 children with severe neutropenia. No pathogenic variants were identified in either gene. Bone marrow aspiration with cytomorphology, performed as part of the initial investigations, was carried out in 32 (67%) children, with 1 sample being inadequate for analysis. This relatively high percentage reflects pre-guideline clinical practice, when indications for bone marrow evaluation in chronic neutropenia were less clearly standardized and largely dependent on individual clinical judgment, and were generally performed to exclude underlying hematologic disease or in cases of severe neutropenia, particularly when congenital neutropenia or bone marrow failure was part of the differential diagnosis. In 7 of 22 (32%) children with AIN, the bone marrow was hypercellular with myeloid hyperplasia; in 2 of 22 (9%), it was hypocellular with myeloid hypoplasia, and in the remaining cases, it was normal. Among the 25 children with CIN, three had hypocellular marrow with myeloid hypoplasia, while the remaining cases demonstrated normal cellularity and myelopoiesis. A 5-year-old boy with isolated severe neutropenia and positive indirect antineutrophil antibodies developed mild bicytopenia (neutropenia and normocytic anemia) during follow-up. Eight months after the initial presentation, bone marrow aspiration revealed acute lymphoblastic leukemia (5).

Microbiological and Radiological Assessments

During the initial diagnostic workup, regardless of prior antimicrobial therapy, pharyngeal swabs were obtained from 46 (96%) children, of whom 37/46 (80%) were negative. The most common isolates were *Streptococcus pneumoniae* (N=3) and *Enterobacter cloacae* (N=2). Urine cultures were obtained from 30 (63%) participants and were positive in three cases (two *E. coli* and one *Klebsiella oxytoca*). Blood cultures were obtained from 7

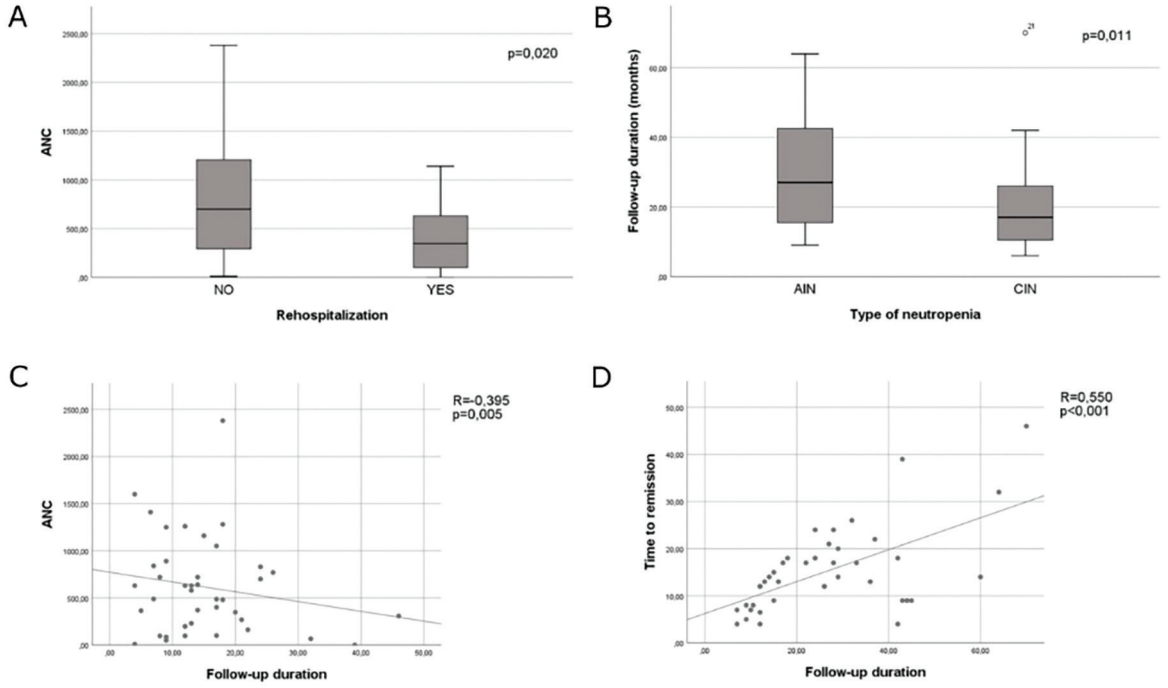
children, and all were negative. Radiological assessments were performed in 20 cases, and pneumonia was diagnosed in three cases.

Follow-up and Clinical Outcomes

The median follow-up time was 21 months (IQR 12.00–32.75). During the neutropenia period, rehospitalization occurred in 15 (31%) participants for a total of 27 admissions: eight were hospitalized once, four twice, one three times, and two four times. The most common reasons for readmission were high fever (N=11), followed by unspecified respiratory infections (N=6), otitis (N=4), gastroenteritis (N=2), pneumonia (N=1), cellulitis (N=1), Covid 19 (N=1), and suspected mastocytosis (N=1). Initial ANC was significantly negatively correlated with repeated admissions, time to remission, and total follow-up duration, whereas no relationship was observed between ANC and age (Supplementary Tables 1 and 2). Scatterplots were generated to depict the relationships between continuous variables and highlight statistically significant associations (Figure 1).

G-CSF therapy was administered in 11 (23%) children during 17 hospitalizations, with an average duration of 2.8 days (IQR 2.00-3.00), and resulted in a significant increase in ANC in all cases. In selected patients, G-CSF was used primarily as a functional test of bone marrow reserve and neutrophil response, in addition to its occasional therapeutic use during clinically significant infectious episodes. The decision was based on individual clinical judgment in the pre-guideline era, when standardized protocols for its use had not yet been established. Antibiotic therapy was administered to 10 (21%) children for 12 episodes of infection. None of the children received prophylactic antibiotics.

Forty participants (83%) achieved spontaneous remission, with a median time to remission of 13.5 months (IQR 9.00-18.00). To further characterize the disease pattern, we compared the main clinical and laboratory features of AIN and CIN. The Mann–Whitney U test demonstrated a statistically



AIN=Autoimmune neutropenia; ANC=Absolute neutrophil count; CIN=Chronic idiopathic neutropenia.

Figure 1. A. Median absolute neutrophil count at diagnosis according to rehospitalization status; B. Median follow-up duration (months) for cases with autoimmune and chronic idiopathic neutropenia; C. Relationship between absolute neutrophil count at diagnosis and follow-up duration; D. Relationship between time to remission and follow-up duration.

Table 2. Clinical and Laboratory Comparison of Cases With Autoimmune Neutropenia and Chronic Idiopathic Neutropenia

Variable	Type of neutropenia	N	C (25-75)	Mann–Whitney U test	P
Median age (IQR) at onset of neutropenia (months)	AIN	22	8 (5.375-19.50)	245.00	0.522
	CIN	25	8 (4.665-11.00)		
Median time (IQR) to referral (months)	AIN	21	1.23 (0.185-4.00)	210.50	0.250
	CIN	25	0.50 (0.23-1.58)		
Median ANC (IQR) at diagnosis (μL)	AIN	22	374 (82.25-832.50)	224.00	0.277
	CIN	25	488 (279.50-830.00)		
Median time (IQR) to remission (months)	AIN	20	14 (9.00-19.50)	176.50	0.524
	CIN	20	12 (8.00-18.00)		
Median (IQR) follow-up duration (months)	AIN	22	27.5 (15.75-43.00)	156.50	0.011*
	CIN	25	17 (9.75-27.00)		

ANC=Absolute neutrophil count; AIN=Autoimmune neutropenia; CIN=Chronic idiopathic neutropenia; IQR=Interquartile range; N=Number; C (25–75)=Median (25th–75th percentile); P=Statistical significance (*P<0.05).

significantly longer follow-up period for AIN cases than for CIN cases (Figure 1B). No significant differences were observed between the groups in terms of age and ANC at diagnosis, time to referral to a hematologist, or time to remission (Table 2).

Discussion

Childhood chronic neutropenia represents a heterogeneous clinical entity and is a frequent indication for pediatric hematology referral (6). Recent expert recommendations have aimed to standardize its diagnostic evaluation and management (7–10).

This study aimed to present institutional experience with the diagnostic work-up, management strategies, and follow-up of children with chronic neutropenia. Because the European guidelines were published only in April 2023 and adequate follow-up of our cases required a defined observation period, we concluded the study at the end of 2021. The retrospective cohort comprised 48 children who were consecutively treated at a tertiary care pediatric hospital. The sex distribution was approximately equal. The median age at presentation was 7.75 months, and 90% were younger than 5 years. This notably early age presentation is consistent with the findings of Angelino et al., who reported that 72% of cases occurred in children under 2 years of age (11). In our study, the median time from the first detection of neutropenia to referral to a hematologist was short (1 month) and was not influenced by the severity of neutropenia, reflecting the preferences and experience of the primary care pediatrician in most cases. In contrast, Nagalapuran et al. (12) reported a short median referral time of 2 months, which was inversely correlated with the severity of neutropenia.

The median ANC at diagnosis was below 500/μL (487/μL), with the majority of children presenting with severe neutropenia (52%), followed by moderate (27%) and mild (21%) forms. The median ANC in similar published studies varies from 314/μL for AIN and 518/μL for CIN (11), to 600/μL (13), 732/μL (14), and 827/μL (15). The proportion of children with mild neutropenia varies across these studies, ranging from 12% (11), 25.5% (13), 26.1% (14), and 29% (12) to 45% (15). The presence of comorbidities or a positive family history was infrequent in the present study. No drug or vaccine was identified as the etiological cause. In 52% of our cases, neutropenia was accompanied by fever or infection, whereas in the remaining cases, it was detected incidentally. The reported frequencies of post-infectious neutropenia vary widely in the literature, ranging from 33.3% (10), 53.9% (14), and 58.5% (13) to 96.5% (15). However, most of these studies included children with both acute and chronic neutropenia,

making it difficult to compare the overall results. The only small-scale study on chronic neutropenia comparable to ours evaluated 29 children (16), including 15 with CIN, 7 with AIN, and 7 with congenital neutropenic syndromes. Among these, 5 of the 7 patients with AIN (71%) and 10 of the 15 patients with CIN (67%) experienced infections requiring hospitalization. The mean age at onset, mean ANC at presentation (0.77 vs. 0.99 years; 291/μL vs. 549/μL, respectively), and mean duration of neutropenia did not differ significantly between AIN and CIN. Infections were more frequent in patients with CIN, who also had a longer average follow-up.

In our cohort of 22 AIN and 25 CIN cases, age at presentation, ANC at onset, and duration of neutropenia did not differ significantly between the groups. Although AIN cases were followed for a longer period, no notable differences were observed in the frequency of infections or rehospitalizations.

Overall, comparison with previously published cohorts reveals several similarities and differences. The median age at presentation in our study was consistent with reports indicating that chronic neutropenia predominantly affects infants and young children. Similarly, the overall benign clinical course and high rate of spontaneous remission are in agreement with prior studies of both AIN and CIN. However, the proportion of patients presenting with severe neutropenia in our cohort was higher than in some reports, which may reflect referral bias to a tertiary care center and the inclusion of more clinically significant cases. Reported infection rates and causes of neutropenia also vary widely in the literature. While some studies describe a predominance of post-infectious cases, particularly when acute and chronic neutropenia are combined, cohorts focusing exclusively on chronic neutropenia, such as ours, generally report lower rates of severe infectious complications. The differences across studies are likely influenced by variability in inclusion criteria, definitions of chronicity, and referral practices.

AIN has a heterogeneous etiology and may present as an isolated condition (primary AIN) or in association with other autoimmune disorders, infection, drug exposure, malignancy, or occasionally, vaccination (secondary AIN) (1, 17). Primary AIN is the most clinically significant form of acquired neutropenia in children. It typically presents during the first years of life and is driven by autoantibodies directed against human neutrophil antigens (HNA), leading to neutrophil destruction through complement-mediated lysis or splenic phagocytosis of antibody-coated neutrophils (17). The clinical course is largely benign, with few infections, and spontaneous resolution occurs in approximately 90% of affected children within a few years (18). Positive indirect antibody identification supports the diagnosis of AIN in appropriately aged children with a typical clinical history. Antineutrophil antibodies can be detected in 52% to 74% of suspected AIN patients at the first screening and 84% with repeated antibody tests (17, 19).

We identified antineutrophil antibodies in 47% of the cases tested. This slightly lower rate may reflect the use of the MAIGA assay, which has been reported to be less sensitive than the indirect granulocyte immunofluorescence test (GIFT). As GIFT remains the most widely used and validated method for antineutrophil antibody detection, repeated testing in a reference laboratory is recommended when AIN is suspected despite initial negative results (1). Importantly, the limited sensitivity of available assays introduces diagnostic uncertainty and raises the possibility of misclassification between AIN and CIN in antibody-negative cases. This distinction is further complicated by evidence suggesting that AIN and CIN may represent a continuum of the same underlying disorder rather than strictly separate entities. In this context, some patients classified as CIN may represent cases of AIN with undetectable antineutrophil antibodies. Therefore, our findings comparing AIN and CIN should be interpreted with caution, as overlap between these groups cannot be excluded.

As part of the initial diagnostic workup, we performed bone marrow aspiration in 67% of the children. In most cases, the bone marrow

was normocellular, with an adequate reserve of mature neutrophils, and no pattern specifically associated with AIN or CIN was observed. It is well established that bone marrow findings are not diagnostic for AIN. The marrow may be hypercellular or normocellular with myeloid hyperplasia, but it can also appear completely normal. In clinically severe cases, evidence of maturation arrest may be observed (20). Prior to the European guidelines, there was often uncertainty about whether a bone marrow evaluation was necessary. Currently, bone marrow examination is reserved for second-line assessment. Bone marrow aspiration with morphology, flow cytometry, and cytogenetics should be performed in pediatric patients with severe or moderate chronic neutropenia, except in primary AIN with positive antineutrophil antibodies or drug-induced neutropenia; in suspected AIN with negative antibody tests accompanied by recurrent infections; and before the initiation of G-CSF therapy (1).

Congenital neutropenia is a rare cause of isolated neutropenia and is typically associated with impaired production and/or release of neutrophils from the bone marrow. It may occur as an isolated defect or in combination with extra-hematologic manifestations or underlying immunodeficiency/immune dysregulation (1, 21). Many germline forms carry an increased predisposition to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). Clinically, congenital neutropenia often presents with severe, recurrent bacterial infections beginning in early infancy (4, 22, 23). Genetic testing is essential to confirm the diagnosis, assess the risk for MDS/AML, and provide appropriate genetic counseling to the family (1).

In our study, genetic testing was performed in a limited number of patients (N=7) and was restricted to *ELANE* and *HAX1* genes because broader genetic panels were not available at the time, which may have led to underdiagnosis of congenital neutropenia. Current European guidelines recommend comprehensive genetic testing, including NGS panels, particularly when second-level investigations are inconclusive. Furthermore, in young children with a family history of severe neutropenia, typical congenital anomalies, or recurrent severe infections, genetic testing should be expedited and performed after first-level investigations (1). However, the likelihood of a substantial number of undiagnosed congenital cases in our cohort is low, given that 83% of children achieved spontaneous remission during follow-up.

We identified one patient with a genetic syndrome associated with a *MAGEL2* variant and neutropenia present from the age of one month. *MAGEL2* variants have not been reported to be associated with neutropenia; therefore, a causal relationship cannot be established in this case. This finding nonetheless highlights the importance of broad genetic evaluation in patients with suspected congenital neutropenia, particularly when clinical features suggest an underlying syndromic condition.

In terms of clinical course, 31% of cases with chronic neutropenia experienced rehospitalizations, and only 21% required antibiotic therapy. Since these children were under regular clinical supervision and instructed to contact their hematologist for any episode of fever, it is unlikely that febrile events or infections were unrecorded. Short G-CSF therapy was administered in 23% of the cases. None of the children received prophylactic antibiotics or prolonged G-CSF treatment. This observation of a mild clinical course in pediatric AIN/CIN is consistent with previous reports, which similarly described severe infections as uncommon (13, 16, 18). The low incidence of serious infections in these children may be explained by their preserved bone marrow reserves. During a median follow-up of 21 months, 83% of the patients in the present study achieved spontaneous remission, with a median time to remission of 13.5 months. Patients with a lower ANC at diagnosis experienced a longer time to remission than those with a higher ANC.

Our study has several limitations. First, the relatively small sample size limits the statistical power and restricts the ability to detect smaller differences between subgroups. Second, the retrospective design carries the risk of both selection bias and information bias, as the data were dependent on

the completeness and accuracy of medical records. Furthermore, outpatient cases were excluded due to highly heterogeneous approaches in primary care, lack of structured follow-up, and absence of essential clinical data; this may have introduced additional selection bias and limited the external validity and generalizability of our findings. As a result, most analyses are descriptive, and caution is warranted when extrapolating these findings to larger populations. Therefore, comparisons with published studies were used to provide context and highlight trends without overinterpretation. Despite these limitations, our findings underscore the need for standardized guidelines for children with chronic neutropenia. A national study is currently underway to compare clinical practice before and after the publication and broader implementation of consensus-based European guidelines.

Conclusion

Chronic isolated neutropenia in children, including AIN and CIN, generally follows a mild clinical course, with severe infections being uncommon. Most children achieve spontaneous remission within 13.5 months. Lower ANC at diagnosis is associated with more frequent rehospitalizations, longer time to remission, and longer follow-up. The recent European guidelines provide standardized recommendations for the diagnosis and management of pediatric neutropenia, helping to harmonize care and reduce variability in clinical practice.

What is Already Known on This Topic:

Chronic isolated neutropenia typically presents during early childhood. Severe infections are uncommon due to preserved bone marrow reserves, and most children achieve spontaneous remission within 1–2 years. Congenital forms of isolated neutropenia can be associated with syndromes and an increased risk of hematologic malignancies. Until the recent European guidelines, clinical approaches to children with neutropenia were heterogeneous and often guided by individual physician experience.

What This Study Adds:

This is a rare study on pediatric chronic neutropenia that provides a direct comparison of AIN and CIN. It showed similar age at presentation, ANC at diagnosis, and time to remission between the two groups, while a lower ANC predicted more frequent rehospitalizations, longer time to remission, and longer follow-up. The study highlights the importance of the European guidelines in standardizing the diagnosis, follow-up, and management of children with neutropenia.

Authors' Contributions: Conception and design: JR; Acquisition, analysis and interpretation of data: MA, AĐ, and SŠ; Drafting the article: JR and AĐ; Revising it critically for important intellectual content: JR and SŠ; Approved final version of the manuscript: JR, MA, AĐ, and SŠ.

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

References

1. Fioredda F, Skokowa J, Tamary H, Spanoudakis M, Farruggia P, Almeida A, et al. The European Guidelines on Diagnosis and Management of Neutropenia in Adults and Children: A Consensus Between the European Hematology Association and the EuNet-INNOCHRON COST Action. Hemasphere. 2023;7(4):e872. Erratum in: Hemasphere. 2023;7(5):e897. doi: 10.1097/HS9.0000000000000872.
2. Dale DC. How I manage children with neutropenia. Br J Haematol. 2017;178(3):351-63. doi: 10.1111/bjh.14677.
3. James RM, Kinsey SE. The investigation and management of chronic neutropenia in children. Arch Dis Child. 2006;91(10):852-8. doi: 10.1136/adc.2006.094706.
4. Coates TD. Evaluation of neutropenia in children and adolescents. UpToDate [Internet]. 2025 Aug [cited 2025 Nov 18]. Available from: https://www.uptodate.com/contents/evaluation-of-neutropenia-in-children-and-adolescents.
5. Roganovic J, Ricci E, Polychronopoulou S, Fioredda F. Isolated neutropenia preceding acute lymphoblastic leukemia in children. Pediatr Blood Cancer. 2022;69(7):e29518. Epub 2021 Dec 16. doi: 10.1002/pbc.29518.
6. Reynolds S, Ainsworth L, Wan Ariffin E. The child with neutropenia. Paediatrics and Child Health. 2023;33(8):235-9. doi: 10.1016/j.paed.2023.05.003.
7. Frater JL. How I investigate neutropenia. Int J Lab Hematol. 2020;42(Suppl 1):121-32. doi: 10.1111/ijlh.13210.
8. Newburger PE, Dale DC. Evaluation and management of patients with isolated neutropenia. Semin Hematol. 2013;50(3):198-206. doi: 10.1053/j.seminhematol.2013.06.010.
9. Thomas AE, Simpson LA. A step-by-step approach to paediatric neutropenia. Paediatr Child Health. 2017;27(11):511-6. doi: 10.1016/j.paed.2017.07.006.
10. Walkovich K, Boxer LA. How to approach neutropenia in childhood. Pediatr Rev. 2013;34(4):173-84. doi: 10.1542/pir.34-4-173.
11. Angelino G, Caruso R, D'Argenio P, Calò Carducci FI, Pascone R, Lanciotti M, et al. Etiology, clinical outcome, and

laboratory features in children with neutropenia: analysis of 104 cases. Pediatr Allergy Immunol. 2014;25(3):283-9. doi: 10.1111/pai.12177.

12. Nagalapuram V, McCall D, Palabindela P, Howard TH, Bemrich-Stolz C, Lebensburger J, et al. Outcomes of Isolated Neutropenia Referred to Pediatric Hematology-Oncology Clinic. Pediatrics. 2020;146(4):e20193637. doi: 10.1542/peds.2019-3637.
13. Ozdemir ZC, Kar YD, Kasaci B, Bor O. Etiological causes and prognosis in children with neutropenia. North Clin Istanb. 2021;8(3):236-42. doi: 10.14744/nci.2020.65624.
14. Akyol Ş, Tüfekçi Ö, Yılmaz Ş, Ören H. Clinical spectrum of pediatric neutropenia: mostly benign, but not to be overlooked. Turk J Pediatr. 2025;67(4):465-72. doi: 10.24953/turkjpediatr.2025.6175.
15. Tantawy AA, Sallam TH, Ibrahim DM, Sallam MT, Ragab IA. Pathogenesis and prognosis of neutropenia in infants and children admitted in a university children hospital in Egypt. PediatrHematol Oncol. 2013;30(1):51-9. doi: 10.3109/08880018.2012.743199.
16. Wan C, Yu HH, Lu MY, Lee JH, Wang LC, Lin YT, et al. Clinical manifestations and outcomes of pediatric chronic neutropenia. J Formos Med Assoc. 2012;111(4):220-7. doi: 10.1016/j.jfma.2010.12.003.
17. Cattaneo A, Porretti L. Laboratory testing for the diagnosis of neonatal and pediatric immune neutropenias: a narrative review. Pediatric Medicine. 2022;5. doi: 10.21037/pm-21-43.
18. Farruggia P, Dufour C. Diagnosis and management of primary autoimmune neutropenia in children: insights for clinicians. Ther Adv Hematol. 2015;6(1):15-24. doi: 10.1177/2040620714556642.
19. Porretti L, Farruggia P, Colombo FS, Cattaneo A, Ghilardi R, Mirra N, et al. Diagnostic value of cell bound and circulating neutrophil antibody detection in pediatric neutropenia. Pediatr Blood Cancer. 2018;65(4). doi: 10.1002/pbc.26904.
20. Capsoni F, Sarzi-Puttini P, Zanella A. Primary and secondary autoimmune neutropenia. Arthritis Res Ther. 2005;7(5):208-14. doi: 10.1186/ar1803.
21. James RM, Phillips B. Fifteen-minute consultation: A guide to managing a child with a new finding of neutropenia. Arch Dis Child Educ Pract Ed. 2019;104(6):282-5. Epub 2019 Mar 4. doi: 10.1136/archdischild-2019-316829.
22. Donadieu J, Fenneteau O, Beaupain B, Mahlaoui N, Chantelot CB. Congenital neutropenia: diagnosis, molecular bases and patient management. Orphanet J Rare Dis. 2011;6:26. doi: 10.1186/1750-1172-6-26.
23. Dufour C, Miano M, Fioredda F. Old and new faces of neutropenia in children. Haematologica. 2016;101(7):789-91. doi: 10.3324/haematol.2016.142760.

Supplementary Material

Supplementary Table 1. Correlations Between Age at Diagnosis, Absolute Neutrophil Count at Diagnosis, Time to Remission, and Follow-up Duration

Variable	Age	ANC	Time to remission	Follow-up duration
Age	1.000	r_s=0.091 P=0.539	r_s=0.171 P=0.290	r_s=0.139 P=0.346
ANC	–	1.000	r_s=-0.112 P=0.492	r_s=-0.395* P=0.005
Time to remission	–	–	1,000	r_s=0.550** P<0.001
Follow-up duration	–	–	–	1.000

ANC=Absolute neutrophil count; rs=Spearman's correlation coefficient; P=Statistical significance (*P<0.05; **P<0.01).

Supplementary Table 2. Differences in Absolute Neutrophil Count Between Groups According to Rehospitalization

Variable	Rehospitalization	N	C (25-75)	Test	P
ANC	No	27	700 (280-1250)	171.500	0.020*
	Yes	21	348 (98.5-630)	–	–

ANC=Absolute neutrophil count; N=Number; C (25–75)=Median (25th–75th percentile); Mann-Whitney U test; P=Statistical significance (P<0.05).

Original Research Article
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Genomic and Molecular Characterisation of Respiratory Syncytial Virus in the National SARI Surveillance System Across Three Consecutive Seasons in North Macedonia (2022-2025)

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Abstract

Objective. To describe the epidemiological, molecular, and genomic characteristics of respiratory syncytial virus (RSV) detected through the national severe acute respiratory infection (SARI) surveillance system in North Macedonia across three consecutive seasons (2022/2023-2024/2025). **Materials and Methods.** This study analysed SARI surveillance data from seven hospital-based sentinel sites. RSV detection and subgroup determination were performed using reverse transcription quantitative polymerase chain reaction (RT-qPCR), following season-specific testing strategies, which should be considered when interpreting seasonal differences in RSV positivity. Consensus genomes were generated, and phylogenetic analyses were performed using contemporaneous European reference sequences. Amino acid substitutions in the G and F proteins were assessed descriptively. **Results.** Among 1,899 laboratory-tested SARI cases, 53.3% were tested for RSV, of which 24.8% were positive. RSV-A predominated overall, although seasonal shifts in subgroup dominance were observed. RSV infections were concentrated among young children, particularly those aged 0-2 years. Whole-genome sequencing of 85 RSV-positive samples revealed a dynamic seasonal turnover of RSV-A lineages, whereas RSV-B circulation remained largely confined to a single dominant lineage across seasons. Analysis of antigenically important genes showed greater variability in the G gene compared with the more conserved F gene in both subgroups. **Conclusion.** The integration of epidemiological, molecular, and whole-genome data within routine SARI surveillance provides a comprehensive overview of RSV circulation and evolution in North Macedonia. These findings contribute genomic data from an under-represented region and provide a foundation for future evaluation of RSV vaccination and monoclonal antibody effectiveness by detecting viral genetic changes, should such measures be implemented in North Macedonia.

Key Words: Respiratory Syncytial Virus ▪ SARI ▪ Genomic Surveillance ▪ Whole-Genome Sequencing ▪ Molecular Epidemiology.

Introduction

Respiratory syncytial virus (RSV) represents a major public health threat worldwide due to its ability to cause seasonal epidemics and severe respiratory disease, particularly among infants, young children, older adults, and immunocompromised individuals. RSV is responsible for more than 3.6 million hospitalisations and approximately 100,000 deaths annually among children under five years of age worldwide (1).

RSV is an enveloped, negative-sense, single-stranded RNA virus belonging to the *Pneumoviridae* family and *Orthopneumovirus* genus. The viral genome encodes multiple structural and non-structural proteins; however, the fusion (F) and attachment (G) glycoproteins are particularly important due to their central roles in viral entry and immune recognition (2). Both proteins are the main targets of neutralising antibodies and are therefore critical for the development and evaluation of RSV

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preventive strategies, with the F glycoprotein representing a key target of current immunoprophylactic approaches. The F protein mediates fusion between the viral and host cell membranes and is less variable at the sequence level than the G protein. In contrast, the G protein is characterised by extensive genetic diversity and plays a key role in antigenic variation and immune evasion.

Whole-genome sequencing provides critical insights into RSV transmission dynamics, genetic diversity, and the seasonal replacement of circulating strains. In particular, G gene analysis remains central to RSV molecular surveillance, as genotype and lineage assignments are primarily based on sequence variability within this region. Based on antigenic and genetic differences in the attachment (G) glycoprotein, RSV is classified into two major subgroups: RSV-A and RSV-B. Both subgroups co-circulate globally and are further divided into multiple genotypes and lineages; however, one subgroup often predominates during a given season, with shifts in the dominant subgroup frequently observed between consecutive seasons (3). Previous large-scale analyses of RSV genetic diversity have shown that among the numerous genotypes described, RSV-A NA1 (including ON1 viruses) and RSV-B BA genotypes have been the most frequently detected globally in recent decades, coinciding with a reduction in overall genotype diversity since the early 2000s (4).

Lessons learned from previous influenza pandemics and, more recently, from the coronavirus disease 2019 (COVID-19) pandemic, underscore the critical importance of integrated respiratory virus surveillance systems for timely detection and effective public health responses. In North Macedonia, these experiences served as a major impetus for establishing the national SARI surveillance system, which began operating in an initial form during the 2014/2015 season and has progressively expanded and strengthened over time. As a result of this gradual development, the national SARI surveillance system has evolved into a comprehensive framework for monitoring severe respiratory infections, supporting enhanced characterisation of circulating respiratory viruses, and providing a foundation for molecular and genomic surveillance activities.

Publicly available sequence repositories provide an important overview of the geographic distribution of RSV genomic data and reveal substantial heterogeneity in data availability across Europe. In this context, Southeast Europe and the Western Balkans remain less well represented compared with other European regions, and RSV genomic data generated through the national SARI surveillance system in North Macedonia contribute valuable information to improve regional representation and understanding of RSV circulation.

In parallel with advances in RSV surveillance, important progress has also been made in RSV prevention. In the European context, currently authorised immunisation products include the long-acting monoclonal antibody Beyfortus (*nirsevimab*) for infant protection and the monoclonal antibody Synagis (*palivizumab*) for selected children at high risk of severe RSV disease. Vaccination strategies include Abrysvo, authorised for maternal immunisation and older adults, as well as Arexvy and mRESVIA, which are also authorised for older adults. In addition, Enflonsia (*clesrovimab*), another long-acting monoclonal antibody for infant protection, had previously received a positive opinion from the European Medicines Agency Committee for Medicinal Products for Human Use and was subsequently authorised by the European Commission in April 2026 (5). Across Europe, the implementation of these products varies according to national epidemiological and economic considerations; however, access remains limited in many settings, highlighting the ongoing need for RSV surveillance (6).

This study aimed to describe the epidemiological, molecular, and genomic characteristics of respiratory syncytial virus detected through the national SARI surveillance system in North Macedonia across three consecutive seasons (2022/2023, 2023/2024, and 2024/2025).

Materials and Methods

Study Design, SARI Surveillance System, and Case Definition

This study analysed data generated through the national SARI surveillance system in North Macedonia across three consecutive RSV seasons (2022/2023, 2023/2024, and 2024/2025). Within the SARI surveillance framework, seven hospital-based sentinel sites nationwide submit nasopharyngeal swabs collected in universal transport medium to the Virology Laboratory at the Institute of Public Health of North Macedonia, which serves as the national reference laboratory and performs the laboratory component of SARI surveillance.

The SARI surveillance system in North Macedonia was established in its initial form during the 2014/2015 season and was initially implemented primarily for the monitoring of influenza. During the COVID-19 pandemic, the system was expanded to include severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and since 2022, RSV testing has been incorporated into the SARI surveillance framework. Over time, this system has evolved beyond basic molecular detection. Currently, it integrates influenza virus typing and subtyping, RSV subgroup determination, and whole-genome sequencing of a subset of positive samples across respiratory pathogens covered by the surveillance system, thereby supporting the genomic surveillance of these pathogens.

Laboratory testing results generated through SARI surveillance are routinely communicated to the Department for Epidemiology of Infectious Diseases of the Institute of Public Health for surveillance data analysis. SARI cases were defined in accordance with national surveillance protocols and international guidelines, aligned with the World Health Organisation (WHO) surveillance case definition of SARI (7).

RSV Testing Algorithm

All specimens were initially tested using a multiplex RT-qPCR assay for SARS-CoV-2, Influenza A, and Influenza B. RSV RT-qPCR testing was performed and applied according to season-specific testing strategies, as summarised in Figure 1. During the 2022/2023 season, RSV testing was performed based on clinical indication. In the 2023/2024 season, RSV testing was performed on all SARI samples that tested negative for both SARS-CoV-2 and influenza viruses. In the 2024/2025 season, RSV testing was prioritised for patients with SARI who belonged to high-risk age groups, specifically children under four years of age and adults aged 60 years and older.

RSV subgroup determination was performed using RT-qPCR either during initial RSV detection using assays capable of subgroup differentiation or by subsequent testing with an available RT-qPCR assay allowing RSV subgroup identification. Whole-genome sequencing was performed on a selected subset of RSV-positive samples. Priority was given to samples with cycle threshold (Ct) values ≤30, although not all RSV-positive samples meeting this criterion were sequenced. Among the eligible samples, the selection further aimed to include specimens collected at different time points within each RSV season. Because different RT-qPCR assays were used during the study period, the Ct threshold for sequencing selection was applied across heterogeneous assays, and cross-kit Ct equivalence was not formally validated.

To provide context on the representativeness of the sequencing subset, sequenced and non-sequenced RSV-positive samples were compared based on Ct value distribution, age group, collection period, and RSV subgroup (Supplementary Table S1).

Laboratory Methods

Viral RNA extraction was performed using either the QIAamp Viral RNA Mini Kit (Qiagen; REF:52906) or the MagCore Viral Nucleic Acid Extraction Kit High Sensitivity (RBC Bioscience; REF: MVN400-05) (8, 9).

RSV detection was performed by RT-qPCR using commercially available assays, with different kits used during the study period depending on availability, including the RevoDx RSV qPCR

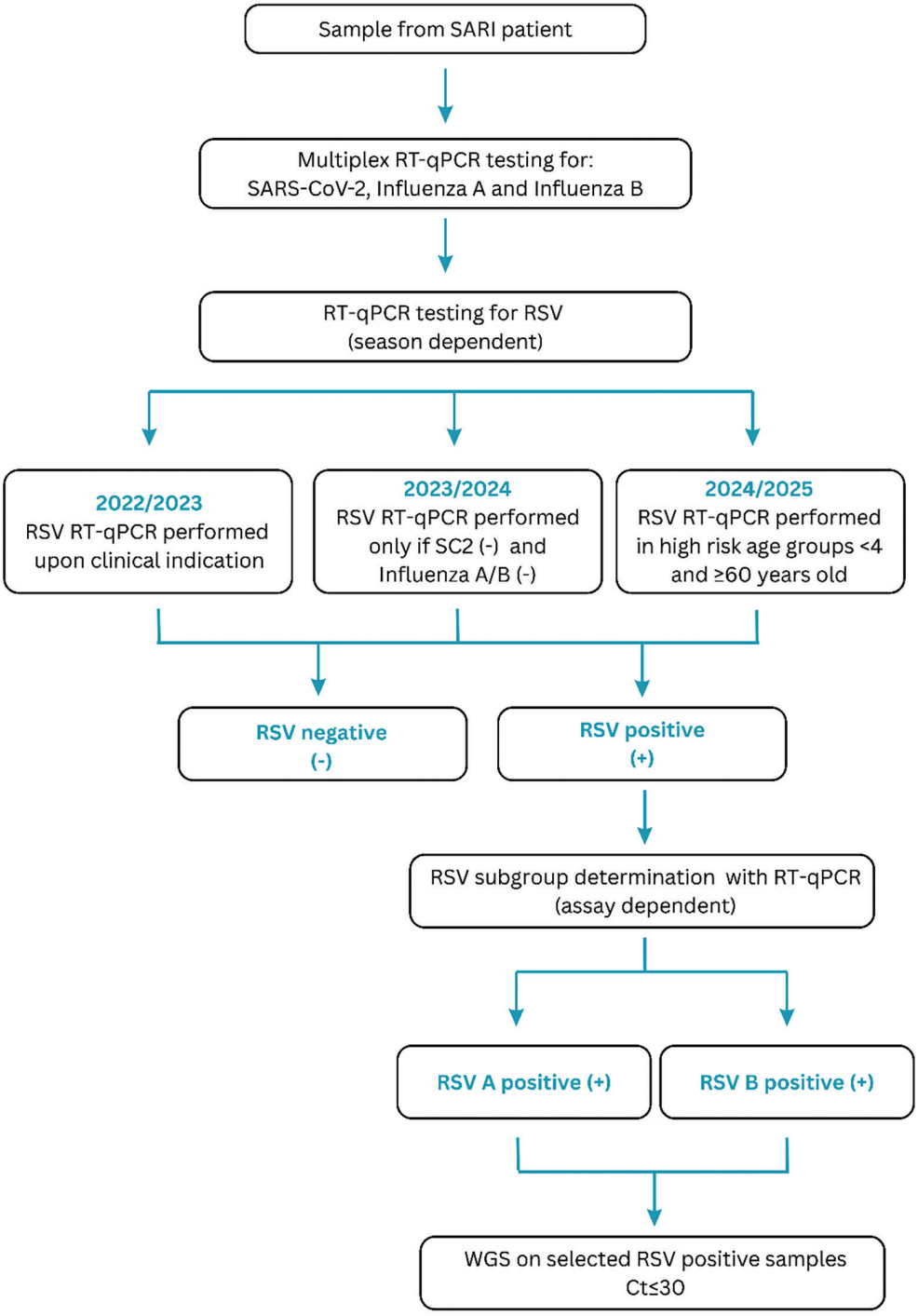


Figure 1. Overview of the RSV testing algorithm applied within the national SARI surveillance system in North Macedonia across three consecutive seasons (2022/2023 – 2024/2025). All respiratory specimens were initially tested for SARS-CoV-2 and influenza A/B using multiplex RT-qPCR. RSV testing criteria differed by season, and RSV-positive samples were subsequently subjected to subgroup determination and whole-genome sequencing.

Kit (Idil Biotech; REF: IP202231-100) (10), FTD Respiratory Pathogens 33 (Fast Track Diagnostics, Luxembourg; REF: FTD-2P.3-64-RUO) (11), RealStar RSV RT-PCR Kit 3.0 (Altona; REF: 193013) (12), and the Respiratory Syncytial Virus Multiplex Real-time RT-PCR Kit (Centers for Disease Control and Prevention - CDC; REF: GR-1365); the Altona and CDC assays additionally enabled RSV subgroup determination. Real-time polymerase chain reaction (PCR) was performed using QuantStudio5 (Applied Biosystems) and CFX96 (Bio-Rad) PCR thermocyclers.

Whole-genome sequencing was performed using a hybridisation-capture-based enrichment approach. Sequencing libraries were prepared using the Illumina DNA Prep with Enrichment kit in combination with the Respiratory Virus Oligos Panel v2 (Illumina) and sequenced on an Illumina MiniSeq platform (13). Library quantification was performed using a Qubit4 Fluorimeter with the Qubit 1X dsDNA High Sensitivity Assay Kit (Invitrogen; Q33231). All assays and kits were used according to the manufacturers' instructions.

Bioinformatic Analysis

Raw sequencing data (FASTQ files) generated from RSV-positive samples were processed for quality control, reference-based genome assembly, and the generation of consensus sequences. During the initial phase of the study, raw read processing and consensus generation were performed using UGENE (v45.0), specifically the *Raw DNA-seq data processing* and *Extract consensus from assemblies* tools (14). Subsequently, a more automated and standardised bioinformatic workflow was implemented, and raw sequencing data were processed using the INSaFLU platform for quality control, reference-based genome assembly, and consensus sequence generation (15). The bioinformatic approach used for each sequence included in the study is indicated in Supplementary Table S2. Reference-based genome assembly was performed using hRSV/A/England/397/2017 (EPI_ISL_412866) and hRSV/B/Australia/VIC-RCH056/2019 (EPI_ISL_1653999) as the reference sequences for RSV-A and RSV-B, respectively.

For phylogenetic analyses, contemporaneous European reference sequences were retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) EpiRSV database to provide epidemiological context. Sequences collected between 29 November 2021 and 7 April 2025 were selected from European countries with publicly available data, with selection guided by the aim of including the same or closely related lineages as those identified among the Macedonian sequences, based on Nextclade lineage assignment. Only sequences with an overall good quality control (QC) status in Nextclade were included. In total, 21 reference sequences were included, comprising 12 RSV-A and 9 RSV-B sequences. The reference sequences were downloaded from GISAID in November 2025, and their accession IDs and corresponding information are provided in Supplementary Table S3.

Multiple sequence alignment of RSV consensus genomes was performed using MAFFT v7.526 (16). Prior to downstream analyses, consensus genome quality was assessed using Nextclade v3.18.1 (17), which evaluates sequence quality based on flagged issues, including missing data (Ns), excess private mutations, mixed sites, frameshifts, and stop codons. No separate study-specific numeric QC threshold was applied; genomes were retained for downstream analyses if assigned an overall QC status of good by Nextclade. Nextclade was also used to assign RSV genetic clades and lineages. Phylogenetic trees were inferred using FastTree v2.1.10 under a generalised time-reversible (GTR) nucleotide substitution model with CAT rate heterogeneity approximation (18). Branch support was assessed using SH-like local support values, as implemented in FastTree. Nodes with SH-like local support values ≥0.80 were considered well supported. Phylogenetic trees were visualised and annotated using the Interactive Tree of Life (iTOL) platform (19).

Ethical Statement

Informed consent was not required, as the study was conducted within the framework of the national SARI surveillance system, and all data and specimens were analysed anonymously in accordance with national surveillance protocols.

Statistical Analysis

Descriptive statistical analyses were performed to summarise the epidemiological and laboratory data. Categorical variables are presented as counts and percentages. No inferential statistical tests were applied, and no predefined level of statistical significance (P-value) was specified, as the study was descriptive. Statistical analyses were performed using Microsoft Excel 2019 (Microsoft Corp.).

Data Availability

All 85 RSV consensus genome sequences analysed in this study are publicly available in the GISAID EpiRSV database. Their GISAID accession IDs and corresponding sample information are provided in Supplementary Table S2.

Results

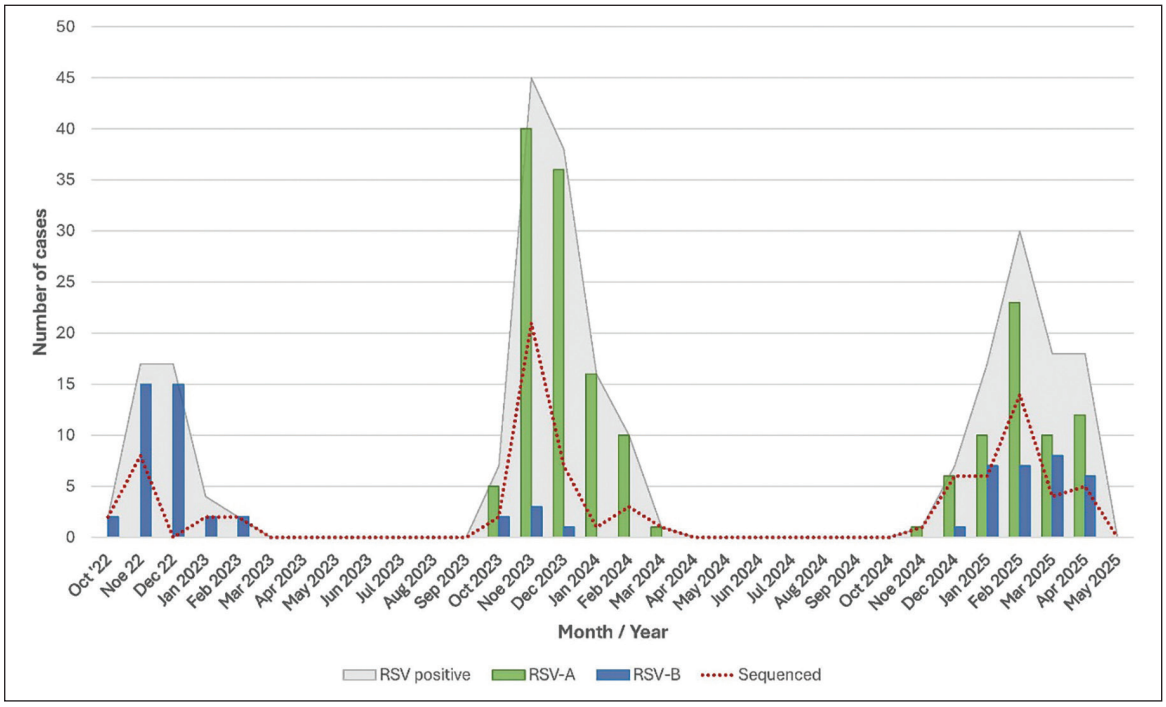
Epidemiological Characteristics of RSV Within the SARI Surveillance System

During three consecutive RSV seasons (2022/2023, 2023/2024, and 2024/2025), a total of 1,899 SARI cases were laboratory-tested within the national SARI surveillance system in North Macedonia, of which 53.3% (N=1012) were tested for RSV. Overall, RSV was detected in 24.8% (N=251) of the tested SARI samples. In the 2023/2024 and 2024/2025 seasons, SARI surveillance was conducted year-round; to ensure uniformity across seasons, all seasonal comparisons in the analysis refer to weeks 40-20.

The 2022/2023 season was the first season in which RSV testing was incorporated into the national SARI surveillance system. RSV-positive cases were identified in all three analysed surveillance seasons, with variations observed in both testing coverage and RSV positivity rates. As RSV testing strategies differed by season, the epidemiological patterns described below, particularly testing coverage and RSV positivity rates, should be interpreted in that context. During the 2022/2023 season, RSV testing was performed for 16.2% (N=94) of all reported SARI cases, of which 44.7% (N=42) tested positive for RSV. In contrast, during the 2023/2024 season, which was characterised by the highest testing coverage, 75.8% (N=589) of SARI cases were tested for RSV, with 20.0% (N=118) of the tested samples yielding positive results. In the 2024/2025 season, 60.9% (*N*=329) of SARI cases were tested for RSV, of which 27.7% (N=91) were RSV-positive. Across all three seasons, the majority of RSV-positive SARI cases were classified as RSV-A, accounting for 67.3% (N=169), whereas RSV-B accounted for 27.9% (*N*=70). RSV subgroup was not determined in 4.4% (N=11) of cases, and one case was identified as a co-infection with both RSV-A and RSV-B.

When analysed by season, distinct patterns of RSV subgroup dominance were observed (Figure 2). During the 2022/2023 season, RSV-B was the predominant subgroup, detected in 85.7% (N=36) of cases. The remaining six RSV-positive cases in 2022/2023 had an undetermined subgroup status, and no RSV-A cases were identified in that season. In contrast, during the 2023/2024 and 2024/2025 seasons, co-circulation of both RSV subgroups was observed, with a clear predominance of RSV-A. In the 2023/2024 season, RSV-A accounted for 90.7% (N=107) of the RSV-positive cases. During the 2024/2025 season, a more balanced distribution between the two RSV subgroups was observed; although RSV-A remained predominant, accounting for 68.1% (N=62) of cases compared to 31.9% (N=29) for RSV-B.

As shown in Figure 2, RSV activity followed a typical seasonal pattern, with the highest activity observed during the winter months. In the 2022/2023 and 2023/2024 seasons, peak RSV activity occurred in November and December, whereas during the 2024/2025 season, a delayed peak was observed in February.



Monthly distribution of laboratory-confirmed RSV-positive SARI cases detected through the national SARI surveillance system in North Macedonia from October 2022 to May 2025. Total RSV-positive cases are shown in grey, with RSV-A and RSV-B cases indicated in green and blue, respectively; in 2022/2023, RSV-positive cases not shown as RSV-B had an undetermined subgroup status. The red dotted line represents the number of RSV-positive samples selected for whole genome sequencing. Interseasonal periods are shown for completeness; however, seasonal comparisons were restricted to weeks 40-20, as described in the Methods. RSV testing differed by season (see Figure 1); therefore, seasonal positivity patterns are not directly comparable.

Figure 2. Seasonal distribution of RSV-positive SARI cases and sequenced samples across three consecutive seasons.

Among the RSV-positive SARI cases, 51.0% (N=128) occurred in males and 49.0% (N=123) in females. RSV-positive cases were predominantly observed among young children, with the highest burden in the youngest age groups. Children aged 0-2 years accounted for 90.0% (N=226) of all RSV-positive cases, followed by those aged 3-4 (4.8%; N=12) and 5-14 years (3.6%; N=9). RSV infections were rare among adults, with one case (0.4%) identified in the 30-64 years age group and three cases (1.2%) among individuals aged 65-79 years, while no RSV-positive cases were detected in the 15-29 or ≥80 years age groups.

Since RSV detections originated from SARI surveillance, cough and fever (≥38°C) were consistently reported among all RSV-positive cases. Beyond these core SARI manifestations, rhinitis/coryza and dyspnoea were commonly observed. Gastrointestinal symptoms (diarrhoea and vomiting/nausea) were less frequent, whereas systemic symptoms such as headache, myalgia, and arthralgia occurred in a smaller proportion of cases. The detailed demographic and epidemiological characteristics of the RSV-positive SARI cases are summarised in Table 1.

Whole-Genome Sequencing

Whole-genome sequencing was successfully performed on 85 RSV-positive samples, representing a subset of cases selected across all three surveillance seasons. Sample selection aimed to ensure temporal coverage by including specimens collected during different months within each season.

Among the sequenced samples, 57 were classified as RSV-A and 28 as RSV-B. The cycle threshold (Ct) values of the sequenced samples ranged from 16 to 30.7. All 85 consensus genomes obtained in this study had an overall good QC status in Nextclade and were therefore included in the

Table 1. Characteristics of RSV-Positive Cases Detected Through the National SARI Surveillance System in North Macedonia, 2022/2023 – 2024/2025

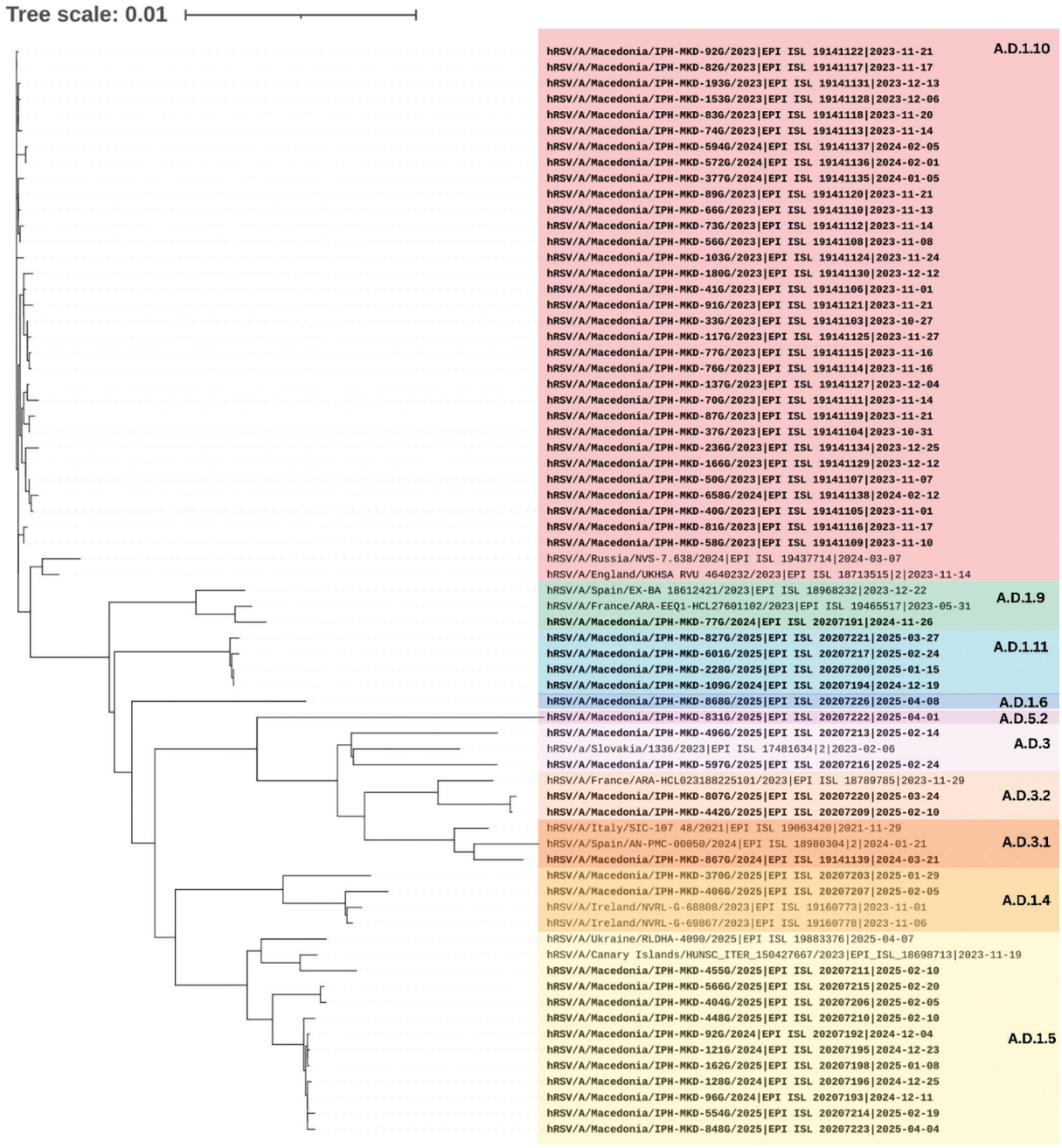
Season	2022/2023	2023/2024	2024/2025	Total
Total tested SARI cases (N)	582	777	540	1899
SARI cases tested for RSV (N; %)	94 (16.2)	589 (75.8)	329 (60.9)	1012 (53.3)
RSV-positive cases (N; %)	42 (44.7)	118 (20.0)	91 (27.7)	251 (24.8)
RSV-positive cases sequenced (N; %)	14 (33.3)	35 (29.7)	36 (39.6)	85 (33.9)
RSV Subgroup (N; %)				
RSV A	0 (0.0)	107 (90.7)	62 (68.1)	169 (67.3)
RSV B	36 (85.7)	5 (4.2)	29 (31.9)	70 (27.9)
RSV A/B coinfection	0 (0.0%)	1 (0.8%)	0 (0.0)	1 (0.4)
Undetermined subgroup	6 (14.3)	5 (4.2)	0 (0.0)	11 (4.4%)
Sex (N; %)				
Male	27 (64.3)	63 (53.4)	38 (41.8)	128 (51.0)
Female	15 (35.7)	55 (46.6)	53 (58.2)	123 (49.0)
Age group (N; %)				
0-2	34 (81.0)	104 (88.1)	88 (96.7)	226 (90.0)
3-4	5 (11.9)	7 (5.9)	0 (0.0)	12 (4.8)
5-14	3 (7.1)	6 (5.1)	0 (0.0)	9 (3.6)
15-29	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
30-64	0 (0.0)	1 (0.8)	0 (0.0)	1 (0.4)
65-79	0 (0.0)	0 (0.0)	3 (3.3)	3 (1.2)
80+	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Symptoms (N; %)				
Cough + fever ≥38°C	42 (100.0)	118 (100.0)	91 (100.0)	251 (100.0)
Rhinitis/Coryza	36 (85.7)	98 (83.1)	70 (76.9)	204 (81.3)
Dyspnoea	37 (88.1%)	105 (89.0)	76 (83.5)	218 (86.9)
Diarrhoea	2 (4.8)	15 (12.7)	8 (8.8)	25 (10.0)
Vomiting/Nausea	11 (26.2)	25 (21.2)	13 (14.3)	49 (19.5)
Headache	4 (9.5)	7 (5.9)	5 (5.5)	16 (6.4)
Myalgia	7 (16.7)	9 (7.6)	4 (4.4)	20 (8.0)
Arthralgia	4 (9.5)	5 (4.2)	5 (5.5)	14 (5.6)

downstream analyses. Accordingly, the same set of 85 genomes was used for both phylogenetic and substitution analyses.

Phylogenetic Analysis

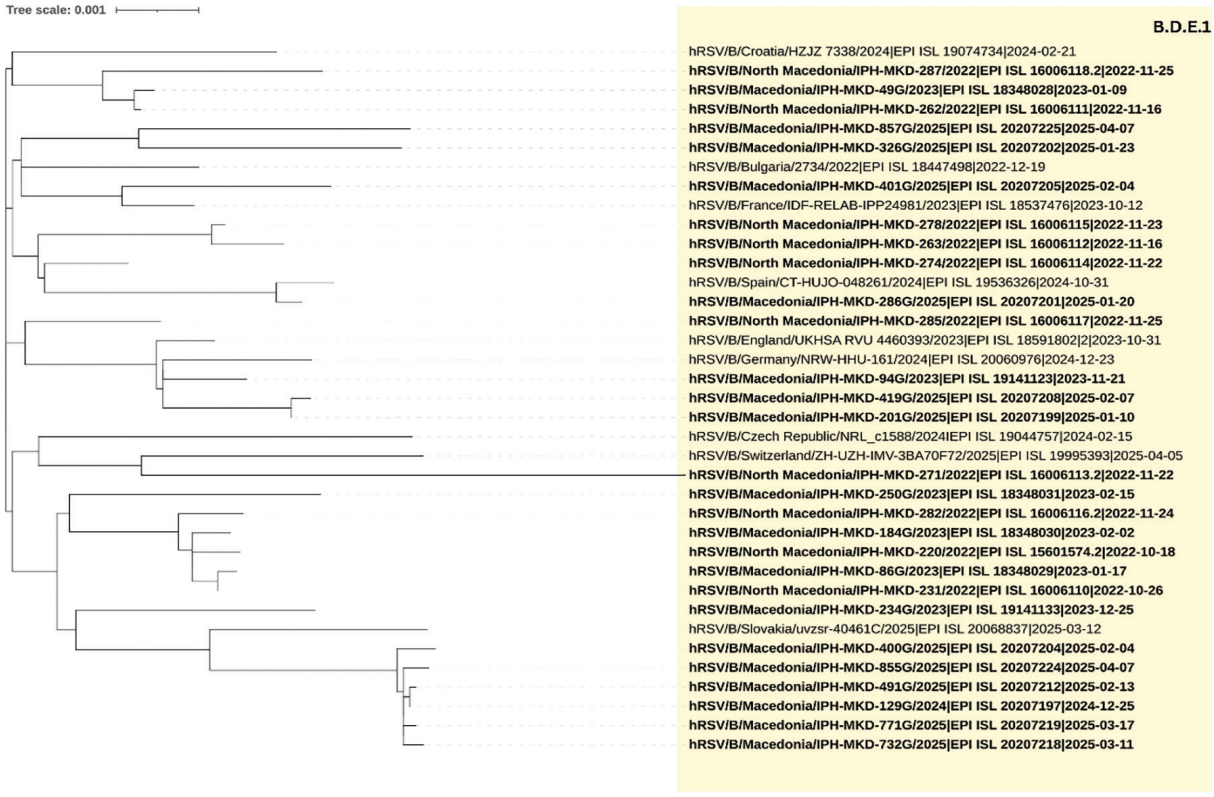
Phylogenetic analysis was performed on 85 RSV whole-genome sequences to describe the genetic relationships of Macedonian RSV strains across the three surveillance seasons, in the context of contemporaneous European reference sequences. As shown in Figure 3, Macedonian RSV-A isolates clustered within the ON1 genotype and demonstrated seasonal differences in clade distribution. No RSV-A cases were identified in the 2022/2023 season; therefore, the RSV-A phylogenetic analysis included only sequences from the 2023/2024 and 2024/2025 seasons. In 2023/2024, almost all RSV-A sequences were assigned to clade A.D.1.10 (32/33; 97.0%), and a single sequence was assigned to A.D.3.1. (1/33; 3.0%). In 2024/2025, a broader clade diversity was observed, with the

predominance of A.D.1.5 (11/24; 45.8%), followed by A.D.1.11 (4/24; 16.7%). Additional clades were detected at lower frequencies, including A.D.1.4 (2/24; 8.3%) and A.D.3.2. (2/24; 8.3%), while A.D.1.9, A.D.3, A.D.3.11, A.D.5.2, and A.D.1.6 were each represented by a single sequence (1/24; 4.2%). Overall, RSV-A showed higher genetic diversity, as reflected by a wider range of detected clades, whereas RSV-B displayed comparatively lower diversity.



Phylogenetic tree of RSV-A whole-genome sequences generated in this study and 12 selected contemporaneous European reference strains. Macedonian isolates cluster within multiple RSV-A genetic lineages across the three seasons, demonstrating lineage turnover between seasons. Distinct genetic lineages are indicated by different colours and labelled alongside the tree. Macedonian isolates are shown in bold. SH-like local support values were computed using FastTree but are not shown on the tree for clarity; values ≥0.80 were considered well supported.

Figure 3. Phylogenetic analysis of RSV-A genomes.



Phylogenetic tree of RSV-B whole-genome sequences generated in this study and 9 selected contemporaneous European reference strains. Macedonian isolates cluster predominantly within a single dominant RSV-B genetic lineage (B.D.E.1) across all three seasons, indicating more stable circulation compared with RSV-A. Genetic lineages are indicated by colour coding and labelled next to the tree. Macedonian isolates are shown in bold. SH-like local support values were computed using FastTree but are not shown on the tree for clarity; values ≥0.80 were considered well supported.

Figure 4. Phylogenetic analysis of RSV-B genomes.

Amino Acid Substitutions in G and F Proteins

Amino acid substitutions in the antigenically important G and F proteins were assessed in RSV-A (N=57) and RSV-B (N=28) whole-genome sequences. Substitutions were summarised according to their frequency across genomes (*n*/*N, %*) and are presented in Table 2. Singleton substitutions were excluded from the main summary to maintain a focus on recurrent substitutions. The bolded numbers in Table 2 indicate the total number of amino acid substitutions identified for each gene and RSV subgroup.

G protein

Across RSV-A genomes (N=57), multiple amino acid substitutions were identified in the G protein, occurring at varying frequencies (Table 2). One substitution, H90Y, was present in all RSV-A genomes (57/57; 100%). Several substitutions were detected at high frequency (≥80%), including I134K, K262E, and I265L (each 56/57; 98.25%), P71L and D284G (each 55/57; 96.49%), Y304H (54/57; 94.74%); S243I (53/57; 92.98%), L142S and T320A (each 51/57; 89.47%), and L101F (46/57; 80.70%). Substitutions detected at intermediate frequency (31-79%) included G224E (44/57; 77.19%), V225A (39/57; 68.42%), A116V, P143L, P234L, and Y273H (each 32/57; 56.14%), L247P, H266Q, and Y297H (each 31/57; 54.39%), and G272D (28/57; 49.12%). Additional substitutions detected at lower frequencies included G224V and T319I (each 12/57; 21.05%), L101S (11/57; 19.30%), V131A (8/57; 14.04%), P120T, N135S, L314P (each 7/57; 12.28%), T113I, V131D, N178G,

Table 2. Frequency Distribution of Amino Acid Substitutions in G and F Genes of RSV-A and RSV-B Genomes

Subgroup / gene	High frequency (80-100%)	Intermediate frequency (31-79%)	Low frequency (0-30%)
RSV-A / G (N=57)	11; H90Y (100%), I134K K262E, and I265L (98.25% each), P71L and D284G (96.49% each), Y304H (94.74%), S243I (92.98%), L142S and T320A (89.47% each), and L101F (80.70%)	10; G224E (77.19%), V225A (68.42%), A116V, P143L, P234L, and Y273H (56.14% each), L247P, H266Q, and Y297H (54.39% each), and G272D (49.12%)	91; highest within this bin: G224V and T319I (21.05% each), L101S (19.30%), V131A (14.04%), P120T N135S, and L314P (12.28% each), T113I, N178G, P276L, V131D, H258Q, and H266L (8.77% each), I83V and E295V (7.02% each), P88S and E295K (5.26% each), and T4N, A57T, I75T, N103T, A122V, I133V, S144I, P217S, P230L, P230T, T245I, G254R, S270P, Y280H, S294P, P298L, P298S, and L310P (3.51% each)
RSV-A / F (N=57)	0	1; S377N (59.65%)	16; V127I (19.30%), L375I (15.79%), T12I (8.77%), A518V and L119H (7.02% each), S276N (5.26%), L3S (3.51%)
RSV-B / G (N=28)	11; A74V and I252T (100% each), S100G, T131A, and I137T (96.43% each), P214S, P221L, and I268T (92.86% each), K256N and S275P (89.29% each), and Y285H (85.71%)	0	49; highest within this bin: S265P (21.43%), E303K (14.29%), S267P (10.71%), and T78P, S138P, T253I, and L284F (7.14% each)
RSV-B / F (N=28)	3; S190N, S211N and S389P (100% each)	1; R42K (46.43%)	13; N105S (21.43%), I292V and C550F (7.14% each)

Note: Only substitutions detected in at least two genomes are shown; singleton substitutions were excluded.

H258Q, H266L, and P276L (each 5/57; 8.77%), I83V, E295V (each 4/57; 7.02%), P88S and E295K (each 3/57; 5.26%), and T4N, A57T, I75T, N103T, A122V, I133V, S144I, P217S, P230L, P230T, T245I, G254R, S270P, Y280H, S294P, P298L, P298S, and L310P (each 2/57; 3.51%).

In RSV-B genomes (N=28), two G protein substitutions, A74V and I252T, were present in all sequences (28/28; 100%). Additional high-frequency substitutions included S100G, T131A, and I137T (each 27/28; 96.43%), P214S, P221I, and I268T (each 26/28; 92.86%), K256N and S275P (each 25/28; 89.29%), and Y285H (24/28; 85.71%). Substitutions detected at lower frequency included S265P (6/28; 21.43%), E303K (4/28; 14.29%), S267P (3/28; 10.71%), and T78P, S138P, T253I, and L284F (each 2/28; 7.14%).

F protein

In RSV-A genomes (N=57), no F protein substitution was detected in ≥80% of sequences. One substitution, S377N, occurred at an intermediate frequency (34/57; 59.65%). Additional substitutions detected at lower frequencies included V127I (11/57; 19.30%), L375I (9/57; 15.79%), T12I (5/57; 8.77%), A518V, and L119H (each 4/57; 7.02%), S276N (3/57; 5.26%), and L3S (2/57; 3.51%). Notably, S276N (3/57; 5.26%) maps within the F protein antigenic site II region targeted by palivizumab.

Among RSV-B genomes (N=28), three F protein substitutions – S190N, S211N, and S389P – were present in all sequences (28/28; 100%). One additional substitution, R42K, was detected at an intermediate frequency (13/28; 46,43%). Lower-frequency substitutions included N105S (6/28; 21.43%) and I292V and C550F (each 2/28; 7.14%). Notably, S211N maps within the prefusion F antigenic site Ø region targeted by nirsevimab, whereas S190N is located at or immediately adjacent to this region.

Discussion

In this study, we describe the epidemiological, molecular, and genomic characteristics of RSV detected through the national SARI surveillance system in North Macedonia across three consecutive seasons. The findings highlight both the substantial

contribution of RSV to severe respiratory illness among hospitalised SARI cases and the dynamic seasonal turnover of RSV subgroups and genetic lineages, while illustrating the added value of integrating genomic approaches into an evolving national surveillance framework.

The epidemiological patterns observed across the three RSV seasons confirm that RSV accounts for a substantial proportion of severe acute respiratory illness among laboratory-tested SARI cases in North Macedonia, while also illustrating how evolving surveillance implementation can shape the observed season-to-season patterns. RSV testing within SARI surveillance has evolved through continuous adaptations designed to optimise case detection among hospitalised SARI patients, and these changes likely influenced both the number of patients tested and the observed RSV positivity. In the first season (2022/2023), RSV testing was newly introduced into the surveillance framework, and as in many settings during an early implementation phase, testing was comparatively limited and more selective. Such selectivity – driven by clinical suspicion – can enrich the tested subset for "typical" RSV presentations and may contribute to higher positivity. This interpretation is consistent with the pattern observed in this study, in which the season with the lowest testing coverage showed the highest RSV positivity, emphasising the potential role of clinician-based selection in identifying patients with a higher probability of RSV infection. In 2023/2024, RSV testing covered a larger share of SARI cases, and the observed RSV positivity was lower. Therefore, season-to-season differences in positivity likely reflect differences in testing practices rather than true changes in RSV circulation in the community. Importantly, although SARI surveillance was conducted year-round in 2023/2024 and 2024/2025, restricting seasonal comparisons to weeks 40-20 strengthens comparability and aligns the analysis with the expected RSV seasonality in temperate climates.

Beyond testing coverage, the seasonal subgroup dynamics observed in North Macedonia align with broader regional patterns and reinforce the value of molecular and subgroup-level surveillance. The predominance of RSV-B in 2022/2023, followed by co-circulation with a clear RSV-A predominance in the subsequent two seasons, reflects a well-documented phenomenon of alternating dominance, commonly attributed to multiple mechanisms, including population-level immunity that accumulates after intense circulation of one subgroup, thereby creating space for the other subgroup in the following season. In addition, ongoing viral evolution and the emergence and spread of fitter lineages can influence the subgroup prevalence and lineage turnover. The concordance between the subgroup pattern observed in North Macedonia and contemporaneous European circulation patterns – as suggested by publicly available sequences on GISAID – supports the interpretation that local RSV epidemiology is embedded within broader regional circulation, likely shaped by shared climatic drivers and population mobility. The timing of peak RSV activity differed between seasons, occurring in November-December in 2022/2023 and 2023/2024 but shifting to February in 2024/2025. This seasonal shift highlights that the timing of RSV activity can vary from year to year and that peak timing should be compared cautiously across seasons.

The age distribution of RSV-positive SARI cases further supports established epidemiological knowledge: severe RSV disease and hospitalisation burden are concentrated in the youngest children, especially those aged 0-2 years. This is biologically plausible, given immunological immaturity, limited prior exposure, and the higher likelihood of severe lower respiratory tract disease in infants and young children, making them more likely to meet SARI criteria and require hospital admission. At the same time, the low number of adult RSV detections should not be interpreted as the absence of adult infection, but rather as a reflection of the SARI-based design of the surveillance platform: adults more frequently experience milder RSV illness that does not necessitate hospitalisation and therefore is less likely to be captured by SARI surveillance. Nonetheless, adults remain epidemiologically relevant as potential reservoirs and transmitters, particularly those in

close contact with children (e.g. parents, caregivers, and grandparents). Finally, the symptom profile must be interpreted in a surveillance-specific manner: cough and fever (≥38°C) were uniformly present because they are integral to the SARI case definition and therefore reflect the inclusion criteria rather than an independent clinical observation. The distribution of additional manifestations – such as rhinitis/coryza and dyspnoea, and less frequent gastrointestinal and systemic symptoms – adds descriptive granularity and complements the demographic and subgroup findings.

The under-representation of RSV genomes from several European countries – particularly in south-eastern Europe – limits the resolution with which regional RSV evolution and cross-border spread can be interpreted from public databases alone. In this context, our contribution of season-resolved whole-genome data from North Macedonia provides timely molecular evidence that local RSV circulation mirrors broader European patterns, while adding the needed geographic coverage to an otherwise sparse area. However, the relatively small number of European reference sequences included for phylogenetic contextualisation may have limited the resolution with which potential cross-border introduction events could be reconstructed. The phylogenies indicate that RSV circulation during 2022/2023 – 2024/2025 is better explained by repeated introductions and turnover of variants than by long-term persistence of a single lineage across seasons, with a notably more dynamic pattern for RSV-A; multiple ON1 sublineages were observed across consecutive seasons, and the dominant genetic groups differed between the 2023/2024 and 2024/2025 seasons. In contrast, RSV-B genomes were largely confined to the BA9 genotype and clustered predominantly within a single lineage (B.D.E.1) across all three seasons.

A methodological limitation of this study relates to the evolving laboratory and analytical approaches applied across the three surveillance seasons. In studies spanning multiple seasons, such refinements are often introduced as surveillance systems develop and analytical capacities expand. In the present study, different RT-qPCR assays were used during the study period, and the Ct values obtained with these assays were used as an operational criterion for prioritising samples for sequencing. However, the Ct threshold for sequencing selection was applied across heterogeneous assays, and the cross-kit Ct equivalence was not formally validated. In addition, two different bioinformatic tools were used for consensus sequence generation, and as they differ in their analytical workflows, a direct comparison of their output characteristics was not feasible. Therefore, potential minor inter-seasonal differences related to assay heterogeneity and bioinformatic processing should be considered when interpreting season-to-season comparisons, phylogenetic relationships, and reported frequencies of amino acid substitutions. The bioinformatic approach used for each Macedonian RSV sequence included in the study is provided in Supplementary Table S2.

An additional consideration when interpreting seasonal differences in lineage composition is that the SARI testing framework changed across the study period, which may have influenced the profile of the sampled and sequenced cases between seasons. In the present dataset, sequenced cases from all three seasons were predominantly concentrated in children aged 0-2 years, who accounted for 90.6% of all sequenced samples, with only a small number of sequences obtained from older age groups (Supplementary Table S1). This distribution does not allow for a meaningful assessment of lineage distribution across age categories. Likewise, because the surveillance platform included only hospitalised cases, the study design does not allow reliable evaluation of whether particular lineages were associated with differences in clinical severity or symptom profiles, as all included patients already met the criteria for severe acute respiratory infection requiring hospital care. This cautious interpretation is further supported by the literature, where data on lineage-specific clinical differences remain limited, and even studies assessing associations between the broader RSV subgroups and disease severity have yielded inconsistent findings, with some suggesting greater severity for RSV-A, others reporting less

favourable outcomes for RSV-B, and others showing no significant subgroup-related difference (20-23). Therefore, although the observed lineage turnover likely reflects true viral circulation dynamics, the contribution of sampling-related differences cannot be excluded, and the findings should be interpreted with appropriate caution. Importantly, both globally successful genetic backgrounds represented in our dataset – RSV-B BA9 genotype and RSV-A ON1 – are defined by duplications in the G gene: a 60-nucleotide duplication in RSV-B first reported in 1999, and a 72-nucleotide duplication in RSV-A reported in 2010. Since their independent emergence, the BA (RSV-B) and ON1 (RSV-A) genotypes have replaced previously circulating RSV variants, suggesting that G-gene duplication confers a selective advantage (24). Because the G protein is exposed to immune pressure, higher variability in this gene is expected. In our dataset, this is reflected by the broader spectrum of G substitutions in RSV-A, whereas RSV-B showed several high-frequency substitutions together with additional low-frequency variants. In comparison, the F gene exhibited fewer substitutions overall in both subgroups, with most changes occurring at low frequencies, consistent with the more conserved nature of the F protein.

An additional limitation of this study is the selection of samples for whole-genome sequencing. Prioritisation of samples with lower Ct values was used as a practical approach to increase the likelihood of successful sequencing while making reasonable use of the available resources. However, this strategy enriched the sequenced subset for samples with higher viral RNA concentrations and may, therefore, have limited its representativeness relative to the broader RSV-positive population. Consequently, bias in lineage representation cannot be excluded if certain viral lineages were more frequently present in samples with lower RNA concentrations and were therefore less likely to be included in the sequencing dataset. A descriptive comparison of sequenced and non-sequenced RSV-positive samples is provided in Supplementary Table S1.

The expanding use of RSV preventive interventions in Europe further strengthens the relevance of integrated RSV surveillance. In the European context, long-acting monoclonal antibodies for infant protection and maternal RSV vaccination are already part of the evolving prevention landscape, while vaccines for older adults have also been authorised. To our knowledge, such preventive interventions were not part of the routine national implementation in North Macedonia at the time of writing. However, if introduced in the future, the established RSV genomic surveillance framework within the national SARI system could provide an important baseline for monitoring possible changes in the RSV burden, age distribution, seasonal dynamics, and circulation of viral variants over time. In our dataset, two RSV-B F substitutions present in all RSV-B genomes, S190N and S211N, mapped at or near the prefusion F antigenic site Ø targeted by nirsevimab, whereas one RSV-A substitution, S276N, was detected within antigenic site II of the F protein, the region targeted by palivizumab (25, 26). Importantly, available data indicate that S211N retains susceptibility to nirsevimab, which is supported both by phenotypic analyses showing preserved neutralisation potency and the OUTSMART-RSV surveillance program, in which S211N variants showed full susceptibility to nirsevimab (IC50 fold change = 1.24 versus reference) (27, 28). In contrast, although S190N is located in close proximity to this antigenically relevant region, its functional significance remains insufficiently characterised. Similarly, although S276N lies within the palivizumab-targeted site II region, the available data do not support an association with reduced palivizumab activity (29). More broadly, the detection of a substitution within a monoclonal antibody binding site should be regarded as noteworthy from a surveillance perspective, while avoiding default assumptions regarding altered binding or functional significance in the absence of phenotypic evidence. In this context, the value of genomic surveillance lies in enabling the timely detection and monitoring of changes with potential relevance,

should preventive interventions be introduced in North Macedonia in the future.

Together, these findings reinforce the importance of sustained RSV genomic surveillance in undersampled European settings, as the inclusion of genomes from North Macedonia improves regional representativeness and strengthens the interpretation of lineage turnover, viral introductions, and subgroup-specific evolutionary patterns across seasons.

Conclusion

This study provides an integrated epidemiological, molecular, and genomic overview of RSV circulation within the national SARI surveillance system in North Macedonia over three consecutive seasons. The findings confirm that RSV is a substantial contributor to severe respiratory disease among hospitalised SARI cases, particularly in young children, and demonstrate marked season-to-season variations in subgroup dominance and lineage composition. Phylogenetic analyses indicate dynamic turnover of RSV-A lineages and more stable circulation of RSV-B within a single dominant lineage, consistent with broader European patterns. Together, these results highlight the value of sustained RSV surveillance that combines epidemiological data with whole-genome sequencing, particularly in undersampled regions. The established surveillance framework provides a robust baseline for future monitoring of RSV evolution and circulation, should pharmacological preventive measures be implemented.

What Is Already Known on This Topic:

Respiratory syncytial virus is a leading cause of severe lower respiratory tract infections and hospitalisation in infants and young children worldwide. RSV circulates in two major subgroups, RSV-A and RSV-B, which co-circulate globally and often show alternating dominance between seasons. Molecular surveillance has demonstrated that contemporary RSV circulation is dominated by several globally successful genotypes, most notably ON1 (RSV-A) and BA9 (RSV-B), both of which are characterised by duplications in the G gene. Whole-genome sequencing has been increasingly applied to understand RSV transmission dynamics, lineage turnover, and genetic diversity. However, RSV genomic data remain unevenly distributed across Europe, with limited representation from south-eastern European countries, constraining the comprehensive regional interpretation of RSV evolution and spread.

What This Study Adds:

This study provided an integrated analysis of RSV epidemiology, molecular characteristics, and whole-genome data across three consecutive seasons within the national SARI surveillance system in North Macedonia. It documents the dynamic seasonal turnover of RSV-A lineages alongside the more stable circulation of RSV-B within a dominant lineage and contributes whole-genome data from an under-represented region of Europe. By embedding genomic analysis within routine SARI surveillance, this study provides new insights into the circulation of RSV subgroups and lineages associated with severe disease in North Macedonia.

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

References

1. Li Y, Wang X, Blau DM, Caballero MT, Feikin DR, Gill CJ, et al. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in children younger than 5 years in 2019: a systematic analysis. The Lancet. 2022 May;399(10340):2047–64. doi:10.1016/S0140-6736(22)00478-0

2. Al-Leimon O, Shihadeh H, Yousef AA, Khraim A, Siwwad R. Respiratory syncytial virus: A review of current basic and clinical knowledge. Qatar Med J. 2024;2024(4):56. doi:10.5339/qmj.2024.56
3. Peret TCT, Hall CB, Hammond GW, Piedra PA, Storch GA, Sullender WM, et al. Circulation Patterns of Group A and B Human Respiratory Syncytial Virus Genotypes in 5 Communities in North America. J INFECT DIS. 2000 Jun;181(6):1891–6. doi:10.1086/315508
4. Cantú-Flores K, Rivera-Alfaro G, Muñoz-Escalante JC, Noyola DE. Global distribution of respiratory syncytial virus A and B infections: a systematic review. Pathogens and Global Health. 2022 Oct 3;116(7):398–409. doi:10.1080/20477724.2022.2038053
5. European Medicines Agency [Internet]. [cited 2026 Apr 22]. Enflonsia European Medicines Agency. Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/enflonsia
6. Rapid scientific advice on protecting infants against respiratory syncytial virus disease for the European 2025/26 winter season [Internet]. European Centre for Disease Prevention and Control (ECDC); 2025 Nov [cited 2026 Mar 20]. p. 18. Available from: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.ecdc.europa.eu/sites/default/files/documents/RSA-winter-season.pdf?utm_source=chatgpt.com
7. WHO surveillance case definitions for ILI and SARI [Internet]. [cited 2025 Dec 22]. Available from: https://www.who.int/teams/global-influenza-programme/surveillance-and-monitoring/case-definitions-for-ili-and-sari
8. QIAamp Viral RNA Mini Kit [Internet]. [cited 2026 Apr 22]. Available from: https://www.qiagen.com/us/products/diagnostics-and-clinical-research/sample-processing/qiaamp-viral-rna-kits
9. MagCore® Viral Nucleic Acid Extraction Kit High Sensitivity [Internet]. [cited 2026 Apr 22]. Available from: https://www.rbcbioscience.com/product-detail39.htm
10. RevoDx Viral Pathogens Idil Biotech [Internet]. Available from: https://www.idilbiotech.com/viral-pathogens/.
11. Fast Track Diagnostics FTD Respiratory pathogens 33 Kit [Internet]. Available from: https://banksiascientific.com.au/wp-content/uploads/2017/10/ftd-2p-3-32_64-validation-v1-2016_11-en.pdf?srsltid=AfmBOoqW4vVOmFDIdivd6lrX9Q_WRQuwHcQzx6qUBK1gWAuUF7VQRB_N
12. Altona RealStar RSV RT-PCR Kit 3.0 [Internet]. Available from: https://altona-diagnostics.com/product/realstar-rsv-rt-pcr-kit-3-0/
13. Illumina Respiratory Virus Oligos Panel v2 [Internet]. Available from: https://emea.illumina.com/products/by-type/sequencing-kits/library-prep-kits/respiratory-virus-oligo-panel.html
14. Okonechnikov K, Golosova O, Fursov M, the UGENE team. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012 Apr 15;28(8):1166–7. doi:10.1093/bioinformatics/bts091
15. Borges V, Pinheiro M, Pechirra P, Guiomar R, Gomes JP. INSaFLU: an automated open web-based bioinformatics suite "from-reads" for influenza whole-genome-sequencing-based surveillance. Genome Med. 2018 Dec;10(1):46. doi:10.1186/s13073-018-0555-0
16. Katoh K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Molecular Biology and Evolution. 2013 Apr 1;30(4):772–80. doi:10.1093/molbev/mst010
17. Aksamentov I, Roemer C, Hodcroft E, Neher R. Nextclade: clade assignment, mutation calling and quality control for viral genomes. JOSS. 2021 Nov 30;6(67):3773. doi:10.21105/joss.03773
18. Price MN, Dehal PS, Arkin AP. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. Poon AFY, editor. PLoS ONE. 2010 Mar 10;5(3):e9490. doi:10.1371/journal.pone.0009490
19. Letunic I, Bork P. Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool. Nucleic Acids Research. 2024 Jul 5;52(W1):W78–82. doi:10.1093/nar/gkae268
20. Laham FR, Mansbach JM, Piedra PA, Hasegawa K, Sullivan AF, Espinola JA, et al. Clinical Profiles of Respiratory Syncytial Virus Subtypes A AND B Among Children Hospitalized with Bronchiolitis. Pediatric Infectious Disease Journal. 2017 Aug;36(8):808–10. doi:10.1097/INF.0000000000001596
21. Midulla F, Nenna R, Scagnolari C, Petrarca L, Frassanito A, Viscido A, et al. How Respiratory Syncytial Virus Genotypes Influence the Clinical Course in Infants Hospitalized for Bronchiolitis. The Journal of Infectious Diseases. 2019 Jan 29;219(4):526–34. doi:10.1093/infdis/jiy496
22. Saravanos GL, Ramos I, Britton PN, Wood NJ. Respiratory syncytial virus subtype circulation and associated disease severity at an Australian paediatric referral hospital, 2014–2018. J Paediatrics Child Health. 2021 Aug;57(8):1190–5. doi:10.1111/jpc.15419
23. Espinosa Y, San Martín C, Torres A, Farfán M, Torres J, Avadhanula V, et al. Genomic Loads and Genotypes of Respiratory Syncytial Virus: Viral Factors during Lower Respiratory Tract Infection in Chilean Hospitalized Infants. IJMS. 2017 Mar 21;18(3):654. doi:10.3390/ijms18030654
24. Hotard AL, Laikhter E, Brooks K, Hartert TV, Moore ML. Functional Analysis of the 60-Nucleotide Duplication in the Respiratory Syncytial Virus Buenos Aires Strain Attachment Glycoprotein. Lyles DS, editor. J Virol. 2015 Aug 15;89(16):8258–66. doi:10.1128/JVI.01045-15
25. Wilkins D, Langedijk AC, Lebbink RJ, Morehouse C, Abram ME, Ahani B, et al. Nirsevimab binding-site conservation in respiratory syncytial virus fusion glycoprotein worldwide between 1956 and 2021: an analysis of observational study sequencing data. The Lancet

Infectious Diseases. 2023 Jul;23(7):856–66. doi:10.1016/S1473-3099(23)00062-2

26. Schickli JH, Whitacre DC, Tang RS, Kaur J, Lawlor H, Peters CJ, et al. Palivizumab epitope–displaying virus-like particles protect rodents from RSV challenge. J Clin Invest. 2015 Apr 1;125(4):1637–47. doi:10.1172/JCI78450
27. Morehouse CA, Ahani B, Aksyuk AA, Brady T, Tuffy KM, Ji H, et al. 2641. Nirsevimab binding-site conservation in RSV F protein between 2015 and 2022: The US OUTSMART-RSV surveillance study. Open Forum Infectious Diseases. 2023 Nov 27;10(Supplement_2):ofad500.2253. doi:10.1093/ofid/ofad500.2253
28. Ahani B, Tuffy KM, Aksyuk AA, Wilkins D, Abram ME, Dagan R, et al. Molecular and phenotypic characteristics of RSV infections in infants during two nirsevimab randomized clinical trials. Nat Commun. 2023 Jul 19;14(1):4347. doi:10.1038/s41467-023-40057-8
29. Adhikari B, Hassan F, Harrison CJ, Dien Bard J, Dunn J, Kehl S, et al. A multi-center study to determine genetic variations in the fusion gene of respiratory syncytial virus (RSV) from children <2 years of age in the U.S. Journal of Clinical Virology. 2022 Sep;154:105223. doi:10.1016/j.jcv.2022.105223

Supplementary Material

Table S1. Comparison of Sequenced and Non-sequenced RSV-Positive Samples by Ct Value Distribution, Age Group, Collection Month, and RSV Subgroup

Variable	Category	Sequenced RSV-positive samples (N=85)	Non-sequenced RSV-positive samples (N=166)
Ct value	<20	14	4
	20-29.9	69	105
	≥30	2	57
Age group	0-2	77	149
	3-4	4	8
	5-14	3	6
	15-29	0	0
	30-64	0	1
	65-79	1	2
	80+	0	0
Month of collection	October 2022	2	0
	November 2022	8	9
	December 2022	0	17
	January 2023	2	2
	February 2023	2	0
	March 2023	0	0
	April 2023	0	0
	October 2023	2	5
	November 2023	21	25
	December 2023	7	31
	January 2024	1	15
	February 2024	3	7
	March 2024	1	0
	April 2024	0	0
	October 2024	0	0
	November 2024	1	0
	December 2024	6	1
	January 2025	6	11
	February 2025	14	16
	March 2025	4	14
	April 2025	5	13
RSV subgroup	RSV-A	57	112
	RSV-B	28	42
	RSV-A/B coinfection	0	1
	Not determined	0	11

Note: The months of collection are presented within the analytical surveillance period used for seasonal comparisons, spanning week 40 of one year to week 20 of the following year.

Table S2. GISAID Accession IDs and Corresponding Sample Information for Macedonian RSV Consensus Genome Sequences Obtained Through National SARI Surveillance During the 2022-2025 Seasons

Virus name	Accession ID	Collection date	Pipeline
hRSV/A/Macedonia/IPH-MKD-137G/2023	EPI_ISL_19141127	2023-12-04	INSaFLU
hRSV/A/Macedonia/IPH-MKD-153G/2023	EPI_ISL_19141128	2023-12-06	INSaFLU
hRSV/A/Macedonia/IPH-MKD-117G/2023	EPI_ISL_19141125	2023-11-27	INSaFLU
hRSV/A/Macedonia/IPH-MKD-103G/2023	EPI_ISL_19141124	2023-11-24	INSaFLU
hRSV/A/Macedonia/IPH-MKD-91G/2023	EPI_ISL_19141121	2023-11-21	INSaFLU
hRSV/A/Macedonia/IPH-MKD-92G/2023	EPI_ISL_19141122	2023-11-21	INSaFLU
hRSV/A/Macedonia/IPH-MKD-89G/2023	EPI_ISL_19141120	2023-11-21	INSaFLU
hRSV/A/Macedonia/IPH-MKD-81G/2023	EPI_ISL_19141116	2023-11-17	INSaFLU
hRSV/A/Macedonia/IPH-MKD-82G/2023	EPI_ISL_19141117	2023-11-17	INSaFLU
hRSV/A/Macedonia/IPH-MKD-76G/2023	EPI_ISL_19141114	2023-11-16	INSaFLU
hRSV/A/Macedonia/IPH-MKD-77G/2023	EPI_ISL_19141115	2023-11-16	INSaFLU
hRSV/A/Macedonia/IPH-MKD-73G/2023	EPI_ISL_19141112	2023-11-14	INSaFLU
hRSV/A/Macedonia/IPH-MKD-74G/2023	EPI_ISL_19141113	2023-11-14	INSaFLU
hRSV/A/Macedonia/IPH-MKD-83G/2023	EPI_ISL_19141118	2023-11-20	INSaFLU
hRSV/A/Macedonia/IPH-MKD-87G/2023	EPI_ISL_19141119	2023-11-21	INSaFLU
hRSV/A/Macedonia/IPH-MKD-658G/2024	EPI_ISL_19141138	2024-02-12	INSaFLU
hRSV/A/Macedonia/IPH-MKD-867G/2024	EPI_ISL_19141139	2024-03-21	INSaFLU
hRSV/A/Macedonia/IPH-MKD-572G/2024	EPI_ISL_19141136	2024-02-01	INSaFLU
hRSV/A/Macedonia/IPH-MKD-594G/2024	EPI_ISL_19141137	2024-02-05	INSaFLU
hRSV/A/Macedonia/IPH-MKD-236G/2023	EPI_ISL_19141134	2023-12-25	INSaFLU
hRSV/A/Macedonia/IPH-MKD-377G/2024	EPI_ISL_19141135	2024-01-05	INSaFLU
hRSV/A/Macedonia/IPH-MKD-180G/2023	EPI_ISL_19141130	2023-12-12	INSaFLU
hRSV/A/Macedonia/IPH-MKD-193G/2023	EPI_ISL_19141131	2023-12-13	INSaFLU
hRSV/A/Macedonia/IPH-MKD-166G/2023	EPI_ISL_19141129	2023-12-12	INSaFLU
hRSV/A/Macedonia/IPH-MKD-40G/2023	EPI_ISL_19141105	2023-11-01	INSaFLU
hRSV/A/Macedonia/IPH-MKD-41G/2023	EPI_ISL_19141106	2023-11-01	INSaFLU
hRSV/A/Macedonia/IPH-MKD-33G/2023	EPI_ISL_19141103	2023-10-27	INSaFLU
hRSV/A/Macedonia/IPH-MKD-37G/2023	EPI_ISL_19141104	2023-10-31	INSaFLU
hRSV/A/Macedonia/IPH-MKD-66G/2023	EPI_ISL_19141110	2023-11-13	INSaFLU
hRSV/A/Macedonia/IPH-MKD-70G/2023	EPI_ISL_19141111	2023-11-14	INSaFLU
hRSV/A/Macedonia/IPH-MKD-58G/2023	EPI_ISL_19141109	2023-11-10	INSaFLU
hRSV/A/Macedonia/IPH-MKD-50G/2023	EPI_ISL_19141107	2023-11-07	INSaFLU
hRSV/A/Macedonia/IPH-MKD-56G/2023	EPI_ISL_19141108	2023-11-08	INSaFLU
hRSV/A/Macedonia/IPH-MKD-77G/2024	EPI_ISL_20207191	2024-11-26	INSaFLU
hRSV/A/Macedonia/IPH-MKD-92G/2024	EPI_ISL_20207192	2024-12-04	INSaFLU
hRSV/A/Macedonia/IPH-MKD-96G/2024	EPI_ISL_20207193	2024-12-11	INSaFLU
hRSV/A/Macedonia/IPH-MKD-109G/2024	EPI_ISL_20207194	2024-12-19	INSaFLU
hRSV/A/Macedonia/IPH-MKD-121G/2024	EPI_ISL_20207195	2024-12-23	INSaFLU
hRSV/A/Macedonia/IPH-MKD-128G/2024	EPI_ISL_20207196	2024-12-25	INSaFLU
hRSV/A/Macedonia/IPH-MKD-162G/2025	EPI_ISL_20207198	2025-01-08	INSaFLU
hRSV/A/Macedonia/IPH-MKD-228G/2025	EPI_ISL_20207200	2025-01-15	INSaFLU

Virus name	Accession ID	Collection date	Pipeline
hRSV/A/Macedonia/IPH-MKD-370G/2025	EPI_ISL_20207203	2025-01-29	INSaFLU
hRSV/A/Macedonia/IPH-MKD-404G/2025	EPI_ISL_20207206	2025-02-05	INSaFLU
hRSV/A/Macedonia/IPH-MKD-406G/2025	EPI_ISL_20207207	2025-02-05	INSaFLU
hRSV/A/Macedonia/IPH-MKD-442G/2025	EPI_ISL_20207209	2025-02-10	INSaFLU
hRSV/A/Macedonia/IPH-MKD-448G/2025	EPI_ISL_20207210	2025-02-10	INSaFLU
hRSV/A/Macedonia/IPH-MKD-455G/2025	EPI_ISL_20207211	2025-02-10	INSaFLU
hRSV/A/Macedonia/IPH-MKD-496G/2025	EPI_ISL_20207213	2025-02-14	INSaFLU
hRSV/A/Macedonia/IPH-MKD-554G/2025	EPI_ISL_20207214	2025-02-19	INSaFLU
hRSV/A/Macedonia/IPH-MKD-566G/2025	EPI_ISL_20207215	2025-02-20	INSaFLU
hRSV/A/Macedonia/IPH-MKD-597G/2025	EPI_ISL_20207216	2025-02-24	INSaFLU
hRSV/A/Macedonia/IPH-MKD-601G/2025	EPI_ISL_20207217	2025-02-24	INSaFLU
hRSV/A/Macedonia/IPH-MKD-807G/2025	EPI_ISL_20207220	2025-03-24	INSaFLU
hRSV/A/Macedonia/IPH-MKD-827G/2025	EPI_ISL_20207221	2025-03-27	INSaFLU
hRSV/A/Macedonia/IPH-MKD-831G/2025	EPI_ISL_20207222	2025-04-01	INSaFLU
hRSV/A/Macedonia/IPH-MKD-848G/2025	EPI_ISL_20207223	2025-04-04	INSaFLU
hRSV/A/Macedonia/IPH-MKD-868G/2025	EPI_ISL_20207226	2025-04-08	INSaFLU
hRSV/B/North Macedonia/IPH-MKD-220/2022	EPI_ISL_15601574.2	2022-10-18	INSaFLU
hRSV/B/North Macedonia/IPH-MKD-231/2022	EPI_ISL_16006110	2022-10-26	Ugene v45.0
hRSV/B/North Macedonia/IPH-MKD-263/2022	EPI_ISL_16006112	2022-11-16	Ugene v45.0
hRSV/B/North Macedonia/IPH-MKD-262/2022	EPI_ISL_16006111	2022-11-16	Ugene v45.0
hRSV/B/North Macedonia/IPH-MKD-274/2022	EPI_ISL_16006114	2022-11-22	Ugene v45.0
hRSV/B/North Macedonia/IPH-MKD-271/2022	EPI_ISL_16006113.2	2022-11-22	INSaFLU
hRSV/B/North Macedonia/IPH-MKD-282/2022	EPI_ISL_16006116.2	2022-11-24	INSaFLU
hRSV/B/North Macedonia/IPH-MKD-278/2022	EPI_ISL_16006115	2022-11-23	Ugene v45.0
hRSV/B/North Macedonia/IPH-MKD-287/2022	EPI_ISL_16006118.2	2022-11-25	INSaFLU
hRSV/B/North Macedonia/IPH-MKD-285/2022	EPI_ISL_16006117	2022-11-25	Ugene v45.0
hRSV/B/Macedonia/IPH-MKD-250G/2023	EPI_ISL_18348031	2023-02-15	INSaFLU
hRSV/B/Macedonia/IPH-MKD-184G/2023	EPI_ISL_18348030	2023-02-02	INSaFLU
hRSV/B/Macedonia/IPH-MKD-86G/2023	EPI_ISL_18348029	2023-01-17	INSaFLU
hRSV/B/Macedonia/IPH-MKD-49G/2023	EPI_ISL_18348028	2023-01-09	INSaFLU
hRSV/B/Macedonia/IPH-MKD-94G/2023	EPI_ISL_19141123	2023-11-21	INSaFLU
hRSV/B/Macedonia/IPH-MKD-234G/2023	EPI_ISL_19141133	2023-12-25	INSaFLU
hRSV/B/Macedonia/IPH-MKD-129G/2024	EPI_ISL_20207197	2024-12-25	INSaFLU
hRSV/B/Macedonia/IPH-MKD-201G/2025	EPI_ISL_20207199	2025-01-10	INSaFLU
hRSV/B/Macedonia/IPH-MKD-286G/2025	EPI_ISL_20207201	2025-01-20	INSaFLU
hRSV/B/Macedonia/IPH-MKD-326G/2025	EPI_ISL_20207202	2025-01-23	INSaFLU
hRSV/B/Macedonia/IPH-MKD-400G/2025	EPI_ISL_20207204	2025-02-04	INSaFLU
hRSV/B/Macedonia/IPH-MKD-401G/2025	EPI_ISL_20207205	2025-02-04	INSaFLU
hRSV/B/Macedonia/IPH-MKD-419G/2025	EPI_ISL_20207208	2025-02-07	INSaFLU
hRSV/B/Macedonia/IPH-MKD-491G/2025	EPI_ISL_20207212	2025-02-13	INSaFLU
hRSV/B/Macedonia/IPH-MKD-732G/2025	EPI_ISL_20207218	2025-03-11	INSaFLU
hRSV/B/Macedonia/IPH-MKD-771G/2025	EPI_ISL_20207219	2025-03-17	INSaFLU
hRSV/B/Macedonia/IPH-MKD-855G/2025	EPI_ISL_20207224	2025-04-07	INSaFLU
hRSV/B/Macedonia/IPH-MKD-857G/2025	EPI_ISL_20207225	2025-04-07	INSaFLU

Table S3. GISAID Accession IDs and Corresponding Information for European RSV Reference Sequences Used in Phylogenetic Analyses

Virus name	Accession ID	Collection date	Country
hRSV/A/Russia/NVS-7.638/2024	EPI_ISL_19437714	2024-03-07	Russian Federation
hRSV/A/England/UKHSA_RVU_4640232/2023	EPI_ISL_18713515.2	2023-11-14	United Kingdom
hRSV/A/Spain/EX-BA_18612421/2023	EPI_ISL_18968232	2023-12-22	Spain
hRSV/A/France/ARA-EEQ1-HCL27601102/2023	EPI_ISL_19465517	2023-05-31	France
hRSV/A/Slovakia/1336/2023	EPI_ISL_17481634.2	2023-02-06	Slovakia
hRSV/A/France/ARA-HCL023188225101/2023	EPI_ISL_18789785	2023-11-29	France
hRSV/A/Italy/SIC-107_48/2021	EPI_ISL_19063420	2021-11-29	Italy
hRSV/A/Spain/AN-PMC-00050/2024	EPI_ISL_18980304	2024-01-21	Spain
hRSV/A/Ireland/NVRL-G-68808/2023	EPI_ISL_19160773	2023-11-01	Ireland
hRSV/A/Ireland/NVRL-G-69867/2023	EPI_ISL_19160778	2023-11-06	Ireland
hRSV/A/Ukraine/RLDHA-4090/2025	EPI_ISL_19883376	2025-04-07	Ukraine
hRSV/A/Canary Islands/HUNSC_ITER_150427667/2023	EPI_ISL_18698713	2023-11-19	Spain
hRSV/B/Croatia/HZJZ_7338/2024	EPI_ISL_19074734	2024-02-24	Croatia
hRSV/B/Bulgaria/2734/2022	EPI_ISL_18447498	2022-12-19	Bulgaria
hRSV/B/France/IDF-RELAB-IPP24981/2023	EPI_ISL_18537476	2023-10-12	France
hRSV/B/Spain/CT-HUJO-048261/2024	EPI_ISL_19536326	2024-10-31	Spain
hRSV/B/England/UKHSA_RVU_4460393/2023	EPI_ISL_18591802	2023-10-31	England
hRSV/B/Germany/NRW-HHU-161/2024	EPI_ISL_20060976	2024-12-23	Germany
hRSV/B/Czech Republic/NRL_c1588/2024	EPI_ISL_19044757	2024-02-15	Czech Republic
hRSV/B/Switzerland/ZH-UZH-IMV-3BA70F72/2025	EPI_ISL_19995393	2025-04-05	Switzerland
hRSV/B/Slovakia/uvzsr-40461/2025	EPI_ISL_20068837	2025-03-12	Slovakia

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Effects of Anti-Gravity Treadmill Training on Functional Outcomes After Total Hip Arthroplasty: A Randomized Controlled Trial

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Abstract

Objective. Total hip arthroplasty is one of the most common orthopedic procedures. When the surgery is successful and followed by high-quality physiotherapy, the patients' quality of life usually improves. This randomized controlled trial aimed to investigate the impact of anti-gravity treadmills on functional outcomes after total hip arthroplasty. **Materials and Methods.** The trial was conducted at the Special Hospital for Medical Rehabilitation of Heart, Lung, and Rheumatic Diseases Thalassotherapia Opatija. The sample consisted of patients admitted for inpatient rehabilitation three months after total hip arthroplasty. A total of 34 subjects were randomly assigned to two groups: an experimental group (N=18) and a control group (N=16). The outcome measures included abductor muscle strength, range of motion, numeric pain rating scale, Timed Up and Go test, and 40-meter Fast-Paced Walk test. **Results.** A statistically significant between-group difference was observed only for hip abduction range of motion, favoring the experimental group (P=0.044). The other measured variables did not show any statistically significant intergroup differences. **Conclusions.** Anti-gravity treadmill training improved hip abduction range of motion but did not demonstrate superiority over conventional rehabilitation in the other assessed functional outcomes.

Key Words: Arthroplasty ▪ Hip ▪ Anti-Gravity Treadmill ▪ Muscle Strength ▪ Pain ▪ Functional Mobility.

Introduction

Total hip arthroplasty (THA) is one of the most common orthopedic procedures. When the surgery is successful and followed by high-quality physiotherapy, patients' quality of life usually improves, and they return to their daily activities more quickly. However, even with proper bone healing and rehabilitation, some patients continue to experience functional limitations and pain. The most common factors are hip muscle weakness, calf muscle weakness, trochanteric bursitis, and anterior knee pain. Among these, hip abductor muscle weakness (1) is the most prevalent and often results in limping, impaired balance, and an increased risk of falling (2). Studies have shown that up to 40% of people over the age of 65 experience at least one fall annually following total hip or knee replacement surgery (3). Although hip replacement surgery is a common and often routine procedure, rehabilitation does not always progress smoothly. Rehabilitation methods and medical equipment are constantly evolving to accelerate functional improvement.

An anti-gravity treadmill (AT) allows exercise with reduced body weight using an airtight chamber to decrease the effects of gravity. The AT enables patients to exercise with minimal stress on the joints, making it possible to reduce pain and enhance muscle strength and cardiopulmonary endurance (4). Mikami et al. demonstrated that

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gait training with the AT had a positive effect on muscle strength, walking endurance, distance walked, oxygen consumption, and patients' perception of their ability to perform tasks independently (5). In another study, the same authors compared traditional rehabilitation with programs that incorporated AT and found improvements in muscle strength, pain reduction, and functional mobility following total hip arthroplasty (6). Overall, AT has been shown to have a positive effect on muscle strength and endurance, pain levels, range of motion, oxygen consumption, and mobility. Despite its potential, research on the role of AT in physiotherapy remains limited, partly due to the high cost of the equipment, which restricts its widespread use.

Our study aimed to investigate the impact of the AT on hip abductor muscle strength as the primary outcome. The secondary outcomes included hip joint range of motion, pain, and functional mobility at three months after THA.

Materials and Methods

The study was conducted between January and June 2023 at the Special Hospital for Medical Rehabilitation of Heart, Lung**,** and Rheumatic Diseases Thalassotherapia Opatija, Croatia. The study protocol was approved by the appropriate institutional ethics committee, and all participants provided written informed consent prior to inclusion in the study (Approval No.01-000-00-63/2-2023).

Initially, 42 participants were assessed for eligibility, and 34 completed the study protocol. The detailed completed CONSORT 2010 flow diagram was assessed in relation to the criteria and is presented in Supplement 1. Eligible participants included adults of all ages and both sexes who underwent THA using the classic cemented lateral surgical approach. The classic lateral approach to THA is a technique consisting of a longitudinal incision and the partial dissection of the gluteus medius and minimus muscles (7).

The exclusion criteria were as follows: revision hip replacement, acute illness, cognitive impairment, and declining participation in the study. All participants began rehabilitation three months post-surgery. Conducting the study before the third month post-surgery was neither technically nor organizationally feasible, as rehabilitation took place in a state-owned inpatient facility, which constrained us to the administrative framework of the public healthcare system.

Patients were randomly assigned to one of two groups: the experimental group (EG) (N=18) received AT therapy in combination with a standard rehabilitation protocol, and the control group (CG) (N=16) followed only the conventional rehabilitation protocol. Randomization was conducted using sealed envelopes labeled "A" and "B." Envelopes marked "A" corresponded to the EG, while "B" indicated the CG. Randomization was performed by an individual who was not involved in any other aspect of the study. The allocation sequence was concealed from the investigators involved in participant recruitment and assessment.

Sample Size Estimation

A dual approach was employed to determine the sample size for this study. First, we considered the number of participants reported in similar studies (5, 8, 9). Second, a power analysis was performed with a significance level of α=0.05 and β=0.20. Assuming a 5-point difference in abductor strength between groups (10), a standard deviation of 4 points, and a ratio between groups of 1. The calculated sample size was 11 participants per group. We enrolled additional participants to ensure an adequate sample size at the end of the study.

Study Design and Interventions

This was a single-center, open-label study conducted in accordance with the Consolidated Standards of Reporting Trials (CONSORT) guidelines (11). No blinding of participants, therapists, or outcome assessors was performed. Both groups received the same core physiotherapy interventions. Each session included 25 minutes of individualized therapeutic exercises focused on breathing, mobility, strength, and balance. In addition, the participants

received 25 min of electrical stimulation of the quadriceps femoris muscle and 25 min of hydrotherapy in a swimming pool, for a total of 75 min of therapy per session. The EG also completed 20 min of daily exercise on an AT (ALTERG VIA, Lifeward CA, Inc., Milmont Drive, Fremont, CA, USA), supervised by a physiotherapist, resulting in a total session duration of 95 min.

All participants were enrolled in a standardized physical therapy program prescribed by a specialist physician and provided through the public health system, which could not be modified; therefore, the anti-gravity treadmill could only be applied as an additional intervention. AT training involved walking in a straight line at a self-selected, comfortable pace—without running, shortness of breath, or pain. The treadmill chamber was set to reduce the effective body weight of the participants to 20% of their actual body weight (slight unloading was applied to reduce the need for handrail support and prevent the use of compensatory mechanisms). This level of body weight support was selected based on clinical considerations to ensure patient safety and tolerance, while minimizing compensatory movement patterns during gait. This level of unloading was considered sufficient to reduce joint loading without substantially altering normal movement mechanics. Given the lack of clear consensus in the literature regarding the optimal degree of unloading, this parameter was chosen as a pragmatic and clinically applicable setting. The determination of training duration and load was guided by the recognition that functional capacity varies among patients at three months postoperatively. Based on extensive clinical experience with orthopaedic patients, the training load was set at a level that participants could tolerate without using compensatory biomechanical mechanisms, such as limping. Both groups underwent therapy daily for three weeks (a total of 15 sessions). Follow-up evaluations were conducted at two time points: baseline and after three weeks (end of therapy).

Outcome Measures

The range of motion (ROM) of the hip joint was assessed using a two-arm goniometer. The measurements included active hip flexion with the knee flexed and active hip abduction. Two physical therapists performed the measurements. Hip abductor strength was measured using a Cybex isokinetic device (HumacNorm, 2009 model) following a standardized protocol (12). The highest recorded peak torque value was used for the analysis (13).

The Numeric Pain Rating Scale (NPRS) was used to assess subjective *pain intensity*. According to Jensen et al. (14), the NPRS categorizes pain intensity as follows: 0 indicates no pain; 1–3, mild pain; 4–6, moderate pain; and 7–10, severe pain.

The Timed Up and Go Test (TUG) measures the time taken by a patient to stand up from a chair, walk 3 meters, turn around, walk back, and sit down. According to Steffen et al. (15), healthy individuals aged 60 to 80 years typically complete the TUG in 10 seconds or less. Barry et al. (16) suggested that a time greater than 13.5 seconds indicates reduced functional mobility and a higher risk of falls.

The 40-Meter Fast-Paced Walk Test (40 mFPWT) evaluates the time required for a patient to walk a 10-meter section as quickly (but safely) as possible, turn around, return to the starting point, and repeat the sequence. Kennedy et al. (17) recommend a performance benchmark of approximately 0.90 seconds per meter for individuals following hip or knee arthroplasty.

Statistical Analysis

Frequencies and relative frequencies were presented for the demographic variables. The normality of the distribution of numerical variables was analyzed using the Kolmogorov-Smirnov test. Numerical variables are presented as medians and interquartile ranges (IQR) and were compared between independent groups using the Mann–Whitney U test due to the non-normal distribution of the variables. Effect sizes were calculated as point-

biserial correlations. Categorical variables were compared between groups using Fisher's exact test. Data entry and processing were performed using Microsoft Office Excel 2016 and Statistica (version 13.5.0.17, 1984-2018 TIBCO Software Inc.). We have calculated the sample size with MedCalc Statistical Software version 16.2.1 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2016). All statistical tests were performed at a significance level of P<0.05. Given the number of outcomes assessed, no formal adjustment for multiple comparisons was applied. Therefore, the results should be interpreted with caution due to the potential risk of type I errors.

Results

The analysis was conducted on participants who completed the study protocol. The demographic characteristics of the patients are presented in Table 1. A total of 34 patients participated in the study, with 18 and 16 assigned to the EG and CG, respectively. The groups did not differ significantly in terms of age, sex, or the frequency of right versus left hip operations, as is also shown in Table 1.

Significant longitudinal improvements from before to after rehabilitation were observed within both the experimental and control groups individually in: abductor muscle strength (EG-P<0.001, CG-P<0.001), hip joint flexion with the knee bent (EG-P<0.001, CG-P=0.002), hip joint abduction (EG-P<0.001, CG-P=0.002), TUG test (EG-P<0.001, CG-P<0.001), and 40mFPWT (EG-P<0.001, CG-P<0.001).

Table 1. Baseline Demographic and Clinical Characteristics of Participants

Variables	Experimental group	Control group	P
Age			
Median (P25–P75)	60.5 (54.0-70.0)	63.0 (56.0-70.0)	0.593*
Gender			
Female (N; %)	8 (45)	10 (62)	0.240†
Male (N; %)	10 (55)	6 (38)	
The operated side			
Left side (N; %)	7 (38)	6 (38)	0.607†
Right side (N; %	11 (62)	10 (62)	

*Mann-Whitney U Test; †Fisher's exact test.

Table 2. Between-group Comparisons of Hip Abductor Muscle Strength Before and After Rehabilitation

Strength of abductors (Nm*)	Median (P25–P75)	MWU test†(P)	Effect size (r_{pb}‡)
Before rehabilitation			
Experimental group	23.0 (11.0-30.0)	129.5	0.10
Control group	25.0 (10.5-36.0)	0.629*	
After rehabilitation			
Experimental group	34.5 (23.0-46.0	126.5	0.12
Control group	35.0 (16.0-47.0)	0.558*	

*Newton meter; †Mann-Whitney U test; ‡Point-biserial correlation coefficient.

The results of the between-group comparisons of hip abductor muscle strength before and after rehabilitation are presented in Table 2.

Although both groups showed an increase in median strength after rehabilitation, the differences between the experimental and control groups remained statistically non-significant (P>0.05).

The results of the between-group comparisons of hip joint flexion range of motion before and after rehabilitation are presented in Table 3.

Both groups demonstrated an increase in median knee flexion angles after rehabilitation, the differences between the experimental and control groups remained statistically non-significant (P>0.05).

Hip joint abduction range of motion comparisons are presented in Table 4. No significant difference was observed between the groups prior to rehabilitation (P=0.162); however, a statistically

Table 3. Between-Group Comparisons of hip Joint Flexion Range of Motion Before and After rehabilitation

Flexion with bent knee (°)	Median (P25–P75)	MWU test* (P)	Effect size (r_{pb}†)
Before rehabilitation			
Experimental group	90.0 (80.0-100.0)	141.5	0.01
Control group	90.0 (77.5-100.0)	0.945	
After rehabilitation			
Experimental group	100.0 (90.0-110.0)	132.5	0.07
Control group	95.0 (90.0-107.5)	0.704	

*Mann-Whitney U test; †Point-biserial correlation coefficient.

Table 4. Between-Group Comparisons of Hip Joint Abduction Range of Motion Before and After Rehabilitation

Abduction in the hip joint (°)	Median (P25–P75)	MWU test* (P)	Effect size (r_{pb}†)
Before			
Experimental group	22.5 (15.0-30.0)	103.0	0.28
Control group	17.5 (12.5-25.0)	0.162	
After			
Experimental group	32.5 (25.0-40.0)	85.0	0.40
Control group	25.0 (17.5-30.0)	0.044	

*Mann-Whitney U test; †Point-biserial correlation coefficient.

significant difference was observed after rehabilitation (P=0.044), with a medium effect size (r_{pb}=0.40).

The results regarding pain before and after rehabilitation are shown in Table 5.

Table 5. Between-group Comparisons of Pain Intensity (Numeric Pain Rating Scale) Before and After Rehabilitation

NPRS (points)	C (25-75 percentile)	U (P*)	Effect size (r_{pb})
Before			
Experimental group	0.0 (0.0-1.0)	124.0	0.13
Control group	0.5 (0.0-2.5)	0.501	
After			
Experimental group	0.0 (0.0-1.0)	118.5	0.17
Control group	0.0 (0.0-1.0)	0.388	

C=Median; *Mann-Whitney U test; r_{pb}=Point-biserial correlation coefficient.

Pain intensity scores on the NPRS were low in both groups at baseline and showed no statistically significant differences between the experimental and control groups (P>0.05).

There was no statistically significant difference in functional mobility between the groups after rehabilitation (Table 6). No significant differences were observed in the TUG test results before and after rehabilitation.

Although the difference in TUG performance between groups did not reach statistical significance (P=0.051), this borderline result may still be clinically relevant. Given the small sample size, the study may have been underpowered to detect modest but meaningful differences in functional mobility. Prior to rehabilitation, three participants

Table 6. Between-Group Comparisons of Functional Mobility (Timed Up and Go Test) Before and After Rehabilitation

TUG (s)	C (25-75 percentile)	U (P*)	Effect size (r_{pb})
Before			
Experimental group	7.65 (6.74-9.05)	112.0	0.22
Control group	9.72 (6.75-10.95)	0.325	
After			
Experimental group	5.35 (5.03-6.70)	87.0	0.39
Control group	6.50 (5.30-8.30)	0.051	

C=Median;*Mann-Whitney U test; TUG=Timed Up and Go Test; r_{pb}=Point-biserial correlation coefficient.

in the EG were at risk of falling (TUG score > 13.5 seconds), whereas no participants were at risk after rehabilitation.

The results showed no statistically significant difference in functional mobility between the groups, as measured by the 40 mFPWT, either before or after rehabilitation (Table 7).

Table 7. Between-Group Comparisons of Walking Performance (40-meter Fast-Paced Walk Test) Before and After Rehabilitation

40 m FPWT (s/m)	C (25-75 percentile)	U (P*)	Effect size (r_{pb})
Before			
Experimental group	0.76 (0.65-0.95)	102.0	0.29
Control group	0.84 (0.68-1.03)	0.152	
After			
Experimental group	0.63 (0.52-0.72)	99.0	0.31
Control group	0.68 (0.61-0.85)	0.125	

C=Median; *Mann-Whitney U test; r_{pb}=Point-biserial correlation coefficient.

Discussion

The results of this study showed no statistically significant differences between anti-gravity treadmill and conventional rehabilitation in tested variables, except for the hip joint abduction range of motion, although the experimental group received a longer total duration of therapy compared to the control group (95 vs. 75 minutes per session). However, when each group was analyzed individually, both showed statistically significant

improvements in all tested variables, except for pain, after rehabilitation.

No significant differences in hip abductor muscle strength were observed between the EG and CG following rehabilitation. Kim et al. (18) have demonstrated the positive effects of AT training on lower extremity muscle strength following femoral fractures. In addition, patients who trained with the AT experienced significantly less postoperative decline in knee muscle strength than those who underwent conventional therapy (6). In contrast, Unlu et al. (19) and Trudelle et al. (20) reported that standard physiotherapy and postural stability exercises can significantly improve abductor strength (by 41.2%) and extensor strength (by 47.8%). The benefits of conventional rehabilitation, including strength, endurance, flexibility, gait, and balance training, three to five months and one year after THA, have also been confirmed by Heiberg et al. (21) and Nankaku et al. (22). No significant differences in hip abductor muscle strength may be attributed to the nature of the anti-gravity treadmill exercise, which reduces body weight load and creates a more relaxed training environment, thus potentially limiting the stimulus needed to build muscle strength.

This study also assessed the ROM of the hip joint. A significant increase in abduction ROM was found in favor of the EG (P=0.044), whereas no statistically significant difference was observed in hip flexion ROM between the groups (P=0.704). The statistically significant finding for hip joint abduction should be interpreted with caution, as multiple outcomes were tested, and no correction for multiple comparisons was applied. Currently, no studies have specifically examined the effect of AT training on hip flexion with the knee bent or in abduction. Evidence supports the positive impact of AT use on ROM in other parts of the musculoskeletal system, including improvements in the ankle following fractures, the knee following tibial plateau fractures and osteoarthritis, and joint mobility post-running injuries and partial knee arthroplasty (23-28).

No statistically significant differences were observed between the groups regarding pain. Notably, 50% of all participants reported no pain before or after rehabilitation, which may explain the modest reduction rate observed. This finding suggests a potential floor effect, limiting the sensitivity of the NPRS to detect differences between groups and reducing its usefulness as an outcome measure in this population. This may be related to the timing of rehabilitation onset, as participants entered the study three months after surgery, when pain levels were typically reduced. Contrary to our results, several studies have reported a reduction in pain following therapy using an AT (22, 29).

In the present study, functional mobility did not differ significantly between the groups after rehabilitation. These findings align with those of Mikami et al., who assessed the impact of AT use two weeks before and after THA in 44 patients and reported no significant group differences (6). However, other studies have identified the beneficial effects of AT training on mobility, functionality, and gait (25, 26, 30), although these effects were observed in the context of conservative management of injuries.

Limitations of the Study

This study has several limitations that should be considered when interpreting the findings. First, the relatively small sample size may have limited the ability to detect significant between-group differences. The open-label design, without blinding of participants, therapists, or assessors, introduces the possibility of performance and expectation bias. Expectation and performance bias cannot be excluded and may have affected internal validity. Although randomization was employed, the single-center design may limit generalizability and does not fully exclude the possibility of selection bias. Also, the presence of a floor effect in pain scores, together with the relatively late initiation of rehabilitation, may have affected both the sensitivity of outcome measures and the generalizability of the findings. Finally, we did not perform a sensitivity intention-to-treat analysis, which could potentially impact the findings.

Conclusions

Anti-gravity treadmill training, when added to standard rehabilitation, was associated with improvements in hip abduction range of motion following total hip arthroplasty, while no clear additional benefit was observed in other assessed outcomes compared with standard rehabilitation alone. Future research with larger samples, balanced treatment exposure, and varying levels of body weight support, including higher levels of unloading than those applied in this study, is needed to further clarify the role of anti-gravity treadmill training in postoperative rehabilitation.

What Is Already Known on This Topic:

To date, studies have examined the effects of anti-gravity treadmill rehabilitation in various clinical populations, including patients with neurological disorders, individuals following femoral fractures, and those with osteoarthritis. The results of these studies indicate the positive effects of AT training on functional mobility, gait, and reduction of lower limb loading. However, despite the available evidence supporting its use in these conditions, no studies have investigated the effects of AT training following THA. Furthermore, no study has compared rehabilitation outcomes using anti-gravity treadmill training with standard rehabilitation protocols in patients after THA.

What This Study Adds:

This study provides data on the effects of rehabilitation using an AT on functional outcomes in patients who have undergone THA compared with standard rehabilitation without the use of an AT. Outcome measures included range of motion in the hip joint, hip abductor muscle strength, the Timed Up and Go Test, the 40-Meter Fast-Paced Walk Test, and pain intensity. The rehabilitation program lasted for three weeks.

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

References

1. Sicard-Rosenbaum L, Light KE, Behrman AL. Gait, lower extremity strength, and self-assessed mobility after hip arthroplasty. J Gerontol A Biol Sci Med Sci. 2002;57(1):M47-51. doi: 10.1093/gerona/57.1.m47.
2. Inacio M, Ryan AS, Bair WN, Prettyman M, Beamer BA, Rogers MW. Gluteal muscle composition differentiates fallers from non-fallers in community dwelling older adults. BMC Geriatr. 2014;14:37. doi: 10.1186/1471-2318-14-37.
3. Hill AM, Ross-Adjie G, McPhail SM, Monterosso L, Bulsara M, Etherton-Beer C, et al. Incidence, risk factors and the healthcare cost of falls postdischarge after elective total hip and total knee replacement surgery: protocol for a prospective observational cohort study. BMJ Open. 2016;6(7):e011139. doi: 10.1136/bmjopen-2016-011139.
4. Malaya CA, Parikh PJ, Smith DL, Riaz A, Chandrasekaran S, Layne CS. Effects of simulated hypo-gravity on lower limb kinematic and electromyographic variables during anti-gravitational treadmill walking. Front Physiol. 2023;14:1141015. doi: 10.3389/fphys.2023.1141015.
5. Mikami Y, Fukuhara K, Kawae T, Kimura H, Ochi M. The effect of anti-gravity treadmill training for prosthetic rehabilitation of a case with below-knee amputation. Prosthet Orthot Int. 2015;39(6):502-6. doi: 10.1177/0309364614532866. Epub 2014 May 12.
6. Mikami Y, Orita N, Yamasaki T, Kamijo Y, Kimura H, Adachi N. The Effect of Simultaneous Antigravity Treadmill Training and Electrical Muscle Stimulation After Total Hip Arthroplasty: Short Follow-Up Time. Ann Rehabil Med. 2019;43(4):474-82. doi: 10.5535/arm.2019.43.4.474. Epub 2019 Aug 31.
7. Xie R, Huang J, Wu Q, Qian YF, Jiang D, Li L, et al. A Comparison of Radiographic Outcomes after Total Hip Arthroplasty between the Direct Lateral Approach and Posterior Lateral Approach with EOS 2D/3D X-Ray Imaging System. Orthop Surg. 2023;15(5):1312-24. doi: 10.1111/os.13726. Epub 2023 Apr 19.
8. Bugbee WD, Pulido PA, Goldberg T, D'Lima DD. Use of an Anti-Gravity Treadmill for Early Postoperative Rehabilitation After Total Knee Replacement: A Pilot Study to Determine Safety and Feasibility. Am J Orthop (Belle Mead NJ). 2016;45(4):E167-73.
9. Cheng YY, Chen CH, Wang SP. Isokinetic training of lower extremity during the early stage promote functional restoration in elder patients with disability after Total knee replacement (TKR) - a randomized control trial. BMC Geriatr. 2024;24(1):173. doi: 10.1186/s12877-024-04778-9.
10. Sekita J, Takahira N, Iwamura G, Watanabe H, Kusaba A, Kondo S. A predictive model for hip abductor strength and knee extensor strength 12 months after total hip arthroplasty with an interaction term. BMC Musculoskelet Disord. 2021;22(1):827. doi: 10.1186/s12891-021-04719-2.
11. EQUATOR Network. Enhancing the QUAlity and Transparency Of health Research [Internet]. Oxford: EQUATOR Network; c2024 [cited 2024 Feb 29]. Available from: https://www.equator-network.org/.
12. CSMi Solutions. 300004D-409 HUMAC NORM user manual [Internet]. Stoughton (MA): CSMi; [cited 2023 Jun 23]. Available from: https://www.yumpu.com/en/document/view/8976833/300004d-409-humac-norm-user-manual-csmi-solutions.

13. Petrc N, Brentin M, Vučković M, Kehler T. Comparison of isokinetic exercise and standard exercise protocol in patients with spondyloarthropathies. J Health Sci. 2024;14(1):56-62. doi: https://doi.org/10.17532//jhsci.2024.2370.
14. Jensen MP, Karoly P, Braver S. The measurement of clinical pain intensity: a comparison of six methods. Pain. 1986;27(1):117-26. doi: 10.1016/0304-3959(86)90228-9.
15. Steffen TM, Hacker TA, Mollinger L. Age- and gender-related test performance in community-dwelling elderly people: Six-Minute Walk Test, Berg Balance Scale, Timed Up & Go Test, and gait speeds. Phys Ther. 2002;82(2):128-37. doi: 10.1093/ptj/82.2.128.
16. Barry E, Galvin R, Keogh C, Horgan F, Fahey T. Is the Timed Up and Go test a useful predictor of risk of falls in community dwelling older adults: a systematic review and meta-analysis. BMC Geriatr. 2014;14:14. doi: 10.1186/1471-2318-14-14.
17. Kennedy DM, Stratford PW, Wessel J, Gollish JD, Penney D. Assessing stability and change of four performance measures: a longitudinal study evaluating outcome following total hip and knee arthroplasty. BMC Musculoskelet Disord. 2005;6:3. doi: 10.1186/1471-2474-6-3.
18. Kim P, Lee H, Choi W, Jung S. Effect of 4 Weeks of Anti-Gravity Treadmill Training on Isokinetic Muscle Strength and Muscle Activity in Adults Patients with a Femoral Fracture: A Randomized Controlled Trial. Int J Environ Res Public Health. 2020;17(22):8572. doi: 10.3390/ijerph17228572.
19. Unlu E, Eksioglu E, Aydog E, Aydog ST, Atay G. The effect of exercise on hip muscle strength, gait speed and cadence in patients with total hip arthroplasty: a randomized controlled study. Clin Rehabil. 2007;21(8):706-11. doi: 10.1177/0269215507077302.
20. Trudelle-Jackson E, Smith SS. Effects of a late-phase exercise program after total hip arthroplasty: a randomized controlled trial. Arch Phys Med Rehabil. 2004;85(7):1056-62. doi: 10.1016/j.apmr.2003.11.022.
21. Heiberg KE, Figved W. Physical Functioning and Prediction of Physical Activity After Total Hip Arthroplasty: Five-Year Followup of a Randomized Controlled Trial. Arthritis Care Res (Hoboken). 2016;68(4):454-62. doi: 10.1002/acr.22679.
22. Nankaku M, Ikeguchi R, Goto K, So K, Kuroda Y, Matsuda S. Hip external rotator exercise contributes to improving physical functions in the early stage after total hip arthroplasty using an anterolateral approach: a randomized controlled trial. Disabil Rehabil. 2016;38(22):2178-83. doi: 10.3109/09638288.2015.1129453. Epub 2016 Jan 10.
23. Cutuk A, Groppo ER, Quigley EJ, White KW, Pedowitz RA, et al. Ambulation in simulated fractional gravity using lower body positive pressure: cardiovascular safety and gait analyses. J Appl Physiol (1985). 2006;101(3):771-7. doi: 10.1152/japplphysiol.00644.2005. Epub 2006 Jun 15.
24. Huang CH, Schroeder ET, Powers C. Antigravity treadmill training during the early rehabilitation phase following unicompartmental knee arthroplasty: A case series. Physiother Theory Pract. 2019;35(5):489-94. doi: 10.1080/09593985.2018.1443535. Epub 2018 Feb 26.
25. Kurz MJ, Corr B, Stuberg W, Volkman KG, Smith N. Evaluation of lower body positive pressure supported treadmill training for children with cerebral palsy. Pediatr Phys Ther. 2011;23(3):232-9. doi: 10.1097/PEP.0b013e318227b737.
26. Liang J, Lang S, Zheng Y, Wang Y, Chen H, Yang J, et al. The effect of anti-gravity treadmill training for knee osteoarthritis rehabilitation on joint pain, gait, and EMG: Case report. Medicine (Baltimore). 2019;98(18):e15386. doi: 10.1097/MD.0000000000015386.
27. Peeler J, Christian M, Cooper J, Leiter J, MacDonald P. Managing Knee Osteoarthritis: The Effects of Body Weight Supported Physical Activity on Joint Pain, Function, and Thigh Muscle Strength. Clin J Sport Med. 2015;25(6):518-23. doi: 10.1097/JSM.0000000000000173.
28. Hesse S. Treadmill training with partial body weight support after stroke: a review. NeuroRehabilitation. 2008;23(1):55-65.
29. Carvalho C, Serrão FV, Mancini L, Serrão PRMDS. Impaired muscle capacity of the hip and knee in individuals with isolated patellofemoral osteoarthritis: a cross-sectional study. Ther Adv Chronic Dis. 2021;12:20406223211028764. doi: 10.1177/20406223211028764.
30. Vincent HK, Madsen A, Vincent KR. Role of Antigravity Training in Rehabilitation and Return to Sport After Running Injuries. Arthrosc Sports Med Rehabil. 2022;4(1):e141-9. doi: 10.1016/j.asmr.2021.09.031.

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The Impact of the Russo-Ukrainian War on Ukrainian Scholarly Publishing and Research Directions: A Bibliometric Analysis

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Abstract

Objectives. Russia's aggression against Ukraine has unfolded in two phases: the onset of the Donbas war and the annexation of Crimea in 2014, and the full-scale invasion in 2022. We examined the changes in Ukrainian scholarly publishing across these disruption periods. **Methods.** Using Web of Science Core Collection and InCites data (1991–2024), we analyzed publication trends in Ukraine and Croatia, disciplinary composition, leading universities, city-level output in occupied territories, and international collaboration. Interrupted time series (ITS) models were specified with breakpoints at 2014 and 2022. **Results.** Ukraine's output increased from 331 articles in 1991 to 12,475 in 2021, then declined by 14.6% to 10,649 in 2024, whereas Croatia's output remained comparatively stable. The segmented ITS showed significant growth before 2014, a steeper upward trend after 2014, and a decline after 2022. Croatia showed continued growth with a smaller, non-significant post-2022 slowdown. In Ukraine, the largest post-2022 declines were observed in Physical Sciences and Social Sciences, while Engineering and Technology appeared more resilient. City-level analyses in occupied territories showed an early break in 2014 in Donetsk and Luhansk, while output linked to Simferopol and Sevastopol was rapidly reclassified after 2014, with records increasingly indexed under Russia. After 2022, collaboration with Russia collapsed, whereas partnerships with Poland, Germany, and the USA expanded. **Conclusion.** Ukrainian scholarly publishing showed phase-specific shifts in output and collaboration patterns that coincided with major geopolitical disruptions.

Key Words: Ukraine ▪ Russo-Ukrainian War ▪ Scientific Publishing ▪ Full-Scale Invasion.

Introduction

Russia's full-scale invasion of Ukraine on February 24, 2022, disrupted nearly all aspects of life, including academia, through displacement, infrastructure loss, and military mobilization (1). A 2022 survey found that 18.5% of researchers had emigrated and 17.6% had left academia, reducing national research capacity by 20%, while damage to scientific institutions and equipment further disrupted research activity (2-4). An estimated 10% decline in scientific output after 2022 has been attributed primarily to human capital loss rather than infrastructure damage (5). Recent studies have documented important war-related changes in Ukrainian academic publishing. These studies show declining productivity, shifts in research priorities toward war-related topics, and increased reliance on international collaborators for statistical, language, and publishing support (6-9). In our previous study, Ukrainian researchers described major wartime barriers to publishing, including limited funding, methodological support needs, language difficulties, and psychological stress (10). In the present study, we extend those findings by examining how such challenges may be reflected in changes in publication output.

Several international organizations have provided support to Ukrainian scientists. Short-term

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grants and scholarships have helped researchers remain connected to the global academic community (8), while newer initiatives have included participation in business incubators focused on post-war recovery projects (7). Our prior research also identified the Giving Voice initiative as an important support mechanism, providing mentorship, editorial assistance, and technical guidance that helped Ukrainian researchers continue publishing and gain international visibility (10, 11). Coordinated by the TRIBE doctoral program at the University of Split School of Medicine, it supports Ukrainian researchers in publishing war-related experiences and the war's impact on scientific work (11).

As a continuation of these efforts, we analyzed Ukrainian publication activity (1991–2024), with particular attention to institutions in temporarily occupied territories, specifically universities in Luhansk, Donetsk, Simferopol, and Sevastopol. These "stolen universities" have faced major disruption since 2014, following the Russian Federation's illegal annexation of Crimea and its support for the so-called Donetsk People's Republic (DPR) and Luhansk People's Republic (LPR) (12). During the occupation, many universities were either relocated to Ukrainian government-controlled areas or taken over by occupation authorities and re-registered under Russian jurisdiction, often continuing to operate under the original institutional names. According to Sych et al., the annexation and war led to the displacement of at least 18 higher education institutions, involving over 3,500 academic staff and approximately 40,000 students (13).

In this context, the present study aimed to describe how the Russo-Ukrainian war changed Ukrainian scholarly publishing and research directions. We examined national output, disciplinary composition, leading universities, international collaborations, and publication activity linked to universities from occupied territories.

Materials and Methods

Study Design and Setting

Our bibliometric study was designed as a descriptive and historical analysis of publication patterns during a period of overlapping systemic disruptions, rather than as a definitive causal evaluation of the effects of war on scientific output. Two major phases of Russian aggression were identified. The first phase began in 2014 with the annexation of Crimea and the war in Donbas, causing major regional disruption in the occupied areas (14). The second phase began in 2022 with the full-scale invasion and remains ongoing, with widespread destruction, displacement, and substantial disruption to daily life, including higher education and research.

Beyond the war, Ukraine's research community experienced COVID-19, which shifted research priorities toward pandemic-related topics and coincided with declines in other areas (15, 16). Following the WHO's March 2020 declaration, we defined the COVID-19 period as 2020–2021 (17), capturing the peak, globally synchronized disruption to higher education and research. Vaccine rollout from late 2020 and throughout 2021 supported gradual reopening and adaptation in many settings.

To capture these major disruptions, we defined four contextual periods: the pre-war baseline (1991–2013), the Donbas war period (2014–2021), the COVID-19 pandemic period (2020–2021), and the full-scale invasion period (2022–2024). These periods serve as historical context markers rather than mutually exclusive analytical regimes. In the interrupted time series (ITS) analysis, breakpoints at 2014 and 2022 were specified for the national series and for analyses of Crimea- and Donbas-affiliated academics, reflecting the 2014 annexation/onset of the Donbas war and the 2022 full-scale invasion. The Donbas war and the COVID-19 pandemic were treated as overlapping background disruptions that may influence publication trends but were not modeled as separate intervention points.

Our analysis starts in 1991, Ukraine's year of independence. Before then, Web of Science indexed institutions in present-day Ukraine under the Soviet Union or the Ukrainian SSR, and records do not reliably distinguish authors across former Soviet republics. Retrospective attribution would require unjustified assumptions based on

bibliometric metadata. Independence is the most defensible starting point for assessing scientific output in a new sovereign state. Low early-1990s counts reflect the post-independence transition and this methodological boundary, not a lack of scientific activity.

Croatia was used as a comparative benchmark rather than a strict counterfactual. Both countries became independent in 1991 and developed post-socialist academic systems with broadly similar trajectories of restructuring and internationalization. Croatia's relative geopolitical stability provides a historically grounded reference for interpreting changes in Ukraine's publication trajectory amid global shifts in publishing and indexing, including the COVID-19 period. The focus on temporal trends rather than absolute publication volume further strengthens the choice of Croatia as a comparator.

Data Sources and Data Collection

The primary sources of bibliometric data were the Web of Science Core Collection (WoS CC) and the InCites Benchmarking & Analytics platform (Clarivate). Data were retrieved using the InCites platform in July 2025 and covered the period from 1991 to 2024. The final dataset included 282,863 publications for Ukraine and 157,943 for Croatia, although these numbers may be subject to minor updates due to ongoing indexing.

To examine how Ukrainian scientific output was distributed across disciplines and which fields were more resilient or vulnerable over time, we classified publications by research field using the InCites Global Institution Profiles Project (GIPP) categories. The categories were Arts & Humanities; Clinical, Pre-Clinical & Health; Engineering & Technology; Life Sciences; Physical Sciences; and Social Sciences. The "Overall" category was excluded to focus the analysis on specific disciplinary trends.

We also assessed institutional scientific productivity for leading Ukrainian universities, identified through the Shanghai and Times Higher Education (THE) rankings. We included the five highest-ranked Ukrainian institutions listed in the 2025 Academic Ranking of World Universities (ARWU – Shanghai Ranking) and the 2025 Times Higher Education (THE) World University Rankings (overall tables). The 2025 rankings were used because the data were retrieved in July 2025, making them the most current rankings available at the time and providing a stable institutional reference set.

Analysis of the scientific production in occupied territories accounted for the complex institutional transformations resulting from the ongoing war. Many Ukrainian universities originally located in occupied territories were relocated to regions under Ukrainian control, where displaced staff continued their work under the umbrella of these relocated institutions, which retained their legal status and preserved institutional continuity under Ukrainian law (e.g., Donetsk National University was relocated to Vinnytsia, and Luhansk Taras Shevchenko National University moved to Poltava). In parallel, occupation authorities continued to operate institutions in the occupied territories under Russian administrative control using the same names. Although these institutions bear the same names, they should not be regarded as identical, as they do not operate under Ukrainian law. To evaluate the impact of these transformations, we analyzed publication outputs from the cities of Crimea (Simferopol and Sevastopol), Donetsk, and Luhansk. To track the publication activity of academic institutions using affiliation of those cities but different countries, we used the WoS platform and the following query structure: AD=(city) AND AD=(country) AND DT=(Article), where "AD" indicates the author's institutional address, "city" was replaced sequentially with Donetsk, Luhansk, Simferopol, and Sevastopol. Because the WoS country field is a database label derived from recorded affiliations (and does not constitute a legal designation) and may change under occupation or administrative reassignment, we ran each city query twice, specifying the country as "Ukraine" and as "Russia," to capture shifts in the country label associated with address records under occupation. The query was

limited to the document type "Article" to ensure consistency with the rest of our dataset.

Finally, to understand how international collaboration patterns evolved over time and which countries were consistently the most active scientific partners for Ukraine, we used the Collaboration tab in the InCites analytics platform and selected "Location" as the primary filter, setting it to Ukraine. This allowed us to identify partner countries and track collaboration trends in publication output from 1991 to 2024. We applied the document type filter to include only "Articles." From the automatically generated list, we selected the 10 most productive collaborating countries.

Statistical Analysis

To examine changes in Ukraine's scientific output around the 2022 full-scale invasion, we conducted an ITS analysis using ordinary least squares regression in R (version 4.5.1; R Core Team, 2025) in RStudio. The outcome was the annual count of WoS-indexed Articles. Models included a continuous time variable (years since 1991) and breakpoint terms for 2014 and 2022 (post-indicators and time-after terms) to estimate level and slope changes at each breakpoint. Model fit was evaluated using R^2 and residual diagnostics, and observed versus predicted values were inspected with vertical markers at the breakpoints. Croatia was included as a comparator series to contextualize Ukraine's trends relative to broader publishing and indexing dynamics. COVID-19 was treated as a contextual background rather than modeled as a separate breakpoint because it overlaps with the war period and broader time-varying publishing and indexing dynamics.

Results

National Publication Trends in Ukraine and Croatia

Scientific publication output in Ukraine increased steadily from 1991 to 2021, from 331 to 12,475 articles annually, with a marked increase between 2019 and 2021 (Figure 1A). Croatia showed a similar upward trend at lower absolute levels, from 434 in 1991 to 6,934 in 2021 (Figure 1B). After the 2022 invasion, Ukraine's growth slowed sharply, falling from 12,475 articles in 2021 to 10,649 in 2024, a decline of 14.6% over this period, while Croatia's output remained relatively stable, declining slightly to 6,540.

Interrupted Time Series Estimates for National Output

Table 1 shows that both countries had strong positive publication trends before 2014. Ukraine experienced a significant acceleration in output after 2014, followed by a significant reversal in trend after 2022, while Croatia showed only a smaller post-2014 increase and no significant post-2022 change.

Disciplinary Composition and Field-specific Disruptions

Analysis of research output by discipline showed that Physical Sciences and Engineering & Technology were the most productive fields, both exhibiting significant pre-2022 growth rates of + 123 and + 71 articles per year, respectively ($P<0.001$) (Figure 2). Physical Sciences output increased sharply in the early post-independence years, surpassing 2,000 annual publications by 1993 before settling into sustained growth. Engineering & Technology followed a similar pattern, with an early surge from a lower baseline. Other fields, Life Sciences (+51/year), Social Sciences (+66/year), Clinical, Pre-Clinical & Health (+32/year), and Arts & Humanities (+23/year), remained at lower levels.

Following the annexation of Crimea in 2014, growth patterns shifted. All scientific fields accelerated, except Physical Sciences, which began to plateau, fluctuating around its peak rather than continuing a strong upward trend.

The COVID-19 pandemic (2020–2021) coincided with declines in Physical Sciences, while Engineering & Technology maintained growth, and other fields remained relatively stable. This period is described for context and was not

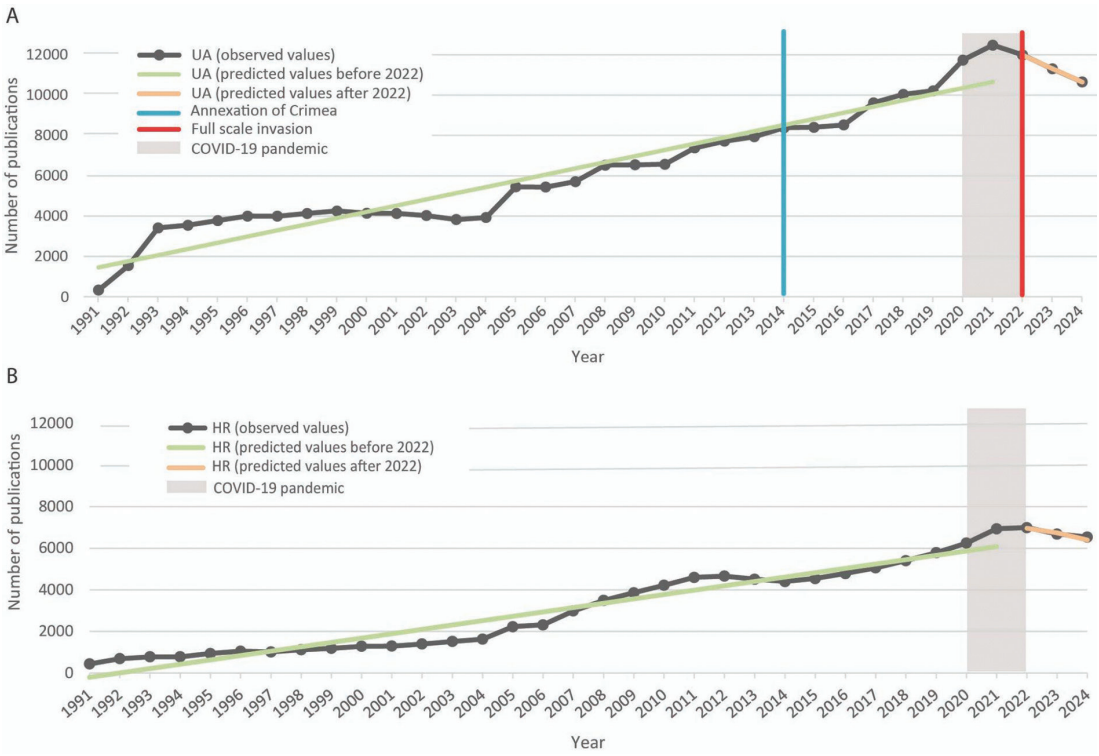

Figure 1. Interrupted time series analysis of scientific publication output in A) Ukraine and B) Croatia (1991–2024), with breakpoints at 2014 (onset of the Donbas war/annexation of Crimea) and 2022 (full-scale invasion).[1]

Table 1. Segmented Interrupted Time Series (ITS) Regression Estimates for Annual WoS-Indexed Article Counts (1991–2024), with Breakpoints at 2014 and 2022

Country	Term	β	SE	CI 2.5%	CI 97.5%	P value	R^2
Ukraine	Baseline slope (pre-2014)	253.5	20.8	210.8	295.9	0	0.963
	Level change 2014	54.9	513.2	-996.2	1106	0.916	0.963
	Slope change 2014	351.7	104.1	138.5	565	0.002	0.963
	Level change 2022	-661.2	793.5	-2286.5	964.2	0.412	0.963
	Slope change 2022	-1276.1	478.5	-2256.2	-296	0.013	0.963
Croatia	Baseline slope (pre-2014)	197.8	14.1	168.9	226.7	0	0.964
	Level change 2014	-299.4	348.7	-1013.7	414.8	0.398	0.964
	Slope change 2014	154.7	70.7	9.8	299.7	0.037	0.964
	Level change 2022	-15.6	539.2	-1120	1088.9	0.977	0.964
	Slope change 2022	-578.6	325.1	-1244.6	87.5	0.086	0.964

Legend: Coefficients are from ordinary least squares segmented regression. Baseline slope is the estimated annual change in articles before 2014. Level changes represent immediate shifts in 2014 and 2022, and slope changes represent changes in annual trends after these breakpoints. β, coefficient estimate; SE, standard error; CI, 95% confidence interval; P, significance test for β = 0; R^2, model fit; N, number of annual observations was 34.

[1] The analysis starts in 1991, the year of Ukraine's independence. Pre-1991 articles from institutions in present-day Ukraine were indexed under the Soviet Union and cannot be reliably disaggregated by national affiliation. Low counts in the early 1990s therefore reflect the post-independence transition and the analytical boundary, rather than an absence of scientific activity.

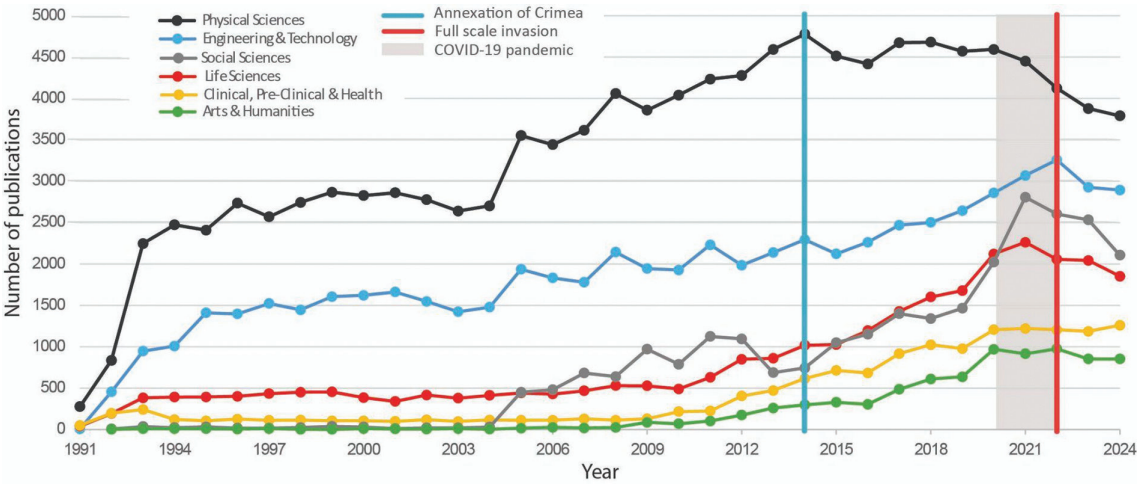

Figure 2. Annual trend in the number of scientific publications by Ukrainian researchers indexed in the Web of Science Core Collection (1991-2024) by scientific discipline.

modeled as a separate intervention because its effects overlap with war-related disruptions and are better treated as a shared background influence captured through the Croatia comparator. ITS analysis incorporating breakpoints in 2014 and 2022 (with 2014 capturing the Donbas phase and 2022 the full-scale invasion) indicated that disciplinary trajectories shifted after 2014 primarily through changes in growth rates (slope) rather than abrupt level drops, with Physical Sciences showing the clearest early flattening. The model also showed estimated post-2022 level changes in several fields, most notably Physical Sciences (–1,232 articles, P=0.014) and Social Sciences (–977, P=0.005), whereas Arts & Humanities showed an estimated increase (+408, P=0.018). Clinical, Pre-Clinical & Health remained close to pre-2022 levels in the post-invasion period. These model-based effects were larger than those visible in raw counts, underscoring the scale of disruption.

Publication Trajectories of Highly Ranked Ukrainian Universities

The publication output of highly ranked Ukrainian universities in the Shanghai (Figure 3A) and THE lists (Figure 3B) was low and fairly stable in 1991, with no institution exceeding about 150 articles (Figure 3). Most universities published fewer than 200 articles annually, and changes during the 1990s were gradual. From 2004 onward, output increased steadily across most institutions, although growth varied markedly. For instance, Ivan Franko National University of Lviv, the top Shanghai-ranked institution, reached around 450 articles (Figure 3A), less than half the output of Taras Shevchenko National University of Kyiv (Figure 3B), which recorded peaks above 1,000 articles. Growth remained modest before 2008, but from 2009 publication output rose consistently until 2022. The full-scale invasion then sharply disrupted this upward trend at nearly all universities, except Bogomolets National Medical University.

Scientific Output in Occupied Territories

The analysis of scientific production in occupied territories focused on Luhansk, Donetsk, Simferopol, and Sevastopol (Figure 4; Supplementary Tables S1 and S2). Because Donetsk and Luhansk were affected by occupation and armed conflict from 2014 onward, city-level changes were interpreted relative to the 2014 breakpoint and, where relevant, the additional 2022 breakpoint used in the same ITS framework as the national analyses. In Luhansk (Figure 4A), Ukrainian-affiliated output showed a significant level drop in 2014 (–12.7, P=0.044), followed by a slope increase after 2014 (+ 5.17/year, P<0.001), but a marked negative slope change after 2022 (–17.95/year, P=0.003). Russia-indexed

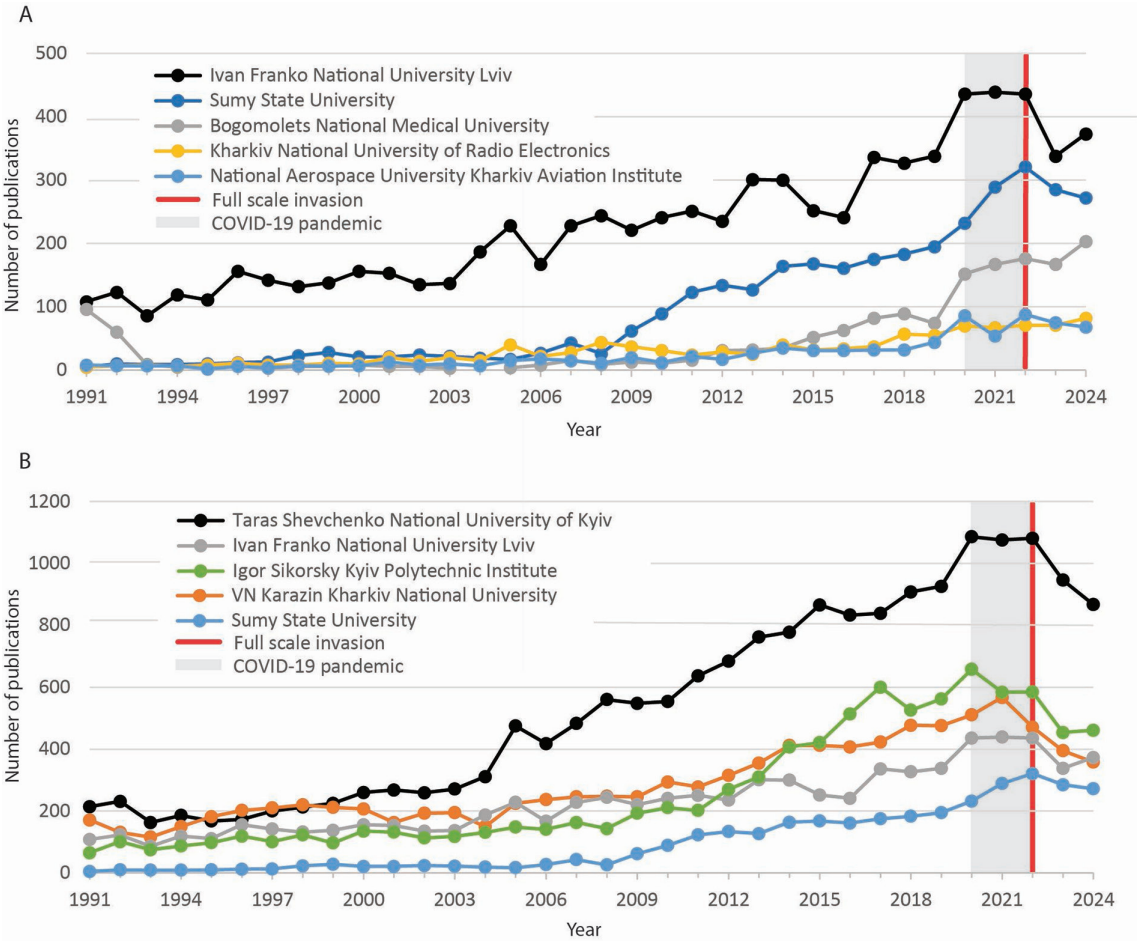

Figure 3. The annual number of publications from highly ranked Ukrainian institutions according to the (A) Shanghai and (B) Times Higher Education ranking lists.

output remained low, with only minor changes over time (Supplementary Tables S1 and S2). In Donetsk (Figure 4B), Ukrainian-affiliated output declined immediately in 2014 (–68.3, P=0.046), while Russia-indexed articles increased sharply after 2014, with both a positive level change (+17.1, P<0.001) and slope increase (+3.10/year, P<0.001). After 2022, Donetsk's Russia-indexed output showed a significant level decrease (–15.6, P=0.009), and the Ukrainian-affiliated series showed a further negative slope change (–64.81/year, P=0.043) (Supplementary Tables S1 and S2).

By contrast, Simferopol (Figure 4C) and Sevastopol (Figure 4D), the main scientific centers of Crimea, followed a different trajectory. After the 2014 annexation, Russia-indexed output rose sharply in both cities, with strong positive post-2014 slope changes (Simferopol: +32.62/year, P<0.001; Sevastopol: +32.92/year, P<0.001), indicating rapid bibliometric substitution. After 2022, these series were clearly disrupted: Simferopol showed a large negative level change (–103.1, P<0.001) and negative slope change (–41.14/year, P=0.006), while Sevastopol showed a significant negative slope change (–47.21/year, P<0.001) (Supplementary Tables S1 and S2). These patterns suggest a clear divergence between Donbas and Crimea: Donbas shows declining Ukrainian-affiliated output with only limited Russia-indexed growth, whereas Crimea shows near-complete bibliometric substitution after 2014, with Russia-indexed output replacing, and at times exceeding, pre-2014 Ukrainian levels.

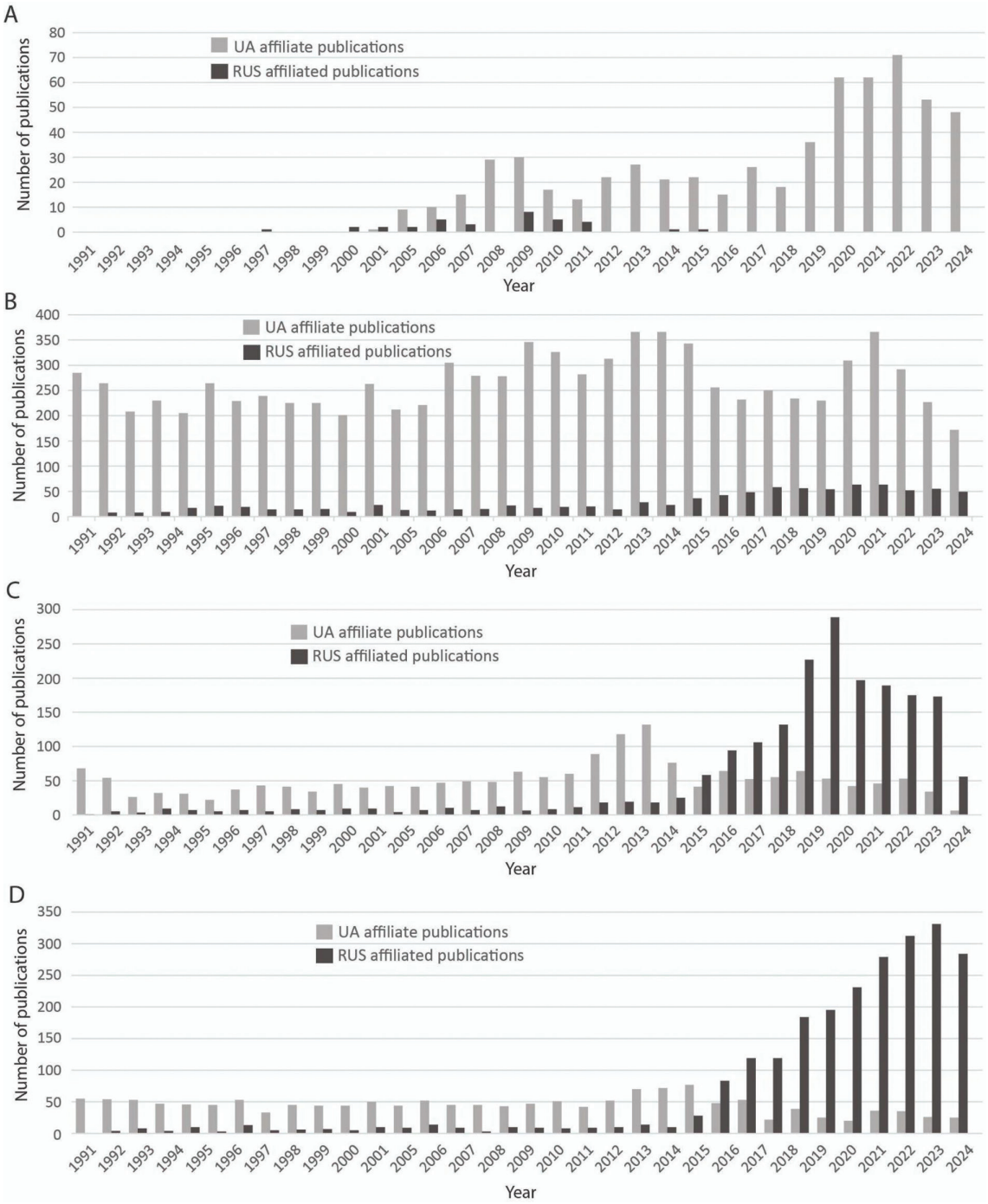

Figure 4. Annual number of publications affiliated with Ukrainian (UA) and Russian (RUS) institutions in cities of occupied territories (1991–2024): (A) Luhansk, (B) Donetsk, (C) Simferopol, (D) Sevastopol.

International Collaboration Patterns

From 1991 to 1994, international co-authored articles by Ukrainian researchers were rare, with fewer than 100 per year (Figure 5). Collaboration with Russia rose rapidly in the early 1990s and remained the largest until 2001, after which Germany and the USA became leading partners. From 2004

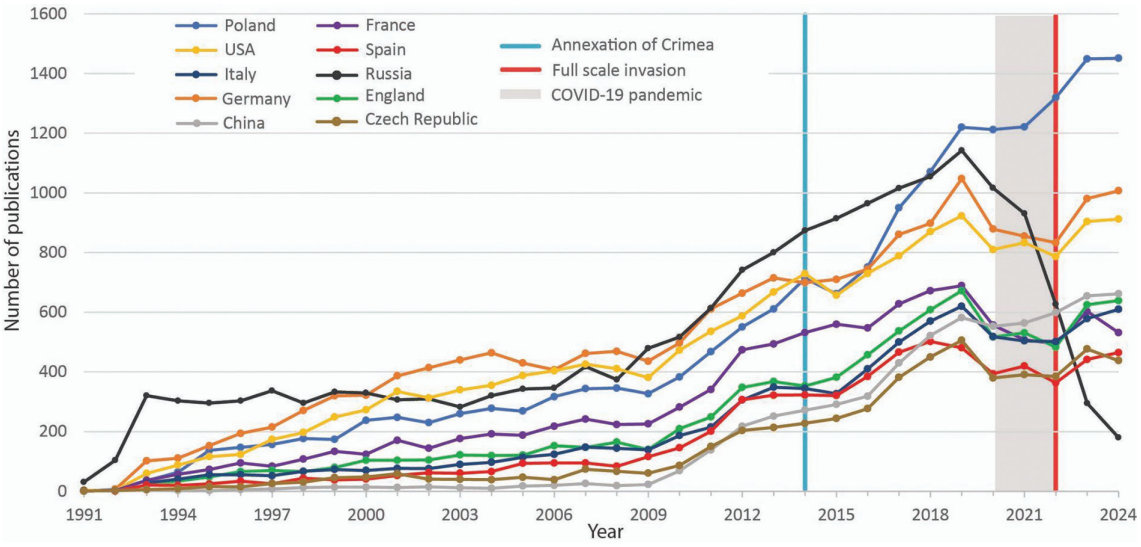

Figure 5. Trends in international research collaborations of Ukrainian scientists with key partner countries (1991–2024).[2]

onward, collaborations with most countries grew steadily. After 2014, collaboration with France, England, and China accelerated, while during COVID-19 (2020–2021), most partnerships stagnated or declined slightly, except in Poland. After 2022, collaboration with Russia fell by an average of 31% compared with the pre-2022 period, dropping from about 900 articles in 2021 to fewer than 200 in 2024. In contrast, Poland became Ukraine's largest partner, increasing by over 220% and exceeding 1,450 joint articles in 2024. Germany (+91%) and the USA (+103%) also expanded cooperation, stabilizing at about 900 to 1,000 articles per year. Other collaborations also increased, including France (+92%), Italy (+168%), England (+153%), Spain (+142%), China (+346%), and the Czech Republic (+211%).

Discussion

Our findings indicate that Russia's aggression produced phase-specific disruptions in Ukraine's research trajectory. Between 1991 and 2021, publication output increased steadily, with accelerated growth after 2014, but this trajectory changed after 2022, when output shifted into sustained decline. Although global factors such as COVID-19, indexing delays, and natural fluctuations may contribute, the timing and magnitude of the downturn are less consistent with a purely global disruption. Because Croatia experienced similar pandemic-era pressures but no war during the period corresponding to Ukraine's wartime years, this comparison suggests that war was the most probable driver. In the segmented interrupted time series analysis, Ukraine showed a significant post-2022 slope reversal, whereas Croatia exhibited only a smaller, non-significant post-2022 slowdown, consistent with war-related displacement, university relocations, infrastructure destruction, and interruption of academic work (2, 5). The ITS estimates also point to a pronounced downturn. Although statistical significance was not reached, likely due to the short post-2022 window, the direction and magnitude align with external evidence on researcher and institutional disruption (5).

The disciplinary analysis highlights both vulnerability and resilience. Fields heavily dependent on laboratories, specialized equipment, and international partnerships, such as the Physical Sciences and Life Sciences, experienced the steepest losses. By contrast, Arts & Humanities and

[2] The analysis starts in 1991, the year of Ukraine's independence. Earlier publications were indexed under the Soviet Union, limiting reliable identification of national collaboration patterns.

Clinical, Pre-Clinical & Health appeared more adaptable, possibly because some publications addressed urgent wartime health, social, and humanitarian needs.

Institutional dynamics also show uneven resilience. In the years following independence, most universities had limited research capacity because of economic hardship and systemic restructuring, with growth emerging only after 2008 during a period of relative stability and gradual modernization. The full-scale invasion reversed these gains across the system, including in Kyiv and Lviv, showing that the impact was nationwide rather than confined to frontline areas. Even leading universities such as Ivan Franko National University of Lviv and Taras Shevchenko National University of Kyiv experienced decline, although they maintained relatively high productivity. Differences between the THE and Shanghai rankings likely reflect their different weighting of publication output (18). An exception was Bogomolets National Medical University, which maintained activity, possibly aided by targeted mentoring and publication support through the Giving Voice initiative (10).

Patterns were especially stark in conflict-affected territories. City-level ITS estimates further support the two-phase pattern. Donetsk and Luhansk show clear changes beginning in 2014, while Crimea displays rapid post-2014 substitution toward Russian-indexed output, with additional disruption after 2022 (Supplementary Tables S1 and S2). In Luhansk and Donetsk, Ukrainian-affiliated publications declined sharply after 2014 and further dropped after 2022, although relocated institutions such as Donetsk National University (Vinnytsia) and Donetsk National Technical University (Pokrovsk) preserved some capacity. By contrast, Crimea displayed bibliometric substitution, as Ukrainian-affiliated output collapsed within two years of annexation while Russian-affiliated publications surged, supported by domestic funding, integration into the Russian Science Citation Index, and targeted re-equipment programs outlined in official Russian policy documents from 2014 onward (19). This divergence underscores two models of disruption: gradual attrition in Donbas versus rapid replacement in Crimea.

The war also reshaped international collaborations. Russia, once Ukraine's leading partner, had largely disappeared from co-authorship networks after 2022, falling below 200 joint papers by 2024. At the same time, partnerships with Poland expanded sharply, surpassing 1,400 publications, while collaborations with Germany, the USA, and other European countries rebounded quickly after an initial wartime dip. Even China strengthened its position. These shifts reflect both the collapse of traditional ties and stronger international support, helping prevent Ukraine's academic isolation (20).

Limitations of the Study

Several limitations should be noted. Bibliometric data are subject to publication lag, which may underestimate recent activity, particularly in 2022 and 2023. In addition, the post-2022 period currently includes only three complete publication years, from 2022 to 2024, which limits longer-term projections and the ability to detect more gradual effects. Reliance on Web of Science also underrepresents locally published work, especially in the humanities and social sciences. This study uses address-based bibliometric data and therefore captures institution- and territory-based output rather than the total productivity of Ukrainian researchers regardless of location. Publications by displaced scholars who re-affiliated abroad are not counted as Ukrainian output, so the observed declines reflect disruptions to nationally affiliated research capacity and may underestimate overall researcher activity. Institutional analysis was also confined to top-ranked universities, which may not capture trends among smaller or regionally focused institutions. Finally, the comparison between Ukraine and Croatia should be interpreted with caution because the two countries differ in the size of their scientific communities and in absolute publication volume. However, our analysis focused on temporal trends and changes in direction over time rather than absolute output levels,

which makes the comparison informative despite these structural differences.

Conclusion

In conclusion, this study demonstrates that the 2022 invasion disrupted decades of scientific progress in Ukraine, with the sharpest losses in infrastructure-dependent fields and occupied regions. Sustained international collaboration, flexible funding, and targeted support for displaced institutions will be critical to preserving Ukraine's scientific capacity and ensuring its reintegration into the global academic system during and after the war.

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

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

References

1. Fiialka S. Assessment of war effects on the publishing activity and scientific interests of Ukrainian scholars. Knowl Perform Manag. 2022;6(1):27-37. doi: 10.21511/kpm.06(1).2022.03.
2. de Rassenfosse G, Murovana T, Uhlbach WH. The effects of war on Ukrainian research. Hum Soc Sci Commun. 2023;10:856. doi: 10.1057/s41599-023-02346-x.
3. Yevstiunina Y, Folhina T, Schmädeke PC. SCIENCE AT RISK Monitoring Report: Ukraine 2022/2023: Threats to science and higher education Berlin: SCIENCE AT RISK Emergency Office; 2023.
4. UNESCO. Analysis of war damage to the Ukrainian science sector and its consequences. 2024. [cited 2026 Apr 11]. Available from: https://unesdoc.unesco.org/ark:/48223/pf0000388803?posInSet=17&queryId=8d77a2e7-8eb9-464f-9998-5281cee9b4bc.
5. Ganguli I, Waldinger F. The impact of the war in Ukraine on science and universities: Centre for Economic Policy Research; 2023. VoxEU. Available from: https://cepr.org/voxeu/columns/impact-war-ukraine-science-and-universities.
6. Guariglia A, Nikolsko-Rzhevskyy A, Talavera O, Zadorozhna O. Research productivity during the Russian war in Ukraine. Public Choice. 2025;205:443-67. doi: 10.1007/s11127-025-01258-5.
7. Qureshi A, Rizwan MS, Ahmad G, Ashraf D. Russia–Ukraine war and systemic risk: Who is taking the heat? Finance Res Lett. 2022;48:103036. doi: 10.1016/j.frl.2022.103036.
8. Zayachkivska O, Smiechowska T, Souchelnytskyi S. The war and science in Ukraine: we can contribute to victory. Proc Shevchenko Sci Soc Med Sci 2022;66(1):14-21. doi: 10.25040/ntsh2022.01.02.
9. Denisova-Schmidt E, Marmilova O. Displacement and Higher Education: A Review of the Research on Ukraine. Soc Sci. 2026;15(1):29. doi: 10.3390/socsci15010029.
10. Shmatkova M, Buljan I, Puljak L, Sapunar D. Experiences of Ukrainian researchers participating in a project assisting in writing and publishing war-related research: a mixed-methods study. Stud High Educ. 2025:1-18. doi: 10.1080/03075079.2025.2605099.
11. Sapunar D, Puljak L. Project Giving Voice – helping authors publish reports about the consequences of War in Ukraine. ST-OPEN. 2023;4(e2023.2319.10.).
12. Plastun AK, S. Stolen Ukrainian universities: An invisible russian weapon. Probl Perspect Manag. 2025;23(2):151-75. doi: 10.21511/ppm.23(2-si).2025.11.
13. Sych T, Panchenko L, Saienko O, Ptakhina O, Ivanov Y. The impact of the forced displacement of universities due to the occupation of certain territories of Ukraine on the effectiveness of their activities: "Top 200 Ukraine" universities ranking. Prob Perspect Manag. 2025;23(2):176-97. doi: 10.21511/ppm.23(2-si).2025.12.
14. Mankoff J. Russia's Latest Land Grab: How Putin Won Crimea and Lost Ukraine. Foreign Affairs. 2014;93(3):60-8.
15. Gao J, Yin Y, Myers KR, Lakhani KR, Wang D. Potentially long-lasting effects of the pandemic on scientists. Nature Commun. 2021;12(1):6188. doi: 10.1038/s41467-021-26428-z.

16. Raynaud M, Goutaudier V, Louis K, Al-Awadhi S, Dubourg Q, Truchot A, et al. Impact of the COVID-19 pandemic on publication dynamics and non-COVID-19 research production. BMC Med Res Methodol. 2021;21(1):255. doi: 10.1186/s12874-021-01404-9.
17. WHO Director-General's opening remarks at the media briefing on COVID-19 - 11 March 2020. Available from: https://www.who.int/news-room/speeches/item/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19---11-march-2020
18. Huang M-H. A Comparison of Three Major Academic Rankings for World Universities: From a Research Evaluation Perspective. J Libr Inform Stud. 2011;9(1):1-25.
19. Russian Government. On the allocation in 2014 of interbudgetary transfers for the financing of education in Crimea. Document No. 13228. [cited 2026 Apr 11]. Available from: http://government.ru/docs/13228/.
20. Hladchenko M. International collaboration of Ukrainian scholars: Effects of Russia's full-scale invasion of Ukraine. arXiv e-prints. 2025;arXiv:2505.20944. doi: 10.48550/arXiv.2505.20944.

Retrospective Study
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Indications and Complications of Total Thyroidectomy in the Management of Thyroid Diseases: A Five-Year Retrospective Study in a Greek Population

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Abstract

Objective. This retrospective study aimed to record, analyze, and evaluate data concerning patients who underwent total thyroidectomy, focusing on the main indications that led to surgical therapy, as well as postoperative complications and oncological outcomes. **Materials and Methods.** A retrospective study was conducted on 312 patients who underwent total thyroidectomy or lobectomy at the First Department of Surgery of Sismanogleio General Hospital in Athens between 2019 and 2024. Demographic data, indications, intraoperative parameters, complications, and histological findings were recorded. Statistical analyses were performed using SPSS v29.0. **Results.** Among the 312 patients, 222 (71.2%) were female, and 90 (28.8%) were male, with a mean age of 54.3±13.3 years. Total thyroidectomy was performed in 96.5% of the cases. The main indications were nodular hyperplasia (31.4%), autoimmune thyroiditis (18.9%), multinodular goiter (12.8%), and papillary carcinoma (17.3%). The overall complication rate was 31.7%, with transient postoperative hypocalcemia being the most common complication (25.3%). Permanent hypocalcemia occurred in 1.9% of patients, hemorrhage in 3.5%, and recurrent laryngeal nerve palsy in 1.3% of patients. Papillary carcinoma was the most frequent malignancy, followed by follicular, medullary, and anaplastic carcinomas. Approximately 20% of patients were diagnosed with incidental carcinoma—mostly papillary microcarcinoma—emphasizing the importance of total thyroidectomy, even for benign thyroid diseases. **Conclusions.** Total thyroidectomy is a safe and effective therapeutic option with low rates of permanent complications. The systematic identification and preservation of the parathyroid glands, intraoperative nerve monitoring, and meticulous hemostasis are key factors for minimizing inadvertent complications and optimizing outcomes.

Key Words: Total Thyroidectomy ▪ Hypocalcemia ▪ Complications ▪ Incidental Carcinoma ▪ Thyroid Surgery.

Introduction

Thyroidectomy is one of the most frequently performed endocrine surgical procedures worldwide. When performed in specialized centers, it is considered a safe and standardized operation; however, postoperative complications—particularly hypocalcemia and recurrent laryngeal nerve (RLN) injury—remain clinically significant.

Although numerous international studies have evaluated the indications and complication rates of total thyroidectomy, contemporary region-specific data reflecting current surgical practice patterns remain limited, particularly in Southeast Europe. Differences in epidemiology, healthcare structure, and institutional protocols may influence operative strategy and reported outcomes. In Mediterranean populations, thyroid nodular disease is highly prevalent, with multinodular goiter affecting up to 20–30% of adults in iodine-sufficient regions. In Greece, the widespread use of high-resolution ultrasonography and fine-needle aspiration has also contributed to the increasing detection of papillary thyroid microcarcinoma and incidental thyroid malignancies (1, 2). These epidemiological characteristics may influence

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both surgical indications and final histopathological outcomes. Furthermore, the optimal extent of thyroid surgery and predictors of postoperative hypocalcemia remain areas of ongoing clinical interest. Hypocalcemia is the most common complication following total thyroidectomy and may result from temporary or permanent impairment of parathyroid gland function. Several studies have demonstrated that the extent of surgical resection, inadvertent parathyroid injury, and patient-related factors may contribute to postoperative calcium disturbances (3, 4).

The surgical management of the thyroid gland has progressed remarkably over the past century. Advances in surgical techniques, anesthesia, and perioperative care have significantly improved the safety of thyroidectomy over the past century, transforming it into a standardized and safe surgical procedure (5, 6). Surgeons must have a thorough understanding of the indications, surgical techniques, and potential complications to ensure the best possible patient outcomes. Thyroidectomy (total or subtotal) remains an important therapeutic option for various benign and malignant thyroid disorders (7). The indication for surgical intervention is based on clinical, imaging, and cytological factors, as well as the patient's symptoms. The main indications for thyroidectomy include large or substernal goiters causing compressive symptoms (dysphagia, hoarseness, or dyspnea), toxic multinodular goiter, hyperthyroidism, and Hashimoto's thyroiditis with a painful or suspicious nodule. Furthermore, the absolute indications for surgery include thyroid nodules with cytological findings suspicious for malignancy (Bethesda categories V–VI) and confirmed thyroid cancer (papillary, follicular, medullary, or anaplastic carcinoma) (8).

The present five-year retrospective study aims to evaluate the indications for thyroidectomy, postoperative complications, independent predictors of hypocalcemia, and histopathological findings in a contemporary Greek tertiary cohort, thereby contributing updated regional data to the existing literature.

Materials and Methods

The study included 312 patients who underwent thyroidectomy or lobectomy at the First Department of Surgery, Sismanogleio General Hospital of Athens, Greece, between 2019 and 2024. All patients were evaluated for demographic data, type of surgery, preoperative assessment, intraoperative techniques, and postoperative complications. Data were collected using a standardized data extraction form that was uniformly applied to all medical records. The variables were retrieved from electronic and written charts, operative reports, anesthesia records, and histopathology reports. The data were collected as part of routine diagnostic evaluations and therapeutic interventions, and their analysis was conducted anonymously using coded identifiers, with full respect for patients' personal information, particularly the patient identification number assigned upon admission to the clinic.

The inclusion criteria were as follows: adult patients (18 years) who underwent total thyroidectomy, completion thyroidectomy, or lobectomy between 2019 and 2024, and patients with complete perioperative and histopathological data. The exclusion criteria included simultaneous thyroidectomy and parathyroidectomy for concurrent pathology of the parathyroid glands, abnormal preoperative calcium levels, the presence of parathyroid disorders affecting calcium homeostasis, and thyroidectomy performed as part of other oncological procedures, such as laryngectomy, in collaboration with the hospital's Otorhinolaryngology Department. Pediatric patients and those with incomplete medical records were also excluded.

The primary outcome of this study was the incidence of postoperative complications, with particular emphasis on postoperative hypocalcemia (both transient and permanent). Secondary outcomes included the identification of risk factors for hypocalcemia, postoperative hemorrhage, recurrent laryngeal nerve injury, histopathological findings, and rates of incidental thyroid carcinoma. Transient hypocalcemia was defined as serum calcium <8.6 mg/dL or the need for calcium/

vitamin D supplementation resolving within 12 months postoperatively. Permanent hypocalcemia was defined as persistence beyond 12 months.

Postoperative hypocalcemia monitoring included serial serum calcium measurements and routine immediate postoperative parathyroid hormone (PTH) assessment in all patients. PTH levels were measured within the first hours after surgery. A value below 15 pg/mL, corresponding to the lower laboratory reference limit, was considered indicative of increased risk for postoperative hypocalcemia and was used to guide the calcium and vitamin D supplementation strategy. Recurrent laryngeal nerve (RLN) palsy was classified as transient if vocal cord mobility recovered within 6 months and permanent if dysfunction persisted beyond that period. Histopathological diagnoses were classified according to World Health Organization (WHO) criteria. Malignancy was confirmed histopathologically in all cases based on the final surgical specimen. TNM staging was determined according to the AJCC 8th edition criteria and was based exclusively on postoperative pathological findings rather than on preoperative clinical assessment.

This study was conducted in accordance with the principles of the Declaration of Helsinki and in compliance with national and institutional data protection regulations. The study design was retrospective and based exclusively on anonymized data obtained from medical records collected during routine clinical care. No identifiable patient information was recorded, and no intervention or deviation from standard clinical practice was performed for the purposes of this study.

Ethics Statement

According to institutional policy, retrospective observational studies using anonymized data do not require formal approval by an institutional ethics committee, nor is written informed consent required. Therefore, ethical approval and individual informed consent were waived.

Statistical Analyses

All data were handled confidentially, and patient privacy was strictly protected throughout the study. Continuous variables were expressed as mean ± standard deviation, and categorical variables as number (percentage). Normality of continuous variables was assessed using the Shapiro–Wilk test. Normally distributed continuous variables were compared using the independent samples t-test, while non-normally distributed variables were analyzed using the Mann–Whitney U test, as appropriate. Univariate analysis was performed using the chi-square or Fisher's exact test for categorical variables. Variables with a P-value <0.10 in the univariate analysis were entered into the multivariate logistic regression model, in order to avoid excluding potentially relevant predictors, in accordance with established methodological recommendations (9, 10). Results were reported as odds ratios (OR) with 95% confidence intervals (CI). Statistical significance was set at P<0.05. Analysis was performed using SPSS v29.0.

Results

The baseline demographic and clinical characteristics are presented in Table 1.

Table 1. Baseline Demographic and Clinical Characteristics of the Study Population (N=312)

Characteristic	Value
Age (years), mean ± SD (range)*	54.3±13.3 (21–84)
Sex†	
Female	222 (71.2)
Male	90 (28.8)
Type of surgery†	
Total thyroidectomy	302 (96.5)
Lobectomy	8 (2.5)
Completion thyroidectomy‡	2 (0.6)
Hospital stay (days), mean±SD (range)*	3.11±0.42 (2–7)
Central lymph node dissection†	55 (17.6)
Lateral lymph node dissection†	5 (1.6)
Accidental parathyroid excision†	100 (32.0)
Parathyroid autotransplantation†	5 (1.6)

*Continuous variables are presented as mean±standard deviation (SD). †Categorical variables are presented as numbers (percentages). ‡Due to >5% residual thyroid parenchyma, as confirmed by postoperative ultrasound.

The study population was predominantly female, and total thyroidectomy represented the main surgical approach. The overall hospital stay was short, reflecting standardized perioperative management. Two patients underwent completion thyroidectomy within two months due to >5% residual thyroid parenchyma, as confirmed by postoperative ultrasound. Both cases involved papillary carcinoma, and radioiodine therapy was subsequently administered. Reoperation was performed in one case due to positive margins in the initial specimen. The overall hospital stay was short, reflecting standardized perioperative management.

This duration reflects the institutional admission protocol, whereby patients were admitted one day prior to surgery for preoperative evaluation, underwent thyroidectomy on the second day, and were discharged on the first postoperative day in uncomplicated cases (11, 12).

The most frequent indications for surgery are presented in Table 2. Benign thyroid diseases, particularly nodular hyperplasia and autoimmune thyroiditis, were the most common indications, while papillary carcinoma was the leading malignant cause. Histopathological examination revealed accidental parathyroid excision in 32% of cases, of which 76% were intrathyroidal. The mean number of identified and preserved parathyroid glands was 2.65±1.05, and autotransplantation of inadvertently removed parathyroid tissue was performed in five patients (1.6%).

Table 2. Indications for Total Thyroidectomy (N=312)

Pathology*	N (%)
Nodular hyperplasia	98 (31.4)
Autoimmune thyroiditis	59 (18.9)
Papillary carcinoma	54 (17.3)
Multinodular goiter	40 (12.8)
Substernal (retrosternal) goiter	30 (9.6)
Toxic multinodular goiter	15 (4.8)
Graves' disease	11 (3.6)
Follicular neoplasm / carcinoma	3 (1.0)
De Quervain's thyroiditis	2 (0.6)

*Histopathological diagnoses were classified according to WHO criteria.

Postoperative complications occurred in 31.7% of patients and are summarized in Table 3. The majority of patients had an uneventful postoperative course.

Table 3. Postoperative Complications

Complication	Number (%)
Transient hypocalcemia*	79 (25.3)
Permanent hypocalcemia†	6 (1.9)
Hemorrhage	11 (3.5)
Wound infection	4 (1.3)
Recurrent laryngeal nerve palsy‡	4 (1.3)
Permanent recurrent laryngeal nerve palsy§	1 (0.3)
30-day mortality	0 (0)

*Transient hypocalcemia was defined as serum calcium <8.6 mg/dL or need for calcium/vitamin D supplementation resolving within 12 months postoperatively; †Permanent hypocalcemia was defined as persistence beyond 12 months; ‡Recurrent laryngeal nerve (RLN) palsy was classified as§ transient if vocal cord mobility recovered within 6 months and permanent if dysfunction persisted beyond that period.

Transient hypocalcemia was the most frequent complication, whereas permanent complications occurred at low rates. Of the 11 patients who experienced postoperative bleeding, six were treated conservatively with observation, transfusion, and intravenous tranexamic acid, while five required reoperation, and only one patient had an identifiable bleeding vessel. Early postoperative PTH measurements were performed in all patients and contributed to risk stratification and postoperative supplementation decisions. During follow-up, 85.3% of patients received prophylactic oral calcium and alfacalcidol supplementation for one month postoperatively until endocrine evaluation. Two patients (0.6%) required readmission due to symptomatic hypocalcemia after discharge. Both cases were managed conservatively with IV calcium supplementation, and no long-term sequelae were observed.

Histopathological findings and TNM staging are shown in Table 4. Papillary thyroid carcinoma was the most common malignancy, and most tumors were diagnosed at an early stage, whereas advanced stages were rare.

Table 4. Histopathological Findings and TNM Staging

Histological Diagnosis	Number (%)
Papillary carcinoma	140 (45)
Follicular carcinoma	6 (1.9)
Medullary carcinoma*	4 (1.3)
Hürthle cell carcinoma*	2 (0.6)
MALT lymphoma*	2 (0.6)
Anaplastic carcinoma*	1 (0.3)
Benign lesions	157 (50.3)
TNM Staging†	
pT1a	85 (27)
pT1aN1	2 (0.6)
pT1b	22 (7)
pT1N1	4 (1.2)
pT2	25 (8)
pT2N1	7 (2.2)
pT3	4 (1.2)
pT3N1	5 (1.5)
pT4 (pT4aN1)	1 (0.3)

*Rare malignancies (medullary, anaplastic carcinoma, and MALT lymphoma) are reported descriptively due to small case numbers; †TNM staging was performed according to the AJCC 8th edition.

Multivariate analysis was performed to identify predictors of postoperative hypocalcemia, and the results are presented in Table 5. It identified total thyroidectomy and inadvertent parathyroid excision as independent predictors of postoperative hypocalcemia.

Table 5. Multivariate Logistic Regression Analysis for Predictors of Postoperative Hypocalcemia

Variable*	Odds Ratio (OR)	95% Confidence Interval‡	P-value†
Total thyroidectomy (vs less extensive surgery)	1.92	1.01-3.65	0.050
Inadvertent parathyroid excision	3.84	1.78-8.29	0.001
Female sex	1.21	0.52-2.81	0.680
Age (>55 years)	1.08	0.59-1.96	0.810
Malignant pathology	1.34	0.71-2.54	0.360

*Multivariate analysis was performed using logistic regression; †Variables with P<0.10 in univariate analysis were entered into the model; ‡Results are presented as odds ratios (OR) with 95% confidence intervals (CI); †Statistical significance was set at P<0.05.

Univariate analysis demonstrated a significant association between nodular hyperplasia and postoperative hemorrhage, whereas no other variables showed statistical significance. The results of this analysis are presented in Table 6.

Table 6. Univariate Analysis of Factors Associated With Postoperative Hemorrhage

Variable*	P-value
Nodular hyperplasia	0.033
Sex	0.74
Age (>55 years)	0.68
Extent of surgery	0.59
Malignant pathology	0.81

*Univariate analysis was performed using the chi-square or Fisher's exact test, as appropriate. Multivariate analysis was not conducted due to the small number of hemorrhagic events.

Discussion

This five-year retrospective study describes the outcomes and complications of thyroid surgery at a single center in Greece, focusing on the indications, complications, and histopathological outcomes of total thyroidectomy. The most notable finding of this study is the relatively high rate of incidental thyroid carcinoma (approximately 20%) identified in patients undergoing surgery for presumed benign thyroid disease. This observation is consistent with contemporary literature and reflects the increasing detection of papillary microcarcinoma in the era of widespread ultrasonography and fine-needle aspiration (13,14). From a clinical perspective, this finding supports the role of total thyroidectomy in selected patients, particularly in populations with a high prevalence of multinodular thyroid disease, as it allows complete histopathological evaluation and may reduce the need for reoperation in cases of occult malignancy (15, 16).

The majority of patients were female, consistent with international data, as thyroid diseases have a well-documented female predominance (10). The mean hospital stay was approximately 3 days; however, the overwhelming majority of patients were

discharged on the first postoperative day. Although early 24-hour discharge protocols have been widely adopted in high-volume endocrine centers, the observed three-day hospitalization primarily reflects institutional admission logistics rather than prolonged postoperative recovery. Effective postoperative hospitalization was approximately 24 hours in uncomplicated cases. The mean age corresponds to the typical demographic affected by both benign and malignant thyroid disorders. Total thyroidectomy was the treatment of choice, highlighting the increasing predominance of complete gland removal over lobectomy, in accordance with contemporary guidelines (17, 18). The rate of central lymph node dissection aligns with the current, more selective approach: prophylactic central dissection is not universally recommended for all cN0 patients but should be individualized according to the residual disease risk, histological subtype, and surgical expertise (19).

The overall postoperative complication rate was consistent with global series (20-22), with the vast majority being transient and mild. Transient hypocalcemia was the most frequent complication, mainly resulting from temporary ischemia or minor trauma to the parathyroid glands. Permanent hypocalcemia was rare and within internationally acceptable limits. Postoperative hemorrhage and recurrent laryngeal nerve (RLN) injury were also very rare. The rates of permanent hypocalcemia and permanent RLN palsy fall within internationally reported ranges of 1–3% and 0.5-2%, respectively, in high-volume endocrine centers (22). Intraoperative nerve monitoring, visual identification of the RLN, and meticulous hemostasis are now considered standard practices for minimizing these risks (23). The proportion of malignant thyroid disease in this retrospective series is consistent with international surgical reports, in which differentiated thyroid cancer (DTC) accounts for 10–25% of operated cases (13). Papillary thyroid carcinoma (PTC) was the most common histological type, while follicular and micropapillary variants were less frequent, confirming the well-known global predominance of PTC (15, 24). TNM staging revealed a predominance of early-stage tumors, in accordance with the modern trend toward early diagnosis, mainly due to the extensive use of ultrasonography and fine-needle aspiration (FNA). The low rate of advanced stages reflects both the favorable biological behavior of DTC and the benefits of early surgical intervention (25, 26). The strong correlation between cytological findings suspicious for malignancy (Bethesda category V) and malignant cytology (Bethesda category VI) and final histology underscores the diagnostic reliability of preoperative FNA (27).

Among the 61 patients with preoperatively confirmed malignancy, total thyroidectomy was performed in 98.3% of the cases. Although oncological outcomes were favorable and the majority of malignant cases were diagnosed at an early stage, formal survival analyses were not performed, as long-term follow-up was not a predefined objective of this retrospective study. Consequently, conclusions regarding overall or disease-free survival cannot be drawn from the present data.

Multivariate logistic regression analysis was performed to identify independent predictors of postoperative hypocalcemia. Total thyroidectomy, as the extent of surgery, was independently associated with an increased risk of postoperative hypocalcemia. Inadvertent parathyroid excision of at least one gland was identified as the strongest independent predictor. Although advanced intraoperative fluorescence-guided techniques, such as indocyanine green angiography, may enhance parathyroid identification, they were not routinely available at our center during the study period. Female sex showed a higher incidence of hypocalcemia (28.8% vs. 16.9%) but did not reach statistical significance. According to the literature (27, 28), the higher frequency of hypocalcemia in women may be related to hormonal influences on parathyroid hormone secretion, anatomical differences, and genetic variations in calcium signaling pathways (29). In addition, women have higher rates of vitamin D and calcium deficiency, especially postmenopause, which may increase their vulnerability to postoperative hypocalcemia (30, 31). Given the complex anatomy of the region, the systematic identification of all parathyroid glands can be

technically challenging and may increase the risk of devascularization or trauma (32). The optimal technique involves careful subcapsular dissection (33) with ligation of the terminal branches of the thyroid arteries close to the true thyroid capsule, thereby preserving parathyroid vascularization without requiring exhaustive identification of all four glands (33, 34). Extensive exploration aimed at identifying all glands may paradoxically increase the risk of injury and is best avoided when recognition is difficult or time-consuming (34-36). At our institution, immediate postoperative PTH measurement was routinely performed in all patients and used in conjunction with serial calcium monitoring to guide supplementation and discharge planning. This approach aligns with contemporary evidence supporting early PTH-guided management to identify patients at risk for hypocalcemia and facilitate safe postoperative care (22, 35). Early PTH and calcium monitoring, meticulous dissection respecting parathyroid vascularity, autotransplantation of accidentally removed glands, and prophylactic calcium/vitamin D supplementation in high-risk patients can reduce symptoms, hospital stays, and readmission rates (18). Although the present study was not primarily designed to stratify complications according to surgical indication, nodular hyperplasia was significantly associated with postoperative hemorrhage in the univariate analysis. Increased vascularity and tissue friability in hyperplastic glands may render hemostasis more technically demanding.

Postoperative hemorrhage occurred in 3.5% of patients, consistent with international data (37). To explore the factors associated with postoperative bleeding, a univariate analysis was performed to examine patient- and pathology-related variables. A statistically significant association was identified between histopathological diagnosis and postoperative hemorrhage. Specifically, patients operated for nodular hyperplasia demonstrated a higher incidence of postoperative bleeding compared with other indications. This finding may be attributed to increased gland vascularity and tissue friability, commonly observed in nodular hyperplasia, which can render meticulous hemostasis more technically demanding. Due to the relatively small number of hemorrhagic events, multivariate analysis was not performed for this outcome, and results should be interpreted with caution.

Interestingly, approximately 20% of patients who underwent surgery for benign indications were found to have incidental malignancy—mostly papillary microcarcinoma (microPTC, pT1a)—upon histopathological examination. This finding aligns with international data on incidental carcinomas, typically reported in 10–20% of thyroidectomy specimens for benign disease (22). Non-incidental papillary microcarcinomas were included within the group of preoperatively diagnosed papillary carcinoma cases, whereas incidental microcarcinomas were identified exclusively in patients operated for benign indications. Our observed rate is at the higher end of the spectrum. It reflects several factors: (1) extensive tissue sampling and detailed histopathological analysis (38), (2) the high prevalence of multinodular goiter and Hashimoto's thyroiditis in the Greek population (both associated with incidental PTC), (3) the near-universal use of total thyroidectomy, allowing complete histological examination (39), and (4) the recent era characterized by increased diagnostic sensitivity and widespread use of ultrasound and FNA (14), contributing to the recognized phenomenon of "overdiagnosis." The high rate of incidental PTC underlines the need to fully inform patients preoperatively, as there is a substantial likelihood of post-surgical cancer diagnosis (40). This consideration affects the extent of the initial surgery, lymph node assessment, and postoperative management (Tg/TSH monitoring and possible radioiodine therapy). Therefore, total thyroidectomy remains the most appropriate endocrine and oncologic surgical approach, even for benign thyroid diseases, ensuring both complete cure and oncologic safety.

In recent years, minimally invasive techniques such as radiofrequency ablation (RFA), microwave ablation (MWA), and nano-pulse ablation have emerged as alternatives for selected benign nodules and low-risk microcarcinomas. These approaches may offer reduced procedural morbidity

and shorter recovery times (41, 42). However, they do not allow for complete histopathological evaluation and may not address multifocal or occult malignancy. In such epidemiological settings, total thyroidectomy continues to provide definitive oncologic management.

Comparative studies suggest that initial total thyroidectomy may reduce the need for reoperation compared to the staged approach (lobectomy followed by completion thyroidectomy), thereby minimizing the cumulative surgical risk (15, 16). Contemporary ATA guidelines emphasize individualized decision-making regarding the extent of surgery in low-risk differentiated thyroid carcinoma, balancing oncologic adequacy and complication risk (15).

The present study reflects contemporary thyroid surgical practice in Greece, where multinodular goiter and autoimmune thyroiditis remain highly prevalent. These epidemiological characteristics may partly explain the high rate of incidental papillary microcarcinoma observed in our cohort. Furthermore, the near-universal adoption of total thyroidectomy at our center provides insight into institutional surgical strategy in a Mediterranean population, where bilateral nodular disease is common. The rate of incidental carcinoma in our cohort lies at the upper range of internationally reported series and highlights the potential oncologic relevance of total thyroidectomy in patients initially operated for benign disease. The study period reflects the modern diagnostic era, characterized by the widespread use of high-resolution ultrasonography and fine-needle aspiration, potentially influencing surgical indications and incidental detection rates. The findings of this study have important clinical implications. First, the high rate of incidental carcinoma underscores the need for thorough preoperative patient counseling, as a significant proportion of patients operated on for benign disease may ultimately be diagnosed with malignancy. Second, the identification of modifiable surgical risk factors highlights the importance of surgical expertise and standardized techniques in reducing complication rates. Finally, these results support the use of total thyroidectomy as a definitive surgical approach in appropriately selected patients, balancing oncological adequacy with acceptable morbidity.

Limitations of the Study

This study has several limitations. First, it was conducted at a single tertiary care center, which may limit the external validity and generalizability of the findings to other clinical settings or populations. This study is limited by its retrospective design and relatively short follow-up period, which does not allow for the assessment of long-term oncological outcomes or quality-of-life measures. Healthcare utilization parameters, such as readmission rates, outpatient visits, and cost analysis, were not systematically assessed, limiting the evaluation of the economic impact of thyroidectomy in our setting (11, 23). Furthermore, neither institutional review board (IRB) approval nor written informed consent was obtained. In addition, conclusions regarding rare malignancies, such as medullary or anaplastic carcinoma, should be interpreted with caution due to the small number of cases.

Conclusions

Total thyroidectomy remains a safe and effective surgical procedure when performed in specialized centers. The main indications remain either benign thyroid disorders, such as nodular hyperplasia and autoimmune thyroiditis, being the most common, or papillary carcinoma, which is the predominant malignant indication. Postoperative hypocalcemia was identified as the most frequent complication, and it was primarily transient and self-limiting. Severe complications were extremely rare. Inadvertent parathyroid excision and the extent of surgery were identified as the strongest independent predictors of postoperative hypocalcemia, highlighting the importance of meticulous surgical technique and preservation of parathyroid gland vascularization. The relatively high rate of incidental thyroid carcinoma observed in this study underscores the importance of performing total thyroidectomy even in benign thyroid conditions,

as it ensures complete removal of potential occult malignancies, provides a definitive cure for thyroid disease, and maintains a low rate of postoperative complications or recurrence. Overall, these findings support the role of total thyroidectomy as a reliable and effective surgical approach for the management of thyroid diseases when appropriately indicated.

What Is Already Known on This Topic:

Total thyroidectomy is one of the most well-established procedures in general and endocrine surgery. The indication for surgical intervention is based on clinical, imaging, and cytological factors, as well as the patient's symptoms. The main indications for thyroidectomy include large or substernal goiters causing compressive symptoms, toxic multinodular goiter, hyperthyroidism, and Hashimoto's thyroiditis with a painful or suspicious nodule. Furthermore, the absolute indications for surgery include thyroid nodules with cytological findings suspicious for malignancy (Bethesda categories V–VI) and confirmed thyroid cancer.

What This Study Adds:

Approximately 20% of patients were diagnosed with incidental carcinoma—mostly papillary microcarcinoma—emphasizing the importance of total thyroidectomy, even for benign thyroid diseases. In our retrospective study, postoperative hypocalcemia was identified as the most frequent complication, and it was primarily transient and self-limiting. Severe complications, such as permanent hypoparathyroidism or recurrent laryngeal nerve injury, were extremely rare. Multivariate analysis identified the extent of surgery (total thyroidectomy) and the inadvertent removal of at least one parathyroid gland as the strongest independent predictors of hypocalcemia. Additionally, patients with nodular hyperplasia had higher bleeding rates, possibly due to increased vascularity and tissue friability, making hemostasis more challenging.

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References

1. Pazaitou-Panayiotou K, Iliadou PK, Chrisoulidou A, Mitsakis P, Doumala E, Fotareli A, et al. The increase in thyroid cancer incidence is not only due to papillary microcarcinomas: a 40-year study in 1 778 patients. Exp Clin Endocrinol Diabetes. 2013;121(7):397-401. doi: 10.1055/s-0033-1345125.
2. Voulgari PV, Venetsanopoulou AI, Kalpourtzi N, Gavana M, Vantarakis A, Hadjichristodoulou C, et al. Thyroid dysfunction in Greece: Results from the national health examination survey EMENO. PLoS One. 2022;17(3):e0264388. doi: 10.1371/journal.pone.0264388.
3. Christou N, Mathonnet M. Complications after total thyroidectomy. J Visc Surg. 2013;150(4):249-56. doi: 10.1016/j.jviscsurg.2013.04.003.
4. Sosa JA, Bowman HM, Tielsch JM, Powe NR, Gordon TA, Udelsman R. The importance of surgeon experience for clinical and economic outcomes from thyroidectomy. Otolaryngol Head Neck Surg. 1998;228(3):320-30. doi: 10.1097/00000658-199809000-00005.
5. Alvarado R, McMullen T, Sidhu SB, Delbridge LW, Sywak MS. Minimally invasive thyroid surgery for single nodules: an evidence-based review of the lateral mini-incision technique. World J Surg. 2008;32(7):1341-8. doi: 10.1007/s00268-008-9554-4.
6. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2016;26(1):1-133. doi: 10.1089/thy.2015.0020.
7. Lorente-Poch L, Sancho J, Muñoz-Nova J, Sánchez-Velázquez P, Sitges-Serra A. Defining the syndromes of parathyroid failure after total thyroidectomy. Gland Surg. 2015;4(1):82-90. doi: 10.3978/j.issn.2227-684X.2014.12.04.
8. Policeni BA, Smoker WRK, Reede DL. Anatomy and embryology of the thyroid and parathyroid glands. Semin Ultrasound CT MRI. 2012;33(2):104-14. doi: 10.1053/j.sult.2011.12.005.
9. Bursac Z, Gauss CH, Williams DK, Hosmer DW. Purposeful selection of variables in logistic regression. Source Code Biol Med. 2008;3:17. doi: 10.1186/1751-0473-3-17.
10. Hosmer DW, Lemeshow S, Sturdivant RX. Applied logistic regression. 3rd ed. Wiley; 2013. doi: 10.1002/9781118548387.
11. Perera AH, Patel SD, Law Nk, Thyroid surgery as a 23-hour stay procedure. Ann R Coll Surg Engl. 2014;96(4):284-8. doi: 10.1308/003588414X13814021679997.
12. Chen Z, Zhao Q, Du J, Wang Y, Han R, Xu C, et al. Risk factors for postoperative hypocalcaemia after thyroidectomy: A systematic review and meta-analysis. J Int Med Res. 2021;49(3):0300060521996911. doi: 10.1177/0300060521996911.
13. de Carlos J, Ernaga A, Irigaray A, Pineda JJ, Echegoyen A, Salvador P, et al. Incidentally discovered papillary thyroid microcarcinoma in patients undergoing thyroid surgery for benign disease. Endocrine. 2022;77(2):325-32. doi: 10.1007/s12020-022-03089-6. Epub 2022 May 31.
14. Alevizaki M, Papageorgiou G, Rentziou G, Saltiki K, Marafelia P, Loukari E, et al. Increasing prevalence of papillary thyroid carcinoma in recent years in Greece: the majority are incidental. Thyroid. 2009;19(7):749-54. doi: 10.1089/thy.2008.0421.

15. Bartsch DK, Dotzenrath C, Vorländer C, Zielke A, Weber T, Buhr HJ, et al. Current Practice of Surgery for Benign Goitre-An Analysis of the Prospective DGAV StuDoQ|Thyroid Registry. J Clin Med. 2019 Apr 8;8(4):477. doi: 10.3390/jcm8040477.
16. Kazaure HS, Sosa JA. Surgical Hypoparathyroidism. Endocrinol Metab Clin North Am. 2018;47(4):783-96. doi: 10.1016/j.ecl.2018.07.005. Epub 2018 Oct 12.
17. Fagin JA, Wells SA Jr. Biologic and clinical perspectives on thyroid cancer. N Engl J Med. 2016;375(11):1054-67. doi:10.1056/NEJMra1501993.
18. Tzortzis AS, Antonopoulos I, Pechlivanidou E, Chrysikos D, Pappas N, Troupis T. Anatomical variations of the superior thyroid artery: a systematic review. Morphologie. 2023;107(358):100597. doi: 10.1016/j.morpho.2023.03.002.
19. Lang BH, Chan DT, Chow FC. Visualizing fewer parathyroid glands may be associated with lower hypoparathyroidism following total thyroidectomy. Langenbecks Arch Surg. 2016;401(2):231-8. doi: 10.1007/s00423-016-1386-3.
20. Docimo G, Ruggiero R, Casalino G, Del Genio G, Docimo L, Tolone S. Risk factors for postoperative hypocalcemia. Updat Surg. 2017;69(2):255-60. doi: 10.1007/s13304-017-0452-x. Epub 2017 Apr 25.
21. Spencer MT, Kennedy EP, Ramirez M, Campbell DA, Chen DW, Sim MW, et al. Impact of Preoperative Education on Clinical Outcomes Following Major Head & Neck Surgery: A Systematic Review. OTO Open. 2025;9(4):e70177. doi: 10.1002/oto2.70177.
22. Edafe O, Antakia R, Laskar N, Uttley L, Balasubramanian SP. Systematic review and meta-analysis of predictors of post-thyroidectomy hypocalcaemia. Br J Surg. 2014;101(4):307-20. doi: 10.1002/bjs.9384.
23. Bergenfelz A, Jansson S, Kristoffersson A, Mårtensson H, Reihnér E, Wallin G et al. Complications to thyroid surgery: results as reported in a database from a multicenter audit comprising 3,660 patients. Langenbecks Arch Surg. 2008;393(5):667-73. doi: 10.1007/s00423-008-0366-7.
24. Tuttle RM, Ahuja S, Avram AM, Bernet VJ, Bourguet P, Daniels GH, et al. Controversies, Consensus, and Collaboration in the Use of 131I Therapy in Differentiated Thyroid Cancer: A Joint Statement from the American Thyroid Association, the European Association of Nuclear Medicine, the Society of Nuclear Medicine and Molecular Imaging, and the European Thyroid Association. Thyroid. 2019;29(4):461-70. doi: 10.1089/thy.2018.0597.
25. Pacini F, Fuhrer D, Elisei R, Handkiewicz-Junak D, Leboulleux S, Luster M, et al. 2022 ETA Consensus Statement: What are the indications for post-surgical radioiodine therapy in differentiated thyroid cancer? Eur Thyroid J. 2022;11(1):e210046. doi: 10.1530/ETJ-21-0046.
26. Lukinović J, Bilić M. Overview of Thyroid Surgery Complications. Acta Clin Croat. 2020;59(Suppl 1):81-6. doi: 10.20471/acc.2020.59.s1.10.
27. Chisholm EJ, Kulinskaya E, Tolley NS. Systematic review and meta-analysis of the adverse effects of thyroidectomy combined with central neck dissection as compared with thyroidectomy alone. Laryngoscope. 2009;119(6):1135-9. doi: 10.1002/lary.20236.
28. Chorath K, Luu N, Go BC, Moreira A, Rajasekaran K. ERAS Protocols for Thyroid and Parathyroid Surgery: A Systematic Review and Meta-analysis. Otolaryngol Head Neck Surg. 2022;166(3):425-33. doi: 10.1177/01945998211019671. Epub 2021 Jun 15.
29. Tan YH, Du GN, Xiao YG, Guo SQ, Wu T, Chen PZ, et al. The false thyroid capsule: new findings. J Laryngol Otol. 2013;127(9):897-901. doi: 10.1017/S0022215113001667. Epub 2013 Aug 8.
30. Baldassarre RL, Chang DC, Brumund KT, Bouvet M. Predictors of hypocalcemia after thyroidectomy: results from the nationwide inpatient sample. ISRN Surg. 2012;2012:838614. doi: 10.5402/2012/838614. Epub 2012 Jul 15.
31. Caulley L, Johnson-Obaseki S, Luo L, Javidnia H. Risk factors for postoperative complications in total thyroidectomy: A retrospective, risk-adjusted analysis from the National Surgical Quality Improvement Program. Medicine (Baltimore). 2017;96(5):e5752. doi: 10.1097/MD.0000000000005752.
32. Slijepcevic N, Zivaljevic V, Marinkovic J, Sipetic S, Diklic A, Paunovic I. Retrospective evaluation of the incidental finding of 403 papillary thyroid microcarcinomas in 2466 patients undergoing thyroid surgery for presumed benign thyroid disease. BMC Cancer. 2015;15:330. doi: 10.1186/s12885-015-1352-4.
33. Taterra D, Wong LM, Vikse J, Sanna B, Pękala P, Walocha J, et al. The prevalence and anatomy of parathyroid glands: a meta-analysis with implications for parathyroid surgery. Langenbecks Arch Surg. 2019;404(1):63-70. doi: 10.1007/s00423-019-01751-8. Epub 2019 Feb 14.
34. Avram AM, Zukotynski K, Nadel HR, Giovanella L. Management of Differentiated Thyroid Cancer: The Standard of Care. J Nucl Med. 2022;63(2):189-95. doi: 10.2967/jnumed.121.262402. Epub 2021 Aug 19.
35. Riordan F, Murphy MS, Feeley L, Sheahan P. Association between number of parathyroid glands identified during total thyroidectomy and functional parathyroid preservation. Langenbecks Arch Surg. 2022;407(1):297-303. doi: 10.1007/s00423-021-02287-6. Epub 2021 Aug 18.
36. Sitges-Serra A, Ruiz S, Girvent M, Manjón H, Dueñas JP, Sancho JJ. Outcome of protracted hypoparathyroidism after total thyroidectomy. Br J Surg. 2010;97(11):1687-95. doi: 10.1002/bjs.7219.
37. Qin Y, Sun W, Wang Z, Dong W, He L, Zhang T, et al. A Meta-Analysis of Risk Factors for Transient and Permanent Hypocalcemia After Total Thyroidectomy. Front Oncol. 2021;10:614089. doi: 10.3389/fonc.2020.614089.
38. Thomusch O, Machens A, Sekulla C, Ukkat J, Brauckhoff M, Dralle H. The impact of surgical technique on

postoperative hypoparathyroidism in bilateral thyroid surgery: a multivariate analysis of 5846 consecutive patients. Surgery. 2003;133(2):180-5. doi: 10.1067/msy.2003.61.

39. Rosato L, Avenia N, Bernante P, De Palma M, Gulino G, Nasi PG, et al. Complications of thyroid surgery: analysis of a multicentric study on 14,934 patients operated on in Italy over 5 years. World J Surg. 2004;28(3):271-6. doi: 10.1007/s00268-003-6903-1. Epub 2004 Feb 17.
40. Tongol MC, Mirasol R. Incidence and risk factors for post-thyroidectomy hypocalcemia. J ASEAN Fed Endocr Soc. 2016;31(1):30-6. doi: 10.15605/jafes.031.01.06.
41. Effraimidis G, Sazakli E, Karapanou O, Saltiki K, Michalaki M. Active surveillance for low-risk papillary thyroid microcarcinoma: a web-survey on clinician readiness for change. Eur Thyroid J. 2025;14(2):e250013. doi: 10.1530/ETJ-25-0013.
42. Cabanillas ME, McFadden DG, Durante C. Thyroid cancer. Lancet. 2016;388(10061):2783-95. doi: 10.1016/S0140-6736(16)30172-6. Epub 2016 May 27.

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Propranolol in the Treatment of Infantile Hemangiomas: A Single-Center Experience

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Abstract

Objective. Infantile hemangiomas (IH) are common benign vascular tumors of infancy that typically undergo gradual spontaneous regression over several years, although a minority require treatment. Propranolol, a non-selective β-adrenergic antagonist, is the first-line therapy for complicated or high-risk IH, demonstrating both efficacy and a favorable safety profile. **Materials and Methods.** We retrospectively analyzed 37 infants treated with oral propranolol at a single center. Patients were monitored in-hospital during treatment initiation and at regular, outpatient visits. The response was assessed using clinical measurements, ultrasonography, and standardized photographs. **Results.** Most patients had solitary lesions predominantly located in the head region. Complete regression occurred in 26 patients (70.3%) and partial regression in 11 (29.7%). Treatment duration ranged from 2 to 24 months (mean 10.1 months). Adverse events were rare and mild, including one case of hypoglycemia and one of transient somnolence. **Conclusion.** Oral propranolol is a safe and effective first-line therapy for IH, particularly when initiated early during the proliferative phase.

Key Words: Infantile Hemangioma ▪ Propranolol ▪ Pediatric ▪ Vascular Tumor.

Introduction

Infantile hemangiomas (IH) represent the most common benign tumors in infancy, with an estimated prevalence of 3–10% (1). They are rarely present at birth and typically become clinically apparent within the first weeks of life. The clinical course of IH generally follows a well-defined pattern, progressing through an initial growth phase (proliferative phase), followed by stabilization (plateau), and subsequent gradual spontaneous regression. While the majority of cases remain clinically benign, approximately 12% are associated with complications that require specialist evaluation and management (2).

Over the past decade, propranolol has emerged as the preferred therapeutic option for problematic IH, despite the relative scarcity of large randomized controlled studies (3). Although widely accepted as first-line therapy, reports describing institutional clinical experience remain valuable for documenting treatment outcomes and safety in clinical practice. Such data may contribute to optimizing treatment protocols and monitoring strategies in pediatric patients.

In the present study, we describe our institutional experience with oral propranolol therapy, and evaluate treatment response and safety in pediatric patients receiving an oral propranolol solution for up to 24 months.

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Materials and Methods

A retrospective study included 37 children (13 males and 24 females) with IH who were consecutively treated with systemic propranolol over a three-year period (January 1, 2015 to December 31, 2017) at the Department of Pediatrics, Clinical Hospital Centre Rijeka, Croatia. Data were collected from the integrated hospital information system (IBIS) and patient medical charts.

Baseline pretreatment evaluations included laboratory tests—complete blood count, blood glucose, liver and renal function, and thyroid function—as well as electrocardiography. Propranolol was administered as a hydrochloride oral suspension, always with a feed, starting at a daily dose of 1 mg/kg—divided into two doses—for the first week to assess patient tolerance. All infants were hospitalized for the first two days of initial treatment for close monitoring. Post-initiation bedside monitoring of blood pressure and heart rate was performed using a noninvasive monitor, and fingerstick blood glucose levels were measured at baseline (before the first dose) and 2 hours after the first three administrations. After the first week, the dose of propranolol was increased to 2 mg/kg/day for the second week, followed by a final maintenance dose of 3 mg/kg/day.

The patients underwent regular outpatient follow-up every two weeks and were weighed monthly to allow dose adjustment of propranolol as required. Treatment efficacy was assessed using standard measurements (for superficial lesions), measurements obtained by serial ultrasonography (for deep and combined lesions), and evaluation of digital photographs by two independent readers for all visible lesions.

Ethics Statement

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of the Faculty of Health Studies, University of Rijeka, Croatia (Class: 602-11/18-01; No.: 2133-61-04-18-01; approval date: March 26, 2018).

Statistical Analyses

Statistical analysis of quantitative data was conducted with descriptive statistics. Continuous variables are presented as mean ± standard deviation and range, and categorical variables as counts and percentages. Minimum and maximum values were reported where relevant. All statistical analyses were performed using Microsoft Excel 2016.

Results

Of the 37 patients, 31 (83.8%) presented with a solitary IH, while 6 (16.2%) had multiple lesions (five with two and one with three hemangiomas). Lesions were most located in the head region (21, 47.8%), followed by the trunk—back (8, 18.2%), chest wall and abdominal wall (3 each, 6.8%), the extremities—upper (6, 13.6%) and lower (1, 2.3%), and the gluteal region (2, 4.5%). Most IH had a widest diameter of 1–3 cm (27, 61.4%). Nine lesions (20.4%) measured >3–6 cm, four (9.1%) were ≤1 cm, and one (2.3%) was >6 cm; lesion size was not documented in three patients (6.8%). Regarding hemangioma type, 7 lesions were superficial (2 flat and 5 elevated), 9 were deep, and 28 were mixed.

The primary indication for propranolol therapy was rapid lesion growth in 33 patients (89.2%). Two patients (5.4%) had complicated (ulcerated) IH, and one patient each (2.7%) was treated due to the risk of functional impairment (periocular location) and for cosmetic reasons. Propranolol therapy was initiated before 3 months of age in 12 patients (32.4%), and between 3 and 6 months in 25 patients (67.6%). The mean age at treatment initiation was 3.7±2.1 months (range 35 days – 6 months).

Treatment duration was ≤6 months in seven patients (18.9%), >6–12 months in 24 patients (64.9%), >12–18 months in one patient (2.7%), and >18 months in five patients (13.5%). The mean duration of propranolol therapy was 10.1±5.9 months (range 2 – 24 months). Complete regression of IH was observed in 26 patients (70.3%), while partial regression occurred in 11 patients (29.7%). No patient demonstrated a lack of response to propranolol (Figure 1).

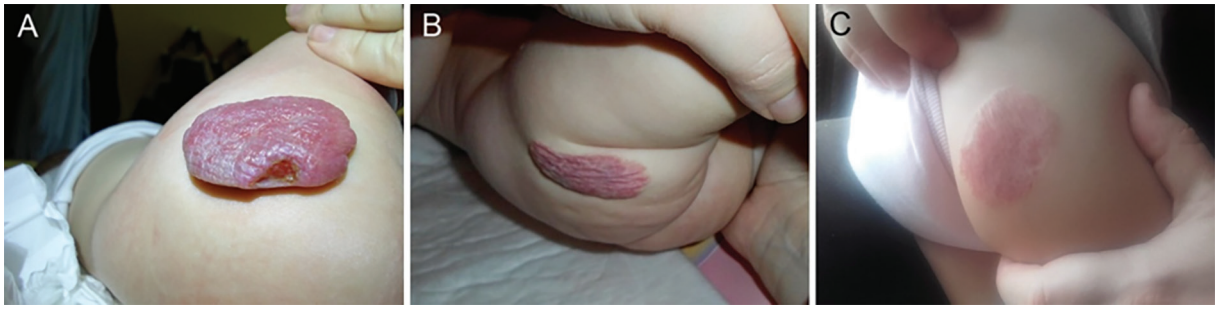

Figure 1. A. Infantile hemangioma in a 4-month-old female infant. B. After 4 months of propranolol therapy. C. After 8 months of propranolol therapy, showing almost complete regression. [Case reproduced from our patient; reproduced with permission from Reference 4.]

Table 1. Patient Baseline Characteristics, Hemangioma Features, and Propranolol Therapy

Patients (N=37; %)	
Gender (N; %)	
Male	13 (35.1)
Female	24 (64.9)
Age at treatment initiation, months (N; %)	
0 – 3	12 (32.4)
3 – 6	25 (67.6)
Number of lesions (N; %)	
Solitary	31 (83.8)
Multiple	6 (16.2)*
Hemangiomas (N=44;%)	
Location (N; %)	
Head	21 (47.8)
Trunk	14 (31.8)
Extremities	7 (15.9)
Gluteus	2 (4.5)
Size (widest diameter, cm) (N; %)	
≤1	4 (9.1)
1–3	27 (61.4)
>3–6	9 (20.4)
>6	1 (2.3)
Not documented	3 (6.8)
Type (N; %)	
Superficial	7 (15.9)
Deep	9 (20.5)
Combined	28 (63.6)
Treatment (N=37; %)	
Reason for initiation (N; %)	
Rapid growth	33 (89.2)
Complications (ulceration)	2 (5.4)
Location	1 (2.7)
Cosmetic reasons	1 (2.7)
Duration, months (N; %)	
≤6	7 (18.9)
>6–12	24 (64.9)
>12–18	1 (2.7)
>18	5 (13.5)
Response (N; %)	
Complete regression	26 (70.3)
Partial regression	11 (29.7)
Adverse effects (N; %)	
Yes	2 (5.4)
No	35 (94.6)

*Six patients had more than one hemangioma; total number of hemangiomas = 44.

Mild hypoglycemia was recorded in one patient (2.7%), and transient somnolence in another (2.7%); no additional adverse events were observed (Table 1). No differences were observed between male and female patients in lesion location, size, indication for propranolol therapy, age at treatment initiation, treatment duration, therapeutic response, or adverse events.

Discussion

In this retrospective study, oral propranolol demonstrated high effectiveness and an excellent safety profile in the treatment of IH. Complete regression was observed in 70.3% of patients, with the remaining 29.7% showing partial regression, and no patient failed to respond. These findings are consistent with previous studies reporting

propranolol as the first-line therapy for complicated or high-risk IH (3, 4). Propranolol, a non-selective β-adrenergic antagonist, exerts its effects in IH via multiple mechanisms. It induces vasoconstriction, resulting in early color and volume changes; inhibits angiogenic pathways, including downregulation of vascular endothelial growth factor (VEGF) and other pro-angiogenic factors; and promotes endothelial cell apoptosis and reduced proliferation, contributing to involution of vascular tissue (5).

In addition to oral propranolol, several other treatment options for IH have been evaluated. Topical β-blockers, such as timolol maleate 0.5% solution, have been increasingly used for superficial IHs, demonstrating comparable efficacy to oral propranolol in selected cases with a lower incidence of systemic side effects, and may be considered particularly for small, uncomplicated superficial lesions (6). Oral selective β-blocker atenolol has been compared with oral propranolol in several randomized trials and systematic reviews, showing similar efficacy in terms of complete remission, reduction in Hemangioma Activity Score (HAS), relapse rates, and overall adverse events, although the certainty of evidence is low (7). Other systemic agents, including corticosteroids, were historically used but are associated with a broader range of adverse effects and lower overall efficacy compared with propranolol (8). In addition, combination approaches such as β-blockers with laser therapy have been explored in network meta-analyses, with some evidence suggesting enhanced outcomes compared with monotherapy (9). In our cohort, oral propranolol was preferred as first-line systemic therapy due to its well-established effectiveness, extensive clinical experience, and favorable overall safety profile, consistent with current practice guidelines.

Early initiation of therapy contributed to favorable outcomes. One third of patients began treatment before 3 months of age, and the remainder between 3 and 6 months. This timing corresponds to the proliferative phase of IH, when intervention is most likely to achieve rapid stabilization and regression. Treatment duration in our study ranged from 2 to 24 months (mean 10.1 months), and prolonged administration was not associated with additional adverse effects, supporting individualized treatment duration based on clinical response.

Propranolol was well tolerated, with only mild hypoglycemia in one patient and transient somnolence in another. No serious adverse events occurred, corroborating the safety profile reported in the literature (3). However, close monitoring during treatment initiation remains essential to promptly detect and manage potential side effects, particularly in infants less than 2 months of age due to an increased risk of hypoglycemia.

Lesion characteristics in our cohort were similar to those reported in previous studies, with the majority being solitary and located in the head region (1, 2). Most lesions were small to medium in size (≤3 cm in 70.5%) and mixed-type hemangiomas predominated (63.6%). The primary indication for therapy was rapid lesion growth in 89.2% of patients, reflecting current clinical practice in selecting patients for systemic therapy.

Our study has several limitations. Its retrospective design and relatively small sample size may restrict the generalizability of the findings. Being a single-center study, patient selection could be influenced by referral patterns and institutional practices, introducing potential selection bias. Data collection relied on existing medical records, which may have led to incomplete or inconsistent documentation (information bias). The absence of a control group prevents direct comparison with alternative treatment strategies and limits the ability to draw causal inferences. Despite these limitations, our results contribute to the growing body of evidence supporting propranolol as a safe and effective therapy for IH, and provide practical guidelines on dosing schedules, monitoring strategies, and treatment duration in clinical practice.

Conclusion

Oral propranolol is highly effective for the treatment of IH, particularly when initiated early in the proliferative phase. The therapy is well tolerated, with minimal adverse effects, and can achieve

complete or substantial regression in the majority of patients. These findings support the continued use of propranolol in pediatric practice.

What Is Already Known on This Topic:

IH are common benign vascular tumors of infancy that usually regress spontaneously but may require treatment when complicated or high-risk. Oral propranolol is established as the first-line therapy for complicated IH, with proven efficacy in inducing regression and a favorable safety profile.

What This Study Adds:

This single-center retrospective study confirms high rates of complete or partial regression of IH with oral propranolol in a pediatric setting. The findings support early initiation of therapy and individualized treatment duration, with minimal adverse effects observed.

Authors' Contribution: Conception and design: JR; Acquisition, analysis and interpretation of data: AĐ, AK and JR; Drafting the article: AĐ and JR; Revising it critically for important intellectual content: JR; Approved final version of the manuscript: AĐ, AK and JR.

References

1. Léauté-Labrèze C, Hoeger P, Mazereeuw-Hautier J, Guibaud L, Baselga E, Posiunas G, et al. A randomized, controlled trial of oral propranolol in infantile hemangioma. N Engl J Med. 2015;372(8):735-46. doi: 10.1056/NEJMoa1404710.
2. Hoeger PH, Harper JI, Baselga E, Bonnet D, Boon LM, Ciofi Degli Atti M, et al. Treatment of infantile haemangiomas: recommendations of a European expert group. Eur J Pediatr. 2015;174(7):855-65. doi: 10.1007/s00431-015-2570-0.
3. Tan X, Guo S, Wang C. Propranolol in the Treatment of Infantile Hemangiomas. Clin Cosmet Investig Dermatol. 2021;14:1155-63. doi: 10.2147/CCID.S332625.
4. Roganović J, Kolombo I. Dojenačkihemangiomi. [Infantile hemangiomas]. Medicina Fluminensis. 2016;52(4):444-51. doi:10.21860/medflum2016_4ro2. [in Croatian].
5. Storch CH, Hoeger PH. Propranolol for infantile hemangiomas: insights into the molecular mechanisms of action. Br J Dermatol. 2010;163:269–274. doi:.1111/j.1365-2133.2010.09848.x.
6. Han K, Wei J, Zheng H, Li X, Zhang A, Ma Y, et al. Efficacy and safety of oral propranolol or topical timolol for the treatment of superficial infantile hemangiomas. J Craniofac Surg. 2024;35(3):e254-7. doi: 10.1097/SCS.0000000000010001.
7. Meza Viteri V, Aranibar L. Oral atenolol compared to oral propranolol for infantile hemangioma. Medwave. 2023;23(11):e2753. English, Spanish. doi: 10.5867/medwave.2023.11.2753.
8. Izadpanah A, Izadpanah A, Kanevsky J, Belzile E, Schwarz K. Propranolol versus corticosteroids in the treatment of infantile hemangioma: a systematic review and meta-analysis. Plast Reconstr Surg. 2013;131(3):601-13. doi: 10.1097/PRS.0b013e31827c6fab.
9. Fei Q, Lin Y, Chen X. Treatments for infantile Hemangioma: A systematic review and network meta-analysis. EClinicalMedicine. 2020;26:100506. doi: 10.1016/j.eclinm.2020.100506.

Case Report
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Euglycemic Ketoacidosis: An Overlap of Diabetic and Non-diabetic Causes

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Abstract

Objective. Ketoacidosis is the most common cause of high anion gap metabolic acidosis in acute medical settings. Although bedside detection is straightforward, identifying the underlying aetiology may be challenging, particularly in patients with diabetes, in whom multiple mechanisms may coexist. **Case Report.** We report a case of a middle-aged woman with type 1 diabetes who presented with vomiting and reduced oral intake. Initial investigations demonstrated high anion gap metabolic acidosis with severe ketoacidosis (ketones 4.8 mmol/L), hypoglycaemia (glucose 3.3 mmol/L), and profound acute kidney injury (eGFR 5 ml/min/1.73 m² from a baseline of 47 ml/min/1.73 m²). She was initially managed for diabetic ketoacidosis (DKA) with hypoglycaemia using a fixed-rate insulin infusion; however, no clinical or biochemical improvement was observed. Re-evaluation of her clinical presentation and investigations by the multidisciplinary team prompted consideration of other differential diagnoses, such as euglycemic DKA, starvation ketosis, and metabolic acidosis secondary to acute tubular necrosis. Targeted treatment for these causes of metabolic acidosis was initiated, and remarkable clinical and biochemical improvements were noted within 48 hours, suggesting an overlapping metabolic pathophysiology. The patient made a full recovery and was discharged after 10 days. **Conclusion.** This case highlights the diagnostic complexities that may be encountered in managing ketoacidosis, especially in patients with diabetes, and underscores the importance of correctly identifying the different causes of ketoacidosis. It draws the attention of acute and general physicians to the possibility of overlapping metabolic processes in the aetiology of ketoacidosis and that successful treatment often targets all identified causes, rather than a single cause. Additionally, other causes of metabolic acidosis, such as lactic acidosis and acute kidney injury, should be identified and treated.

Key Words: Ketoacidosis ▪ Euglycemic Diabetic Ketoacidosis ▪ Starvation Ketosis ▪ High Anion Gap Metabolic Acidosis ▪ Acute Kidney Injury.

Introduction

Ketoacidosis is the most common cause of high anion gap metabolic acidosis in clinical practice and is often precipitated by diabetes mellitus, starvation, and alcoholism, either in isolation or in combination (1, 2).

In the last two decades following the introduction of sodium-glucose transporter type 2 (SGLT-2) inhibitors in the management of type-2 diabetes, the occurrence of diabetic ketoacidosis (DKA) with normoglycemia, termed euglycemic DKA, has seen an uptick in its reportage in the medical literature. The absence of hyperglycaemia in this condition poses a diagnostic dilemma with starvation ketoacidosis in patients with diabetes presenting with ketoacidosis (3, 4). Other nonketotic contributors to metabolic acidosis - such as renal failure and lactic acidosis - may coexist with ketoacidosis, further confounding the clinical picture. Failure to identify the overlapping causes of acidosis may delay appropriate therapy and adversely affect patient outcomes.

We describe a diagnostically challenging case of multifactorial metabolic acidosis and highlight the key considerations in differentiating overlapping aetiologies, alongside a focused review of the

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literature to enhance clinical reasoning in acute care settings.

Case Description

A middle-aged woman with a medical history of type 1 diabetes mellitus, hypertension, and chronic kidney disease presented to the Emergency Department with a 48-hour history of vomiting, reduced oral intake, and oliguria. Her diabetes was managed with subcutaneous insulin (long-acting (Lantus®) 20 units at night and short-acting (Novorapid®) 8-15 units with meals) and oral metformin 850 mg thrice daily. Importantly, she was not taking any SGLT-2 inhibitors and had never been admitted to the hospital with a diabetes emergency. Due to vomiting and poor oral intake during the intercurrent illness, she omitted her insulin doses and reported home blood glucose readings ranging from 3.0 to 3.9 mmol/L.

On initial assessment, there were signs of clinical dehydration, supported by the findings of a dry buccal mucosa, delayed capillary refill time, and reduced skin turgor. Examinations of the cardiovascular, respiratory, and neurological systems were normal. Her vital signs were also normal (full volume, regular, radial pulse at 80 beats/minute, blood pressure 154/83 mmHg, oxygen saturation 100% inspiring room air, and respiratory rate 18 cycles/minute).

Laboratory investigations revealed severe acute kidney injury (urea 25 mmol/L [baseline 8.3], creatinine 712 µmol/L [baseline 105], eGFR 5 mL/min/1.73 m^2 [baseline 47]) and electrolyte derangement (sodium 130 mmol/L, potassium 6.8 mmol/L). Venous blood gas analysis showed severe high anion gap metabolic acidosis (pH 7.14, bicarbonate 12.2 mmol/L, calculated anion gap 30.6 mEq/L), with ketonemia (4.8 mmol/L), hyperlactataemia (2.5 mmol/L), and hypoglycaemia (3.3 mmol/L). Other blood results on admission are presented in box 1. Bedside urine analysis was positive for ketones and leucocytes, and a sample was sent for culture and sensitivity. Her $HbA1_C$ on admission was 81 mmol/mol, reflecting poor glycaemic control. The previous result from a year earlier was 80 mmol/mol.

Complete blood count:
Haemoglobin – 86 g/L
White cell count – 6.7×10^9/L
Platelet – 240×10^9/L

Liver function tests:
Total bilirubin - 5 umol/L
Aspartate transaminase – 22 IU/L
Alanine transaminase – 15 IU/L
Gamma-glutamyl transaminase – 25 IU/L
Lactate dehydrogenase - 249 IU/L
Alkaline phosphatase - 118 IU/L
Albumin - 34 g/L

Clotting profile:
Prothrombin time - 10.0 s
Activated partial thromboplastin time – 27.0 s
International normalised ratio - 0.9
Activated partial thromboplastin time ratio - 1.1

Electrolytes:
Magnesium - 0.73 mmol/L
Adjusted Calcium - 2.26 mmol/L
Phosphate - 2.50 mmol/L

Others:
Total Protein 60 g/L
C-Reactive protein - 11 mg/L
Procalcitonin - 0.27 ng/mL

Box 1: Blood results on admission.

An initial diagnosis of 'diabetic ketoacidosis (DKA) with hypoglycaemia' was made, and she was commenced on a standard regimen of fixed-dose insulin infusion at 7 units/hour (0.1 units/kg/hour) and 10% dextrose infusion at 125 mls/hour. She was also fluid-resuscitated with a litre of 0.9% sodium chloride intravenously and maintained on 125 mL/hr thereafter. Her clinical diagnosis was later revised to 'euglycemic DKA with acute kidney injury' following further review by the Acute Medical team on call, and her insulin infusion was reduced to 3.5 units/hour (0.05 units/kg/hr) with continuation of 10% dextrose infusion at 100 mls/hr. She was monitored for the next eight

Table 1. Trend of blood gas results (HCO3- - bicarbonate, BE – base excess)

Day	Time	pH	pCO2 (kPa)	pO_2 (kPa)	HCO_3^- (mmol/L)	BE (mmol/L)	Glucose (mmol/L)	Ketones (mmol/L)	Lactate (mmol/L)
0*	23:46	7.14	-	-	12.2	-	3.3	4.8	2.5
1	07:45	7.16	-	-	12.6	-	6.1	2.9	4.9
1	08:38	7.20	-	-	14	–12.7	12.5	-	4.9
1	15:20	7.13	2.47	13.9	-	– 21.0	25	4.4	6.7
1	20:00	7.28	4.23	9.23	16	-	12.3	-	2.9
2	00:00	7.30	-	-	-	-	6.3	0.6	-
2	03:41	7.31	-	-	16	-	9	0.3	-
2	18:30	7.27	4.27	-	15.3	-	12.3	-	2.5
2	22:30	7.32	-	-	-	-	5.5	-	2.0
2	22:37	7.37	3.36	17	16.9	– 9.4	6.0	<0.3	1.7

*Admission.

hours but did not show any appreciable biochemical or clinical improvement (pH 7.16, bicarbonate 12.6 mmol/L, ketone 2.9 mmol/L, lactate 4.9 mmol/L) (See Table 1). She later developed profound hypoglycaemia (1.0 mmol/L), necessitating urgent correction.

Her clinical course remained variable over the next 24-36 hours with labile blood glucose fluctuating between hypo-and hyperglycaemia depending on the treatment strategy (insulin versus glucose infusion) (see Table 1). Maintaining her glucose level within the normal range proved challenging, even with a combination of reduced-dose insulin and dextrose infusion. She was reviewed by the multidisciplinary team (acute medicine, endocrinology, intensive care medicine, nephrology) at various stages during her hospital stay, but a clear diagnosis remained elusive. Several diagnoses were considered, including euglycemic DKA, starvation ketoacidosis, and acute tubular necrosis. Renal biopsy was considered at some point. Her pH remained static, and metabolic acidosis persisted. Meanwhile, her urine sample tested positive for Escherichia coli, suggesting that a urinary tract infection triggered her illness. She was commenced on oral antibiotics (pivmecillinam).

Given the lack of adequate response to insulin therapy and the clinical context of prolonged fasting, an overlapping aetiology was considered. Fixed-dose insulin infusion was permanently discontinued, and her management was revised to maintenance intravenous fluids with Hartmann's solution, reintroduction of her regular subcutaneous insulin at a reduced dose (18 units of Lantus® at night) with the advice to skip her short-acting mealtime doses if hypoglycaemic, and nutritional replenishment orally (vomiting had settled by this time).

This approach resulted in rapid clinical and biochemical improvement by the second and third days of admission, with resolution of acidosis and ketonemia, normalisation of anion gap (18.1mEq/L (calculated)), and gradual recovery of her renal function (Tables 1&2).

She was discharged after 10 days with significantly improved renal function and subsequently demonstrated complete renal recovery to her baseline at follow-up a week after discharge from the hospital (Table 2).

Discussion

The index case reflects the nuances and diagnostic dilemmas faced by clinicians while managing cases of ketoacidosis. Starvation is central to the pathogenesis of ketoacidosis as it lowers the insulin-glucagon ratio in the presence of calorie depletion (3). It induces a catabolic state characterised by increased lipolysis and hepatic ketogenesis to provide ketones as an alternate source of energy for the brain (3). This physiological adaptation may be exaggerated during intercurrent illness,

Table 2. Trend of blood test results (Hb – Haemoglobin, WCC – White cell count, CRP – C-Reactive Protein, Na^+ - Sodium, K^+- Potassium, $HOC3^-$ - Bicarbonate, Cl^- - Chloride, eGFR – estimated glomerular filtration rate)

Day	Hb (g/L)	WCC ($\times 10^9$/L)	CRP (mg/L)	Na^+ (mmol/L)	K^+ (mmol/L)	$HCO3^-$ (mmol/L)	Cl^- (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)	eGFR (mL/min/1.73 m^2)
0*	86	6.7	11	130	6.8	12.2	94	25	712	5
1	-	-	9	128	6.6	12	-	24.1	734	5
2	-	-	-	126	5.7	-	-	22.4	680	5
4	-	-	-	132	4.1	21	97	21.1	623	6
6	85	6.9	-	-	-	-	-	16.2	446	9
8	87	6.9	7	-	-	-	-	10.4	311	13
9	-	-	-	-	-	-	-	9.5	240	18
10†	89	6.6	5	142	4.0	-	-	7.4	226	19
17‡	106	6.2	-	-	3.0	-	-	3.7	103	47

*Admission; †Discharge; ‡Follow-up.

sepsis, malignancy, pregnancy, or vomiting, causing clinically significant ketoacidosis (3).

The most common causes of ketoacidosis in clinical practice are diabetes (including euglycemic DKA), starvation, and alcoholism (1). All three conditions share similar pathophysiological mechanisms, as described above, and may overlap concomitantly in a patient, confounding the clinical diagnosis and delaying appropriate treatment. In the index case, ketoacidosis in a patient with diabetes and missed insulin doses and a clear history of starvation worsened by vomiting convincingly makes the argument for an overlapping pathophysiologic process between euglycemic DKA and starvation ketoacidosis. Therefore, a sound knowledge of the biochemistry of metabolism during starvation is crucial for the Acute and General Physician managing these cases.

While DKA has been extensively studied and reported in the medical literature, the relatively new concept of euglycemic DKA has only gained traction in the last two decades following the introduction of SGLT-2 inhibitors in the management of Type-2 diabetes (4). These medications cause euglycemic DKA through their glucosuric effect, which lowers blood glucose and simulates carbohydrate deficiency, reducing the insulin-glucagon ratio and triggering ketogenesis. They also inhibit ketonuria and directly stimulate pancreatic alpha cells to secrete glucagon (5, 6). Other reported mechanisms for euglycemic DKA include continued use of insulin at the onset of DKA in patients who are fasting and pre-hospital administration of insulin in suspected DKA (4).

The clinical presentation of ketoacidosis (from any cause) is similar: nausea, vomiting, lethargy, and dehydration, which may cause acute kidney injury and metabolic acidosis (7). The latter results from impaired excretion and accumulation of organic acids, such as urea and lactic acid (4, 8). This clinical picture of mixed metabolic acidosis was well portrayed in our patient, who had ketoacidosis, lactic acidosis, and metabolic acidosis due to acute kidney injury (AKI).

There are slight differences in the management of ketoacidosis, depending on the cause. Successful treatment hinges on correctly identifying the underlying pathophysiological processes and aiming to reverse the reduced insulin-glucagon ratio. In starvation ketoacidosis, this is achieved by oral or intravenous carbohydrate replenishment with dextrose-containing fluids. This stimulates endogenous insulin release, which is sufficient to shut down lipolysis and ketogenesis. The management of euglycemic DKA, on the other hand, is a balancing act between calorie replenishment and exogenous insulin supplementation. While these patients are not profoundly insulin deficient and/or resistant as in classical DKA, they still require some exogenous insulin at a reduced dose

of 0.05 units/kg/hour to reverse ketogenesis (9). Metabolic acidosis with pre-renal AKI is treated with isotonic fluid rehydration.

The absence of hyperglycaemia in patients with diabetes presenting with ketoacidosis may distract clinicians from correctly diagnosing and treating euglycemic DKA. This warrants a high index of suspicion when they present to the acute medical take. Misdiagnosing starvation ketoacidosis as euglycemic DKA, on the other hand, and administering insulin risks worsening hypoglycaemia and putting the patient to harm (10). A careful clinical assessment, correct interpretation of biochemical data, and understanding of overlapping metabolic processes will help distinguish between the two and/or reveal an overlap.

In the case of overlap, a combined management strategy targeting both metabolic processes should be deployed. We suggest managing such patients in a high-monitoring unit, initially with isotonic fluid resuscitation, followed by calorie replenishment (orally or intravenously). In patients with diabetes, low-dose insulin infusion should be initiated with close monitoring for glucose derangements. Urine output and fluid balance should be carefully monitored, and electrolytes and vitamins appropriately replaced.

Conclusion

This case underscores the diagnostic complexity that clinicians may encounter in some presentations of ketoacidosis, especially in patients with diabetes, where multiple causes may be at play. It also highlights the importance of a sound knowledge of the pathophysiology of ketoacidosis, biochemistry in starvation, and their application in clinical practice. This will enable the clinician to promptly make the correct diagnosis, including identifying when overlapping processes are in effect, and institute appropriate treatment to improve outcomes.

What Is Already Known on This Topic:

Ketoacidosis is the most common cause of high anion gap metabolic acidosis in clinical practice, often triggered by diabetes, starvation, or alcoholism. There has been increased reporting of euglycemic DKA since the introduction of SGLT-2 inhibitors for the management of diabetes.

What This Study Adds:

Although the detection of ketoacidosis using simple bedside investigations is straightforward, identifying the underlying cause(s) may pose diagnostic challenges. This case draws the attention of clinicians (especially in Acute and General Internal medicine) to the possibility of a mixed aetiology (overlap) of ketoacidosis, including in patients with diabetes, as well as the co-occurrence of other causes of metabolic acidosis. It emphasizes the need to correctly diagnose these patients early and commence appropriate, individualised treatment targeting the different causes, under close monitoring.

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

Patient Consent: Written informed consent was obtained from the patient prior to the preparation of this case report.

References

1. Mostert M, Bonavia A. Starvation Ketoacidosis as a Cause of Unexplained Metabolic Acidosis in the Perioperative Period. Am J Case Rep. 2016;17:755-8. doi: 10.12659/ajcr.900002.
2. Mehta A, Emmett M. Fasting ketosis and alcoholic ketoacidosis. UpToDate. 2023. [cited 2026 Jan 28]. Available from: https://www.uptodate.com/contents/fasting-ketosis-and-alcoholic-ketoacidosis.
3. Palmer BF, Clegg DJ. Starvation Ketosis and the Kidney. Am J Nephrol. 2021;52(6):467-78. doi: 10.1159/000517305. Epub 2021 Jul 19.
4. Shukla R, Shrestha P, Khanal B, Regmi B. Euglycemic Diabetic Ketoacidosis: Clinical Suspicion and Diagnosis. Clin Med Insights Endocrinol Diabetes. 2026;19:11795514261431390. doi: 10.1177/11795514261431390.
5. Nasa P, Chaudhary S, Shrivastava PK, Singh A. Euglycemic diabetic ketoacidosis: A missed diagnosis. World J Diabetes. 2021;12(5):514-23. doi: 10.4239/wjd.v12.i5.514.
6. Rosenstock J, Ferrannini E. Euglycemic Diabetic Ketoacidosis: A Predictable, Detectable, and Preventable Safety Concern With SGLT2 Inhibitors. Diabetes Care. 2015;38(9):1638-42. doi: 10.2337/dc15-1380.
7. Umpierrez GE, DiGirolamo M, Tuvlin JA, Isaacs SD, Bhoola SM, Kokko JP. Differences in metabolic and hormonal milieu in diabetic- and alcohol-induced ketoacidosis. J Crit Care. 2000;15(2):52-9. doi: 10.1053/jcrc.2000.7900.
8. Leblanc M. Acid-base balance in acute renal failure and renal replacement therapy. Best Pract Res Clin Anaesthesiol. 2004;18(1):113-27. doi: 10.1016/j.bpa.2003.08.001.

9. Joint British Diabetes Societies for Inpatient Care (JBDS-IP) Group. The management of diabetic ketoacidosis in adults. Revised Mar 2023. London: Association of British Clinical Diabetologists (ABCD); 2023. [cited 2026 Jan 30]. Available from: https://abcd.care/resource/current/jbds-02-management-diabetic-ketoacidosis-adults?utm_source=chatgpt.com.
10. Hammerbeck H, Holland MR. Starvation ketoacidosis: Treatment pitfalls. J Intensive Care Soc. 2017;18(3):265. doi: 10.1177/1751143716687593. Epub 2017 Jul 24.

Case Report
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Beyond the Spine: A Rare Case of Myxopapillary Ependymoma With Extraneural Metastases

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Abstract

Objective. Myxopapillary ependymoma (MPE) is a rare tumor of ependymal origin that commonly arises within the spinal canal. It is rather benign and slow-growing; however, it tends to recur along the central nervous system (CNS), usually after incomplete resection or tumor seeding due to surgical manipulation. Extraneural metastases of MPE are extremely rare, with only a few documented cases in the literature. **Case Report.** We describe a case of a middle-aged female patient with sacrococcygeal MPE, which was initially misdiagnosed as a pilonidal cyst and subjected to surgical excision. Over the following years, she experienced three local recurrences in the sacrococcygeal region, which were treated with surgery and radiotherapy. Ten years after the initial excision, the patient complained of dyspnea and hemoptysis, and imaging studies revealed multiple right lung nodules. A core biopsy of a lung lesion was performed, and histopathology was consistent with metastatic MPE. The patient was started on chemotherapy with per os temozolomide and has not experienced disease progression for nearly 3 years. **Conclusion.** Extraneural metastases of MPE represent an exceptionally rare and diagnostically challenging entity and should be considered in patients with a history of MPE who present with distant lesions, even years after the initial diagnosis.

Key Words: Myxopapillary Ependymoma ▪ Spinal Neoplasms ▪ Extraneural Metastasis ▪ Central Nervous System.

Introduction

Myxopapillary ependymoma (MPE) is a distinct subtype of ependymoma that originates from ependymal cells within the central canal of the spinal cord (1). It is an exceptionally rare tumor, with an estimated incidence of 0.01 per million individuals, accounting for approximately 27% of spinal cord tumors (1, 2). MPE is predominantly located in the conus medullaris, cauda equina, or filum terminale and is typically encountered in the pediatric population and adults during the third and fourth decades of life, with a slight male predominance (1, 3). This tumor has a high propensity for local recurrence along the central nervous system (CNS), whereas extraneural metastases are extremely rare (2). We present a case of MPE in an adult female patient who presented with secondary lung metastases 10 years after her initial diagnosis.

We aim to enhance the understanding of the diverse clinical manifestations, pathological characteristics, and treatment challenges associated with distant recurrence in MPE, which is very rare and often results from tumor dissemination during prior surgical procedures.

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Case Report

A 45-year-old woman presented with lower back pain for the past month and underwent surgical excision of a sacrococcygeal lesion that was initially presumed to be a pilonidal cyst. No preoperative MRI was performed, and the surgeon proceeded directly to surgical excision without prior biopsy. Histopathological examination of the resected specimen revealed a sacrococcygeal myxopapillary ependymoma (MPE) measuring 35 mm with capsule violation. Following the diagnosis, the patient was placed on regular surveillance with serial magnetic resonance imaging (MRI) of the spine. Postoperative MRI scan of the spine demonstrated no evidence of a residual tumor.

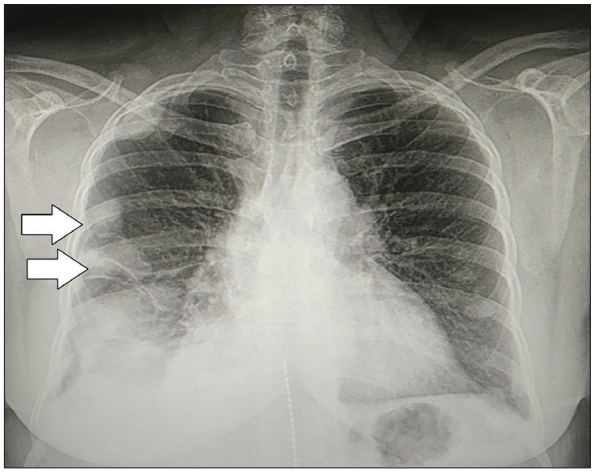

Figure 1. Chest X-ray showing multiple lung nodules in the right middle lobe.

During the following five years, she developed three local recurrences, which were managed with two lumbar spine surgeries and adjuvant radiotherapy (40 Gy delivered in 20 fractions). Post-radiotherapy, she developed chronic L5 radiculopathy associated with muscle pain and motor weakness, which required treatment with opioid analgesics and peripheral nerve stimulation. Ten years after the initial surgical resection, at the age of 55, she presented to our department with progressive dyspnea and episodes of hemoptysis for the past month. Physical examination was unremarkable, and routine laboratory investigations were within normal limits. Chest radiography demonstrated multiple peripheral lung nodules in the right middle lobe (Figure 1).

Computed tomography pulmonary angiography (CTPA) revealed multiple peripheral lung nodules in the right middle lobe, in contact with the pleura (Figure 2A).

A computed tomography (CT)-guided biopsy of a lung nodule was performed, revealing small cuboidal cells with a basophilic cytoplasm, radially arranged around the blood vessels (perivascular pseudorosettes), suggestive of a myxopapillary

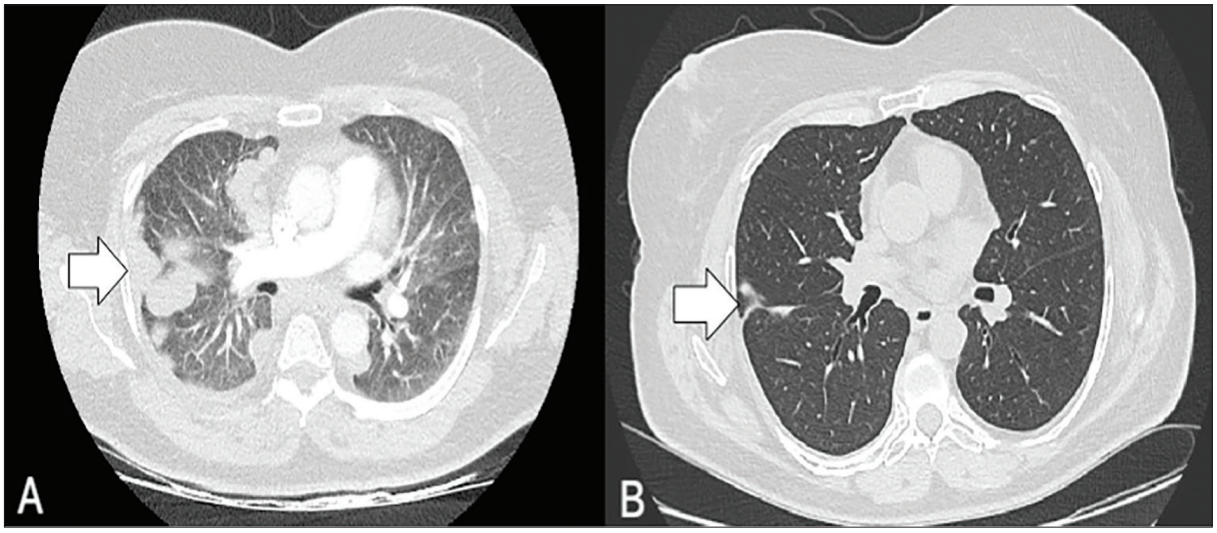

Figure 2. Axial CT scan of the chest demonstrating multiple peripheral lung nodules, as per MPE metastases (white arrow - panel A). Axial CT scan of the chest following 5 cycles of chemotherapy with temozolomide showing a significant disease response (white arrow - panel B).

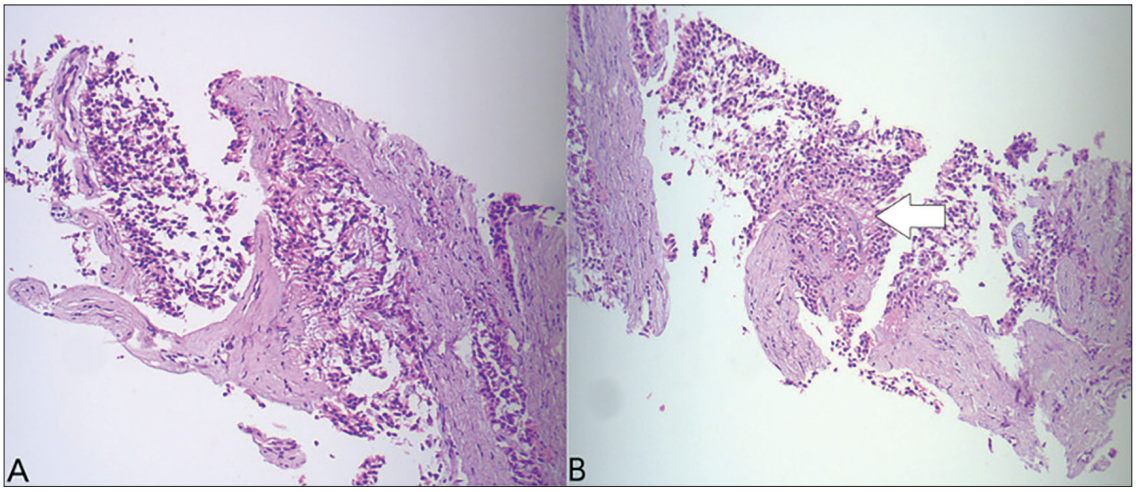

Figure 3. Hematoxylin/eosin staining, × 10 magnification. Radial arrangement of cuboidal to epithelioid elongated glial tumor cells around hyalinized fibrovascular cores in a papillary configuration. Accumulation of basophilic myxoid material around blood vessels (myxoid stroma) is usually observed; however, it is not particularly evident in this case, which is characterized mostly by hyalinization of the papillary cores (panel A). Focal presence of a perivascular pseudorosette, which is the hallmark of ependymomas (white arrow - panel B).

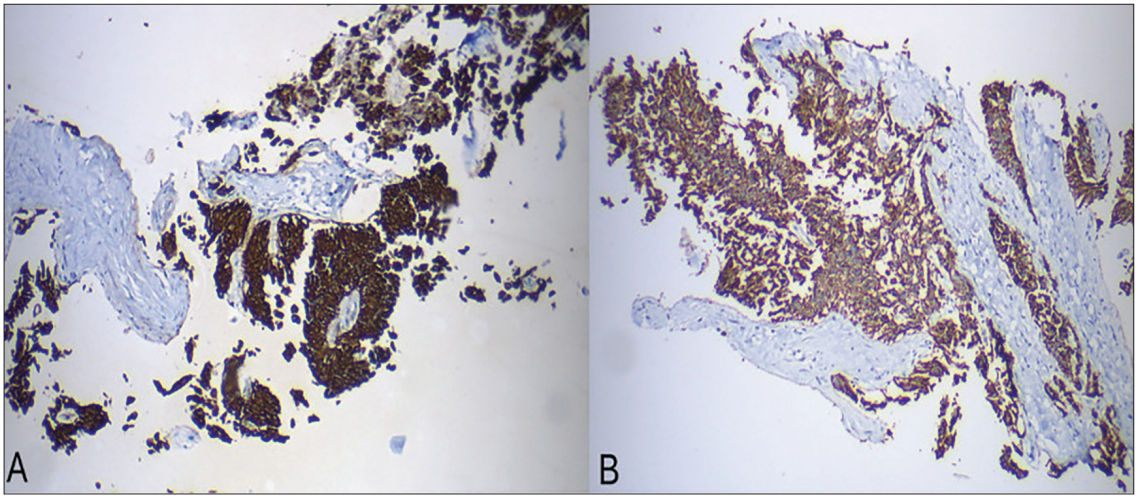

Figure 4. Immunohistochemical examination reveals diffuse positive GFAP (panel A) and CD56 (panel B) staining of the tumor cells.

ependymoma (Figure 3). Immunohistochemically, the cells stained strongly positive for G-FAP and CD56 (Figure 4), focally positive for TTF-1, and negative for chromogranin-A, synaptophysin, EMA, p40, and Napsin A. The Ki67 index was 5%, indicating a low cell proliferation rate. A review of both the initial surgical specimen biopsy and the recent lung nodule biopsy confirmed the diagnosis of metastatic MPE. No additional tissue was available for molecular profiling. Cranial and whole-spine MRI scans were negative for signs of CNS dissemination.

The case was discussed at a multidisciplinary tumor board, and the patient was initiated on chemotherapy with temozolomide (dose: 150 mg/m^2 PO daily on days 1–5 of a 28-day cycle) in October 2021. During treatment, the patient reported complete resolution of dyspnea and hemoptysis. The treatment was well tolerated overall, apart from grade 2 thrombocytopenia and neutropenia

requiring granulocyte colony-stimulating factor (G-CSF) support. Restaging CT scans after 5 cycles of treatment demonstrated a significant size reduction of the lung nodules, indicating a partial response according to the RESIST 1.1 criteria (Figure 2B). The patient is still on treatment with temozolomide and remains free of disease progression for nearly three years. She is under close follow-up with serial craniospinal MRI scans, in order to mitigate the risk of CNS recurrence. Treatment will be continued until disease progression or the development of unacceptable toxicity. In the event that temozolomide must be discontinued, management would be individualized based on disease status. Options would include close surveillance, consideration of targeted therapies, or enrollment in a clinical trial.

Discussion

Myxopapillary ependymoma is a tumor that originates from the ependymal lining of the spinal canal. It is an extremely rare tumor with an annual incidence of 0.01 per million (1, 4), predominantly affecting males during the third and fourth decades of life (5, 6). The most common sites of occurrence include the conus medullaris, cauda equina, and filum terminale regions (1). No specific genetic or environmental risk factors have been identified to date (1). MPE is classified as a grade II tumor according to the recent World Health Organization (WHO) Classification of Tumors of the Central Nervous System and is considered to have low metastatic potential (3, 7).

The clinical presentation of MPE is characterized by vague, nonspecific symptoms, including low back pain radiating to the lower limbs, occasionally accompanied by motor weakness or paralysis, altered sensation, and sphincter or sexual dysfunction (3, 4, 7). The clinical presentation of metastatic MPE varies according to the metastatic site. In our case, the patient presented with hemoptysis, dyspnea, and fatigue due to pulmonary metastases ten years after resection of a sacrococcygeal MPE initially misdiagnosed as a pilonidal cyst.

MRI scans with intravenous contrast remain the gold standard imaging method for visualizing the spinal canal. On MRI, sacrococcygeal myxopapillary ependymoma (MPE) appears as a well-circumscribed lesion with variable enhancement, depending on the presence of hemorrhage or calcifications (7, 8). On CT scan, spinal and extraneural MPEs typically present as hyperdense lesions with homogeneous contrast enhancement (9). In our case, lung metastases of MPE appeared as hyperdense, well-circumscribed nodules on CT.

Due to the lack of distinctive clinical and imaging features, definitive diagnosis relies on a tissue biopsy (7). The histopathological characteristics of MPE include papillary structures composed of cuboidal or columnar cells often embedded within a myxoid stroma, resulting in a characteristic myxopapillary appearance (10). These cells are radially organized around blood vessels, forming unique rosettes or pseudorosettes (8). Immunohistochemical studies reveal consistent expression of ependymal markers, such as GFAP, vimentin, CD99, and S-100, supporting their ependymal origin (11-13). Additionally, positive CD56 staining is observed in some instances (11).

Gross total resection (GTR), while preserving the integrity of the tumor capsule, is the mainstay of treatment for localized disease (3). Adjuvant focal radiotherapy is indicated in cases of subtotal resection (STR), capsule violation, or positive postoperative cerebrospinal fluid (CSF) cytology; however, its role in spinal ependymoma management beyond these indications is not well established (3, 14). Due to the rarity of metastatic MPE, no current guidelines exist for its management; however, various methods are employed, including metastasectomy, chemotherapy, radiotherapy, targeted therapy, and immunotherapy (4, 12-16). Systemic agents used in the management of metastatic MPE with reported durable responses include temozolomide (either as monotherapy or in combination with olaparib), tyrosine kinase inhibitors (TKI) such as imatinib, sorafenib, and pazopanib, and monoclonal antibodies such as bevacizumab and tislelizumab, among others (4, 10, 15-18).

Table 1. Reported Cases of Sacrococcygeal MPE With Extraneural Metastases, Compiled, Modified, and Updated From Mastantuoni et al., 2022 (7), and Jayawardena et al., 2025 (29)

Author/Year	Age/ Sex	Initial level	Metastatic site	Duration (presentation to metastases)	Treatment modality	Outcome (total f/up)
Weiss, 1955 (22, 23)	22/M	Cauda equina	Retroperitoneum, lungs, liver, hilar and tracheobronchial lymph node, mediastinum, IVC, pleura, chest wall, diaphragm, pelvis	10 years	None	Died (10 years)
Sharma, 1956 (7)	29/M	Cauda equina	Lungs, liver, mediastinum, paraaortic lymph node, IVC, pleura	4 years	N/A	N/A
Patterson, 1961 (23, 24)	28/F	Cauda equina	Vertebrae, mediastinum, pleura, lung, hilar lymph nodes	17 years	Radiation therapy	Died (17 years)
Wight, 1973 (7, 23)	20/M	Cauda equina	Para-aortic lymph nodes, pelvis, humerus, rib, pleura	38 years	Cobalt	Died
Bardales, 1994 (12, 25)	19/M	Sacrum	Inguinal lymph nodes	16 years	Resection	N/A
Newton, 1992 (7)	37/M	Cauda equina	Lungs, pleura	<1 year	N/A	N/A
Newton, 1992 (7)	16/M	Cauda equina	Abdomen	22 years	N/A	N/A
Bardales, 1993 (25)	19/M	Sacrum	Abdomen, subcutaneous tissue, inguinal lymph nodes	16 years	N/A	N/A
Graf, 1999 (26)	15/M	Cauda equina/ conus	Lungs, liver, abdominal and mediastinal lymph node, pleura, chest wall	36 years	Cisplatin/ Ifosfamide/ Etoposide	40 years
Rickert, 1999 (7, 27)	55/F	Cauda equina	Lungs	12 years	N/A	N/A
Satti, 2005 (2)	16/F	Gluteus	Lungs	9 years	-Carmustine/ etoposide -Oral etoposide -Radiotherapy	Alive (10 years)
Vega-Orozco, 2011 (7, 28)	22/M	Cauda equina	Inguinal lymph node	18 years	N/A	N/A
Fegerl, 2012 (16, 29)	39/F	Sacrum	Pleural	20 years	-Temozolomide -Imatinib -Sorafenib	Alive
Guzin, 2016 (29, 30)	23/F	Sacrum	Cervix	9 years	Surgical resection	Alive
Ekemen, 2018 (12)	17/F	Sacrum	Inguinal lymph node	19 years	N/A	N/A
Katz, 2018 (31)	38/M	Sacrum	Gluteus	2 years	- Lapatinib/ temozolomide -Radiotherapy -yclophosphamide -Etoposide	Alive (6 years)
Katz, 2018 (31)	76/M	Sacrum	Retroperitoneal lymph nodes	N/A	N/A	Died (4.2 years)
Deniel, 2019 (17, 29)	30/F	Filum terminale	Lung, pleura, mediastinum	13 years	Temozolomide	Died (13 years)
Tapia Rico, 2020 (4, 29)	23/F	Sacrum	Lungs	De novo	-Tislelizumab -Temozolomide -Carboplatin	Alive (4 years)
Bruno, 2020 (21)	32/F	Sacrum	Lungs, gluteus	20 years	-Temozolomide -Platinum/lomustin	Died (23 years)
Mastantuoni, 2022 (7)	9/M	Cauda equina	Paraspinal muscles	30 years	Surgical resection	Alive
Mahalingam, 2023 (10, 29)	28/F	Pelvis	Lungs	20 years	-Carboplatin-etoposide -Temozolomide -Vismodegib -Olaparib/ ctemozolomide	Died
Fecker, 2024 (29, 32)	29/F	Sacrum	Pleura, lung, intravascular space	De novo	None	Died
Riegel, 2024 (29, 33)	24/F	Sacrum	Pleura, lungs, pelvis	11 years	Temozolomide/ lapatinib	Alive
Jayawardena, 2025 (29)	24/M	Lumbar	Lungs	37 years	None	Died (39 years)

MPE exhibits a rather indolent course and a generally favorable prognosis, with a 10-year overall survival rate of approximately 93% after GTR (1, 19). However, literature reports suggest a high rate of local recurrence within the CNS, reported up to 15.5%, particularly after incomplete resection or surgical manipulations (1, 3, 6, 7, 20). Most local recurrences occur during the first 3-6 years after the initial excision, often necessitating subsequent debilitating surgeries that affect the patient's overall health and well-being (3). In our case, the tumor was initially resected under the assumption of a pilonidal cyst, raising the possibility of unplanned capsule disruption and tumor seeding, which may have contributed to the three local recurrences observed during the first five years after diagnosis

Although MPE is typically considered a low-grade neoplasm, distant metastases may occur despite a low Ki-67 proliferation index, suggesting that conventional histologic features do not reliably predict metastatic potential. Proposed mechanisms of dissemination include cerebrospinal fluid spread with secondary extraneural extension and hematogenous dissemination, particularly following repeated surgical interventions. The relatively early development of pulmonary metastases in our patient, occurring ten years after the initial diagnosis compared with the reported average interval of more than 20 years, may reflect the cumulative impact of repeated local recurrences and prior surgical manipulation.

To our knowledge, fewer than 30 cases of adult MPE with extraneural metastases have been reported in the literature since 1955, highlighting the exceptional nature of such occurrences (2, 7, 15, 17, 21). Published cases of myxopapillary ependymoma with extraneural metastases are summarized in Table 1.

Extraneural metastases of MPE have been documented in the lungs, pleura, liver, bones, pelvis, or lymph nodes (2, 7, 15, 17, 21). The mean time from the initial presentation of MPE to extramedullary metastases can exceed 20 years, thus necessitating lifelong follow-up with serial MRI or CT scans (2, 6). However, unlike many reported cases in which metastases developed after two decades or more, pulmonary metastases in our patient were detected ten years after the initial diagnosis, underscoring the variability in the timing of distant recurrence.

Given the risk of both local and distant relapse, long-term surveillance is essential. We suggest annual contrast-enhanced MRI of the brain and entire spine for patients with recurrent disease or prior subtotal resection. CT scans should be considered when extraneural symptoms develop or in patients with multiple recurrences and suspected distant dissemination.

Conclusion

Extraneural metastases of myxopapillary ependymoma are extremely rare. The majority of cases occur due to incomplete surgical excision or violation of the tumor capsule, leading to the dissemination of tumor cells along the spinal canal or to distant extraneural sites. To mitigate the risk of potential tumor seeding and subsequent local or distant recurrence, it is imperative to maintain a high index of suspicion for myxopapillary ependymoma and seek expert pathology consultation in select suspicious cases prior to proceeding with surgery for a pilonidal cyst.

What Is Already Known on This Topic:

Myxopapillary ependymoma (MPE) is a rare, slow-growing spinal tumor that arises from ependymal cells of the filum terminale and cauda equina. It is generally considered a low-grade neoplasm with a favorable prognosis following gross total resection. Despite its indolent behavior, MPE has a recognized tendency for local recurrence, particularly after incomplete excision or capsule violation. Extraneural metastases are extremely uncommon, with only a limited number of cases reported worldwide. When distant spread occurs, the lungs, lymph nodes, bones, and liver are the most frequently involved sites. Due to the rarity of metastatic disease, there are no standardized systemic treatment guidelines, and management is largely based on individual case reports and small series. Therefore, long-term surveillance is recommended, as metastases may develop many years after the initial diagnosis.

What This Study Adds:

This report describes a rare case of myxopapillary ependymoma presenting with extraneural pulmonary metastases ten years after initial diagnosis and multiple local recurrences. This case highlights the potential for very late distant dissemination and underscores the importance of lifelong follow-up in patients with MPE. Additionally, it demonstrates durable disease control with temozolomide chemotherapy, supporting its potential role as a therapeutic option in metastatic MPE when standard treatment strategies are lacking.

Authors' Contributions: Conception and design: AK; Acquisition, analysis and interpretation of data: CR, VR; Drafting the article: SS, VK; Revising it critically for important intellectual content: EB, NC, VK; Approved final version of the manuscript: AK, CR, VR, SS, EB, NC, VK.

Patient Consent: Written informed consent was obtained from the patient for the publication of this case report and any accompanying images.

Ethics Statement: All procedures in this study were approved by the Institutional Review Board of the 251 Air Force General Hospital (Approval No: 2025-354).

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

References

1. Fonseca L, Cicuendez M, Martínez-Ricarte F, Martínez-Saez E, Cordero E, Bescos A. A rare case of an intramedullary metastasis of a myxopapillary ependymoma. Surg Neurol Int. 2019;10:83. doi: 10.25259/SNI-96-2019.
2. Satti M, Firoze M, Malaker K, Hussain M, Maniyar I. Mediastinal myxopapillary ependymoma primary or late metastases of paracoccygeal ependymoma: a case report. Ann Diagn Pathol. 2005;9(4):215-8. doi: 10.1016/j.anndiagpath.2005.04.015.
3. Tabor JK, Ryu B, Schneider D, Sciubba DM, Narayana A, Zlochower A, et al. Multifocal lumbar myxopapillary ependymoma presenting with drop metastasis: a case report and review of the literature. Spinal Cord Ser Cases. 2022;8(1):43. doi: 10.1038/s41394-022-00513-x.
4. Tapia Rico G, Townsend A, Price T, Patterson K. Metastatic myxopapillary ependymoma treated with immunotherapy achieving durable response. BMJ Case Rep. 2020;13(12):e236242. doi: 10.1136/bcr-2020-236242.
5. Shapey J, Barazi S, Bodi I, Thomas N. Myxopapillary ependymoma of the cerebellopontine angle: retrograde metastasis or primary tumour? Br J Neurosurg. 2011;25(1):122-3. doi: 10.3109/02688697.2010.503817.
6. Omerhodžić I, Pojskić M, Rotim K, Splavski B, Rasulić L, Arnautovic KI. MYXOPAPILLARY EPENDYMOMA OF THE SPINAL CORD IN ADULTS: A REPORT OF PERSONAL SERIES AND REVIEW OF LITERATURE. Acta Clin Croat. 2020;59(2):329-37. doi: 10.20471/acc.2020.59.02.17.
7. Mastantuoni C, Tortora F, Tafuto R, Tortora M, Briganti F, Franca RA, et al. Extra-Neural Metastases of Late Recurrent Myxopapillary Ependymoma to Left Lumbar Paravertebral Muscles: Case Report and Review of the Literature. Brain Sci. 2022;12(9):1227. doi: 10.3390/brainsci12091227.
8. Kumar N, Tan WLB, Wei W, Vellayappan BA. An overview of the tumors affecting the spine-inside to out. Neurooncol Pract. 2020;7(Suppl 1):i10-7. doi: 10.1093/nop/npaa049.
9. Albadr F, Albahkali SM, Alahmadi MS, Alsakkaf HM, Al-Habib A, Alkhalidi H, et al. Atypical Imaging of Hemorrhagic Lumbosacral Myxopapillary Ependymoma with Histopathological Correlation: A Case Report. Am J Case Rep. 2020;21:e925449. doi: 10.12659/AJCR.925449.
10. Mahalingam P, Smith S, Lopez J, Sharma RK, Millard T, Thway K, et al. PARP inhibition utilized in combination therapy with Olaparib-Temozolomide to achieve disease stabilization in a rare case of BRCA1-mutant, metastatic myxopapillary ependymoma. Rare Tumors. 2023;15:203636132311523. doi: 10.1177/20363613231152333.
11. Wang M, Wang H, Zhou Y, Zhan R, Wan S. Myxopapillary ependymoma in the third ventricle area and sacral canal: dropped or retrograde metastasis? Neurol Med Chir (Tokyo). 2013;53(4):237-41. doi: 10.2176/nmc.53.237.
12. Ekemen S, Yapicier O, Boler HD, Ince U. An Extremely Rare Case of Back and Hip Pain due to the Metastasis of Late Recurrent Myxopapillary Ependymoma to the Inguinal Lymph Node. J Pathol Transl Med. 2018;52(1):67-70. doi: 10.4132/jptm.2017.11.09.
13. Lamzabi I, Arvanitis LD, Reddy VB, Bitterman P, Gattuso P. Immunophenotype of Myxopapillary Ependymomas. Appl Immunohistochem Mol Morphol. 2013;21(6):485-9. doi: 10.1097/PAI.0b013e318283980a.
14. Akyurek S, Chang EL, Yu TK, Little D, Allen PK, McCutcheon I, et al. Spinal myxopapillary ependymoma outcomes in patients treated with surgery and radiotherapy at M.D. Anderson Cancer Center. J Neurooncol. 2006;80(2):177-83. doi: 10.1007/s11060-006-9169-2.
15. Ilhan A, Furtner J, Birner P, Rössler K, Marosi C, Preusser M. Myxopapillary Ependymoma With Pleuropulmonary Metastases and High Plasma Glial Fibrillary Acidic Protein Levels. J Clin Oncol. 2011;29(30):e756-7. doi: 10.1200/JCO.2011.36.6401.
16. Fegerl G, Marosi C. Stabilization of Metastatic Myxopapillary Ependymoma with Sorafenib. Rare Tumors. 2012;4(3):134-7. doi: 10.4081/rt.2012.e42.
17. Deniel A, Marguet F, Beaussire L, Tobenas-Dujardin AC, Peillon C, Gambirasio MA, et al. TERTp Mutation Detection in Plasma by Droplet-Digital Polymerase Chain Reaction in Spinal Myxopapillary Ependymoma with Lung Metastases. World Neurosurg. 2019;130:405-9. doi: 10.1016/j.wneu.2019.07.111.
18. Bates JE, Peterson CR, Yeaney GA, Walter KA, Lundquist T, Rosenzweig D, et al. Spinal Drop Metastasis in Myxopapillary Ependymoma: A Case Report and a Review of Treatment Options. Rare Tumors. 2014;6(2):79-82. doi: 10.4081/rt.2014.5404.
19. Wahab SH, Simpson JR, Michalski JM, Mansur DB. Long term outcome with post-operative radiation therapy for spinal canal ependymoma. J Neurooncol. 2007;83(1):85-9. doi: 10.1007/s11060-006-9310-2.

20. Newton HB, Henson J, Walker RW. Extraneural metastases in ependymoma. J Neurooncol. 1992;14(2):135-42. doi: 10.1007/BF00177617.
21. Bruno F, Pellerino A, Bertero L, Soffietti R, Rudà R. Long-term survival of a sacro-coccygeal myxopapillary ependymoma with extra-neural metastases: case report and review of the literature. Neurol Sci. 2020;41(7):1955-7. doi: 10.1007/s10072-020-04269-w.
22. Weiss L. A metastasizing ependymoma of the cauda equina. Cancer. 1955;8(1):161-71. doi: 10.1002/1097-0142(1955)8:1<161::aid-cncr2820080123>3.0.co;2-k.
23. Wight DGD, Holley KJ, Finbow JAH. Metastasizing ependymoma of the cauda equina. J Clin Pathol. 1973;26(12):929-35. doi: 10.1136/jcp.26.12.929.
24. Patterson RH, Campbell WG, Parsons H. Ependymoma of the Cauda Equina with Multiple Visceral Metastases: Report of a Case. J Neurosurg. 1961;18(2):145-50. doi: 10.3171/jns.1961.18.2.0145.
25. Bardales RH, Porter MC, Sawyer JR, Mrak RE, Stanley MW. Metastatic myxopapillary ependymoma: Report of a case with fine-needle aspiration findings. Diagn Cytopathol. 1994;10(1):47-53. doi: 10.1002/dc.2840100113.
26. Graf M, Blaeker H, Otto HF. Extraneural metastasizing ependymoma of the spinal cord. Pathol Oncol Res. 1999;5(1):56-60. doi: 10.1053/paor.1999.0056.
27. Rickert CH, Kedziora O, Gullotta F. Ependymoma of the cauda equina. Acta Neurochir (Wien). 1999;141(7):781-2. doi: 10.1007/s007010050376.
28. Vega-Orozco R, Rembao-Bojórquez D, Salmerón-Mercado M, García-Marquez A, Tena-Suck ML. Inguinal lymph nodal metastasis of myxopapillary ependymoma confirmed by fine-needle aspiration cytology, biopsy, and immunohistochemistry: Case report. Diagn Cytopathol. 2011;39(9):689-93. doi: 10.1002/dc.21535.
29. Jayawardena D, Yates J, Clarke E, Sinclair G. Extra-neural metastases of recurrent myxopapillary ependymoma: A patient case and literature review. Surg Neurol Int. 2025;16:182. doi: 10.25259/SNI_190_2025.
30. Guzin K, Bozdag H, Aydin A, Sahin S, Ozkanli S. Uterine Cervix Metastasis of Myxopapillary Ependymoma Originated from the Spinal Cord. Balk Med J. 2016;33(2):235-8. doi: 10.5152/balkanmedj.2015.151101.
31. Yust Katz S, Cachia D, Kamiya-Matsuoka C, Olar A, Theeler B, Penas Prado M, et al. Ependymomas arising outside of the central nervous system: A case series and literature review. J Clin Neurosci. 2018;47:202-7. doi: 10.1016/j.jocn.2017.10.026.
32. Fecker A, Maanum KA, Shahin MN, Hakar M, Wright Iii JM. Myxopapillary Ependymoma Metastasis Mimicking Pulmonary Embolism: An Illustrative Case. Asian J Neurosurg. 2024;19(03):551-5. doi: 10.1055/s-0044-1779293.
33. Riegel DC, Fonkem E, Connelly JM. Treatment of Extraneural Metastases of Myxopapillary Ependymomas With Dose-Dense Temozolomide and Lapatinib. Cureus. 2024;16(8):e67928. doi: 10.7759/cureus.67928.

Meta-Analysis
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A Meta-Analysis of Whole Blood Cholinesterase Activity in Healthy Ruminants Using a Modified Electrometric Measurement Method

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Abstract

Objective. This meta-analysis aimed to compile and analyze WBChE activity using the modified electrometric method to establish normal or baseline enzyme activity values in ruminants (sheep, goats, cattle, and buffalo). **Materials and Methods.** This is a one-group randomized-effect-size meta-analysis model applied to the mean ± SD of WBChE activities in sheep, goats, cattle, and buffaloes. **Results.** Using PRISMA selection, we identified 16 records from five studies that measured WBChE activity in sheep (N=267), goats (N=165), cattle (N=197), and buffaloes (N=31) using a modified electrometric method. The studies included both sexes and were published between 2007 and 2025. The forest plot showed that the WBChE activities of sheep, goats, cattle, and buffaloes were 0.40, 0.32, 0.51, and 0.42 Δ pH/enzymatic reaction incubation time, respectively. Their 95% CI were 0.19-0.60, 0.16-0.49, 0.31-0.70, and 0.39-45, respectively. The weights of the individual records across the four species varied from 0.91% to 14.77%. The I^2 heterogeneity index of 61% was moderate and significant (Q=38.35; P=0.001). Despite the low pseudo-R^2 value (24.24%), the I^2 of subgroup analysis was significant only in sheep (64.2%, Q=14, P=0.016). The funnel plot showed the possibility of publication bias in five imputed studies. However, Egger's regression analysis was not statistically significant (t=1.91, P=0.077). The overall risk of bias in the studies was low. **Conclusion.** This study is the first meta-analysis to establish the reference WBChE activity in healthy ruminants, including sheep, goats, cattle, and buffaloes. This is an additional contribution to the existing literature, as the modified electrometric method is recommended for measuring blood or tissue ChE activity in various animal species. These findings provide valuable reference points for the clinical and toxicological assessment of poisoning by cholinesterase-inhibiting pesticides in these species.

Key Words: Blood Cholinesterase ▪ Insecticide Exposure ▪ Sentinel Species ▪ Organophosphate ▪ Carbamate.

Introduction

Organophosphate (OP) and carbamate (CA) insecticides are used to control ectoparasites in ruminants (1-3). The toxicity of these insecticides in animals is caused by the inhibition of cholinesterase (ChE) activity in the nervous system, either irreversibly (OP) or reversibly (CA) (4, 5). This enzyme-inhibitory action of insecticides leads to the buildup of acetylcholine at synapses and neuromuscular junctions, inducing a toxidrome of cholinergic poisoning, which is characterized by nicotinic, muscarinic, and central nervous system manifestations (4-7). The measurement of blood ChE activity is the key diagnostic assessment of poisoning by OP and CA insecticides (6, 7). Blood ChE activity comprises butyrylcholinesterase (E.C. 3.1.1.8) found in the plasma, serum, and liver, and acetylcholinesterase (E.C. 3.1.1.7) in erythrocytes, and the whole blood ChE (WBChE) activity is the net of these two enzyme entities (6-8).

Ruminants are characterized by low plasma ChE activity compared to erythrocytes (9-13).

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Therefore, measuring WBChE activity in ruminants under field conditions would be beneficial, as it would skip the stage of separating blood constituents by centrifugation, allowing for rapid monitoring of the enzyme activity in cases of insecticide poisoning (13-15). Several studies have advocated the measurement of WBChE activity to assess exposure to OP and CA insecticides in humans and animals (14-19). This would be especially beneficial in ruminants, as whole blood contains the majority of acetylcholinesterase in erythrocytes (>90%) compared to butyrylcholinesterase (8, 9, 11, 13, 20). Studies have also identified ruminants, particularly sheep, as sentinel species for monitoring exposure to ChE-inhibiting insecticides, as these animals are raised close to human agricultural settings where pesticides are commonly used (21-23). Measuring WBChE activity would reflect the functional status of ChE activity in the nervous system following exposure to OP and CA insecticides (13-15, 18).

Various ChE assay methods are available to measure the enzyme activity in biological fluids (9, 20-28). One of the simplest and most reliable ChE methods is the electrometric method of Michel (26, 28), which was modified specifically for ruminants to accommodate the inherently low blood ChE activities (9, 13, 24, 25, 29). Recent studies have used the modified electrometric method to determine WBChE activity (19, 20, 23). WBChE activity reference values are required to assess animal exposure to OP and CA insecticides (6, 7, 9, 12, 13, 25), similar to the reference values used for humans (30).

The aim of the present study was to conduct, for the first time, a meta-analysis of the existing literature that used the modified electrometric method to determine baseline (e.g., 9, 12, 13, 20, 24, 25) WBChE activity in ruminants (sheep, goats, cattle, and buffaloes). This sets the basis for comparing WBChE activity in animals exposed to OP or CA insecticides.

Materials and Methods

Study Approval

Study approvals were provided by the Directorate of Postgraduate Studies and Scholarships at the University of Duhok, Kurdistan Region, Iraq (Number 9327, dated September 10, 2024).

Literature Search

Using the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (31), different databases (PubMed, Google Scholar, Science Direct, Directory of Open Access Journals, Iraqi Academic Scientific Journals, and Iraqi Digital Repository) were searched for the use of the modified electrometric method (9, 13, 20, 23-25, 29, 32, 33) in measuring WBChE activity in ruminants until December 30, 2025. A manual search was also conducted to identify published academic articles and theses in the above-mentioned databases. The main keywords used to retrieve articles were: "whole blood cholinesterase activity + electrometric method + ruminants or sheep, goats, cattle, buffaloes, camels", "whole blood cholinesterase activity + modified electrometric method + ruminants, or sheep, goat, cattle, buffalo, camel", "cholinesterase + modified electrometric method + ruminants", "blood cholinesterase + modified electrometric method + ruminants", "modified electrometric method for blood cholinesterase measurement in ruminants", or "normal whole blood cholinesterase activity in ruminants + modified electrometric method" with continuous refinements of the search as needed. The search also included the citations of articles that qualified for inclusion in the meta-analysis. This study did not impose any language restrictions. Both authors searched the data, and any discrepancies were resolved by consensus with the first author (FKM).

Meta-Analysis Duration and Location

The study was initiated on August 1, 2024, and continued until the conclusion of the analysis on December 30, 2025, at the Department of Pharmacology, College of Pharmacy, University of Duhok, Kurdistan Region, Iraq.

Inclusion Criteria

The published studies or academic theses included in this study comprised records of WBChE activity, as measured by the modified electrometric method, in apparently healthy ruminants. The PRISMA advocated identification, screening, and inclusion criteria were employed to finalize the study selection (Figure 1). Data were extracted from each study to include animal species, sex, number of animals, mean, and standard deviation (SD) of normal or baseline WBChE activity of ruminants not exposed to pesticides (including OP and CA) (9, 13, 20, 23, 24, 29, 32, 33). The unit of enzyme activity was Δ pH per incubation time, which usually ranged between 20 and 40 minutes at 37 °C. The typical steps of the modified electrometric method for measuring WBChE activity in ruminants are shown in Table 1.

Table 1. Steps for the Determination of Whole Blood Cholinesterase (WBChE) Activity in Ruminants*

Assay mixture	Animal blood	Blank
Distilled water	3 ml	3.2 ml
Whole blood	0.2 ml	None
Buffer, pH 8.1: 1.237 g sodium barbital, 0.163 g potassium dihydrogen phosphate, and 35.07 g sodium chloride/L distilled water	3 ml	3 ml
Measure pH1 with a pH meter	pH1	pH1
Acetylcholine iodide 7.1%	0.1 ml	0.1 ml
Incubate in a water bath at 37 °C	30 min	30 min
Measure pH2	pH2	pH2
WBChE activity: Δ pH/incubation time=(pH1–pH2)–Δ pH of blank		

*References: 9, 13.

The initial search identified 100 studies; after applying PRISMA screening, the pool was refined to 16 records drawn from five published studies. The mean WBChE activity values (± SD) for sheep, goats, cattle, and buffaloes were extracted from carefully selected studies (13, 20, 23, 32, 33) and included in the meta-analysis in accordance with PRISMA (Figure 1).

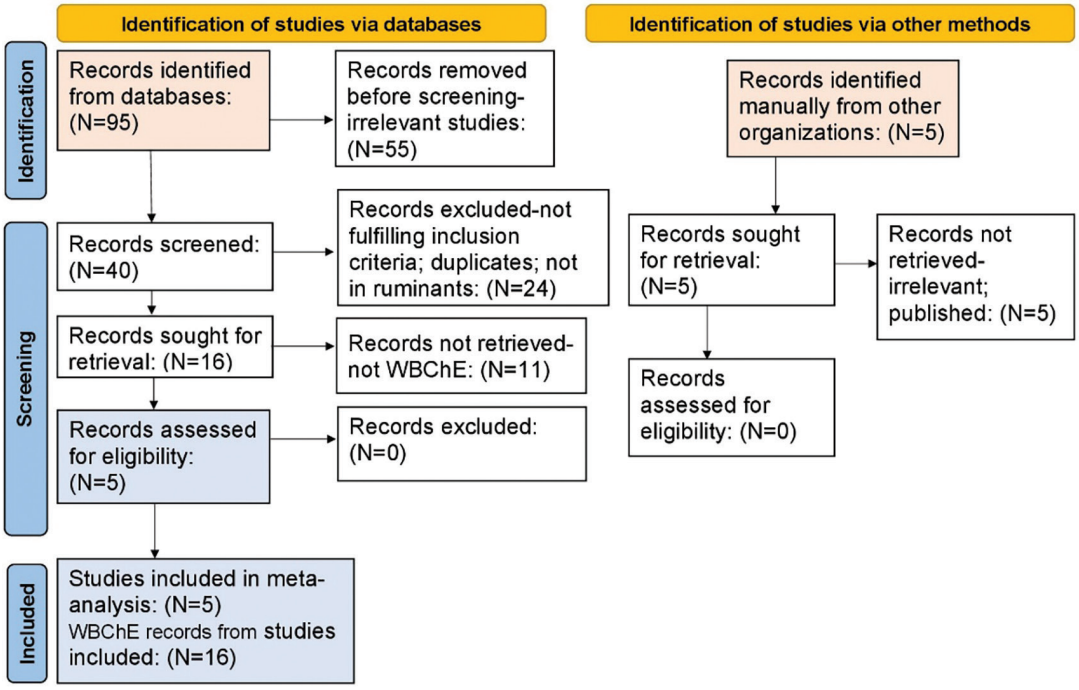

Figure 1. Studies on whole blood cholinesterase (WBChE) activity in ruminants were selected for the meta-analysis following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.

Exclusion Criteria

Studies that did not report normal or baseline WBChE activity in ruminants or those that used other electrometric methods were excluded. Studies that did not report the SD or standard error (SE) of WBChE activity were also excluded. Academic theses or dissertations were excluded because of duplication, as their findings were subsequently published in peer-reviewed journals.

Meta-Analysis and Statistics

This is a single-group randomized-effect-size meta-analysis applied to the mean WBChE activity (±SD) in sheep, goats, cattle, and buffaloes, and was conducted using Meta-Essentials, Version 1.5, from the Erasmus Research Institute of Management (ERIM) in Rotterdam, Netherlands (34). The analyses included a forest plot showing the WBChE effect size, 95% confidence intervals (CI), each study's weight (percentage) in the overall estimate, and the Z-test ($P<0.05$), subgroup analysis, and moderator effect (34-37). Heterogeneity was assessed using Cochran's Q-test ($P<0.10$) and Higgins' I^2, categorized as low (25%), moderate (50%), and high (75%) heterogeneity (35-38). Additionally, a pseudo-R^2 was estimated in the subgroup analysis to quantify the extent to which heterogeneity across studies is explained by subgroup/moderator factors (34, 38). A small R^2 value (e. g., 0.25) explains only a modest portion of the between-study heterogeneity.

Publication Bias

Publication bias was analyzed using a funnel plot, which examines the effect size against the SE, and by Egger's regression test at $P<0.05$ (34, 39, 40). Additionally, Galbraith regression and quantile plots were used to assess data homogeneity and outliers (34, 39, 40).

Risk of Bias

The method of scoring potential bias (risk assessment) in animal studies was applied to the present data by allocating ten questions within six bias items (selection, performance, detection, attrition, reporting, and other problems) (41, 42). Scores of 2, 1, and 0 indicated low, unclear (moderate), and high risk of bias, respectively. Unclear or moderate bias was indicated when a score of one was given to one or more of the sub-questions or when there was "no information" (41, 42). The online Risk of Bias Visualization (robvis) tool was used to assess the scores (43).

Results

Extraction of WBChE Activities of Ruminants from Studies

The literature search identified 100 studies, as shown in the PRISMA flowchart (Figure 1). After applying the inclusion and exclusion criteria, this number was finalized to 16 records of five studies showing WBChE activities as measured by the modified electrometric method in sheep (N=267), goats (N=165), cattle (N=197), and buffaloes (N=31) of both sexes, totaling 660 animals (Table 2). These studies were published between 2007 and 2025.

Meta-Analysis

The forest plots of the single-group random-effects model analysis showed that the WBChE activities of sheep, goats, cattle, and buffaloes (effect sizes) were 0.40, 0.32, 0.51, and 0.42 Δ pH/enzyme reaction incubation time, respectively (Figures 2, 3). Their 95% CI were 0.19-0.60, 0.16-0.49, 0.31-0.70, and 0.39-45, respectively. The weights of the individual records across the four species varied from 0.91% to 14.77% (Figure 2).

Analysis using the Galbraith regression (Z-score against inverse SE) indicated that the WBChE data points were within the lower and upper boundaries of the regression line, with no points below the zero (no effect) line (Figure 4). Furthermore, no outliers were observed in the normal quantile plot (Figure 5).

Table 2. Whole Blood Cholinesterase (WBChE) Activity in Ruminants (Δ pH/ Incubation Time), Measured Using a Modified Electrometric Method

Code	Authors/year	Species	Sex	N	Mean WBChE activity (Δ pH)±SD
A	Mohammad et al., 2007 (13)	Sheep	M	100	0.249±0.105
B		Sheep	F	98	0.257±0.069
C		Goat	M	59	0.234±0.058
D		Goat	F	53	0.252±0.071
E		Cattle	M	103	0.374±0.142
F		Cattle	F	43	0.450±0.075
G	Esmaial et al., 2010 (32)	Sheep	F	10	0,36±0.034
H	Alias, 2018 (33)	Buffalo	F	31	0.42±0.017
I	Ramadhan and Mohammad, 2025 (20)	Sheep	M	33	0.436±0.205
J		Sheep	F	15	0.943±0.260
K		Goat	M	31	0.409±0.202
L		Goat	F	11	0.498±0.282
M		Cattle	M	40	0.693±0.328
N	Ramadhan and Mohammad, 2025 (23)	Sheep	M	11	0.605±0.105
O		Goat	M	11	0.519±0.107
P		Cattle	M	11	0.640±0.099

M=Male; F=Female; WBChE activity: Δ pH/incubation time=(pH1−pH2)−Δ pH of blank.

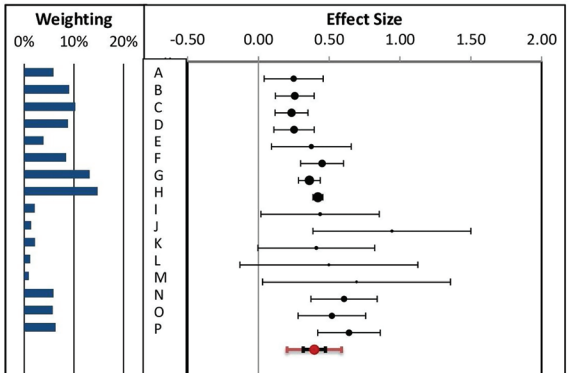

Figure 2. Forest plot of whole blood cholinesterase activity and its weighting in ruminants. The overall effect size was enzyme activity (0.40 Δ pH/incubation time, SE=0.04, 95% CI: 0.32, 0.47; Z value=10.72, P<0.0001).

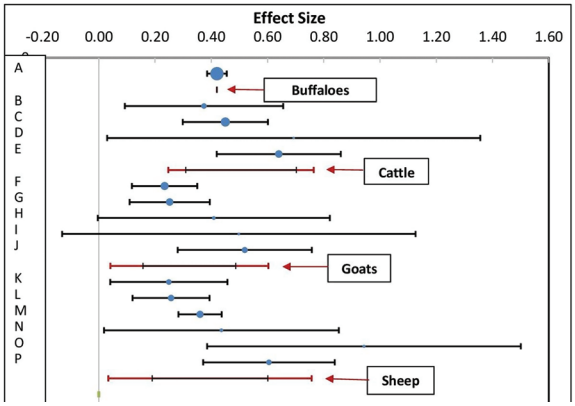

Figure 3. Forest plot of subgroup analysis of whole blood cholinesterase activity (Δ pH/incubation time) in ruminants.

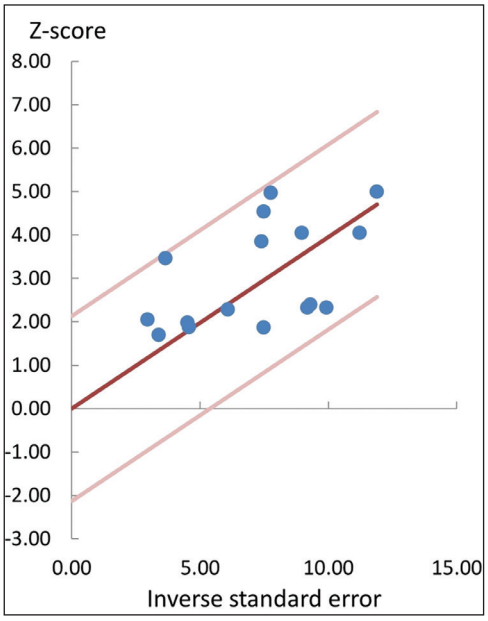

Figure 4. Galbraith regression distribution of whole blood cholinesterase activity in ruminants (Z-score vs. inverse standard error). The zero line represents the no-effect line.

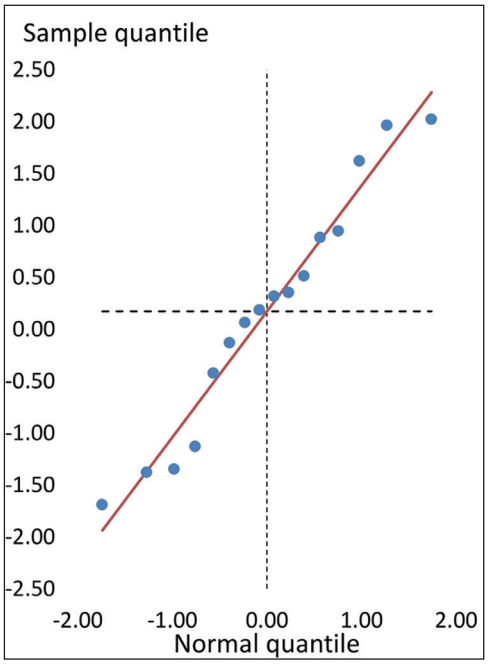

Figure 5. Normal quantile plot of whole blood cholinesterase activity in ruminants.

Data Heterogeneity

The I^2 heterogeneity index of 61% was moderate, with low T^2 (0.01) and T (0.08) values. However, the test of heterogeneity was significant (Q=38.35; P=0.001), indicating heterogeneity in the data, which were therefore subjected to a subgroup analysis.

Subgroup Analysis

Despite the low pseudo R^2 value (24.24%), subgroup analysis showed that WBChE activity (Δ pH) was highest in cattle (0.51), followed by buffaloes (0.42), sheep (0.40), and goats (0.32) (Figure 3). Within subgroups, the heterogeneity values (I^2) in sheep, goats, and cattle were 64.2% (Q=14, P=0.016), 40.2% (Q=6.69, P=0.153), and 16.1% (Q=3.6, P=0.311), respectively. The WBChE activity of buffaloes was not generated by the software because of the single record of WBChE activity in this species. The significant heterogeneity was attributed to the sheep WBChE values, as shown above. The regression analysis of the moderator (sex) on effect size was not significant (F value=0.47, P=0.502, T^2=0.01).

Funnel Plot Examining Publication Bias

The funnel plot (SE versus effect size) showed the possibility of publication bias, since effect size points were not symmetrical within the designated area of the plot, and the five imputed studies were added to the plot to adjust for symmetrical equilibrium (Figure 6). However, Egger's regression analysis was not statistically significant (t=1.91, P=0.077).

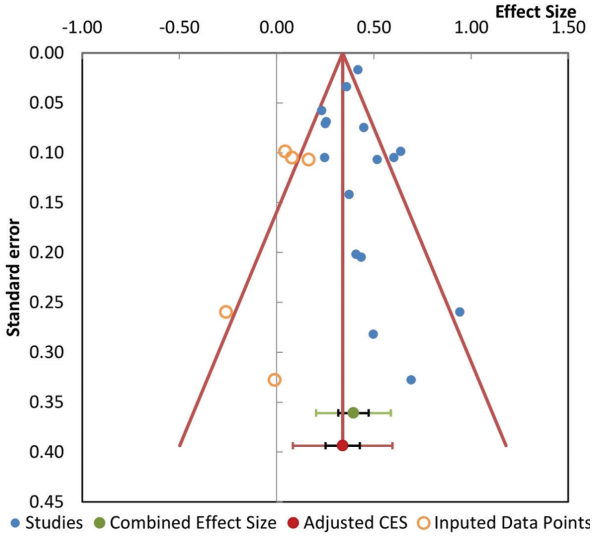

Figure 6. Funnel plot assessing publication bias in studies of whole blood cholinesterase activity in ruminants. Five imputed data points were included to achieve symmetrical distribution.

Risk of Bias

The risk of bias was low in three of the five studies in the present meta-analysis, with total scores of 20/20 (Figure 7). Two studies had a moderate risk of bias, with total scores of 16/20 (allocation of concealed selection and attrition of addressing incomplete data). However, the total score of the studies (18.4 out of 20) indicated a tendency toward a low risk of bias.

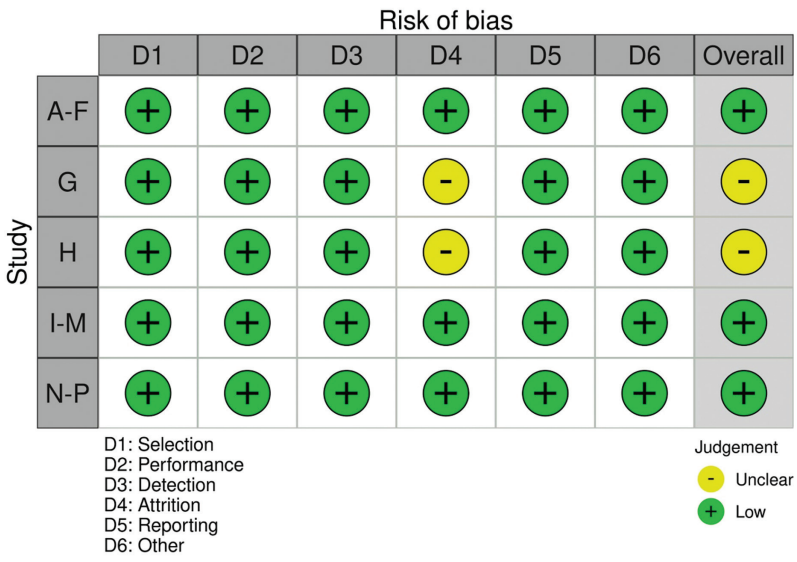

Figure 7. Risk of bias distribution across studies included in the meta-analysis of whole blood cholinesterase activity in ruminants.

Discussion

This study is the first meta-analysis to establish the reference WBChE activity in healthy ruminants, including sheep, goats, cattle, and buffaloes. It is an additional contribution to the existing literature, since the current modified electrometric method is recommended for measuring blood or tissue ChE activity in various animal species (9, 12, 13, 20, 23-25) as well as humans (16, 23, 27, 30) to evaluate exposure to OP and CA pesticides. This method has gained popularity for its simplicity, high throughput, and cost-effectiveness (9, 19, 25, 30). The use of WBChE is preferred in ruminants because of their inherently low plasma ChE activity, which is approximately 10% of the WBChE (8, 9, 11, 13, 20). The use of whole blood for measuring ChE activity is preferred under field conditions and requires minimal laboratory equipment (a pH meter and water bath) (9). Furthermore, WBChE activity suitably mirrors the functional status of ChE activity in the nervous system when assessing exposure to OP and CA pesticides (13-15, 18). Studies have also identified ruminants, particularly sheep, as sentinel species for monitoring exposure to ChE-inhibiting pesticides, as these animals are raised in human agricultural settings where pesticides are commonly used (21-23). Ruminants can be exposed to OP or CA pesticides by grazing on contaminated forage, drinking contaminated water, or inhaling airborne pesticide particles after nearby applications (1, 5, 10, 12, 21, 22).

The ruminant WBChE activities presented in the current meta-analysis could be considered as pilot and initial references, especially in the absence of pre-pesticide exposure values, for normal enzyme activity in sheep, goats, cattle, and buffaloes. Nevertheless, several factors, such as age, animal species, disease conditions, concurrent exposure to other pesticides, and nutritional status, affect blood ChE activity and need to be carefully considered when interpreting pesticide exposure and its adverse effects (7, 26, 28). The pooled WBChE activity values calculated in the meta-analysis differed between ruminant species, with cattle displaying the highest mean effect size (0.51 Δ pH/enzyme reaction time), followed by buffaloes (0.42), sheep (0.40), and goats (0.32). These differences suggest species-specific reference ChE activity levels, that may reflect genetic or physiological variations inherent to each species (8, 9, 12, 13, 20-23). In the present study, the heterogeneity was mainly attributed to the WBChE activity of sheep (I^2=64.2%, Q=14, P=0.016), as the sex effect was not significant using the regression of the moderator on effect size. The ranking of WBChE activity among ruminants is in accordance with previous species-specific reports of WBChE activity in ruminants using electrometric methods (9, 12, 13, 20) or other methods of measuring enzyme activity (8, 10, 11, 21, 22). Understanding these reference values of WBChE activity using the modified electrometric method is crucial for clinical and toxicological assessments of exposure to ChE-inhibiting pesticides, where the enzyme activity deviates from normal states (9, 12, 23-25).

The relatively narrow 95% CI of WBChE activity for each species indicates the precision of the estimates. However, the expected overlap among ruminants (Figures 2, 3) requires cautious differentiation and interpretation of borderline WBChE values.

The low pseudo-R^2 value (24.24%) of WBChE activity across the species was accompanied by a moderate level of heterogeneity (I^2=61%), with no sex effects. The effect size in buffaloes was limited by a single record of WBChE activity, necessitating additional studies in this species using the present modified electrometric method. The subgroup analysis results confirmed the previously reported species variations in blood ChE activities among ruminants. This variation may lead to different interpretations of pesticide exposure, given the expected differences in how ruminant species respond to ChE-inhibiting pesticides (12, 13, 21-25). However, future studies should aim to standardize the modified electrometric method for measuring reference WBChE activity across species and control for animal-related pathophysiological variables. We recommend measuring WBChE activity in both ruminants and humans working in agricultural settings exposed to OP and CA pesticides.

The funnel plot indicated potential publication bias, as asymmetry was observed; however, Egger's regression test was not statistically significant (P=0.077). This borderline result suggests a cautious interpretation of the results; the addition of imputed data points to achieve symmetry hints that unpublished or missing data might impact the overall findings.

Risk of bias assessment revealed that the studies in the present study had a low-risk profile; two studies showed moderate risk, mainly arising from allocation concealment and incomplete data handling issues (Figure 7). However, the high combined quality score (18.4/20) supports the reliability of the studies included in this meta-analysis; however, future research should address these moderate-risk areas to reduce bias further.

Conclusion

This meta-analysis presents reference WBChE activities across four ruminant species using a reliable modified electrometric method, revealing clear species differences while inferring significant inter-study heterogeneity. These findings provide valuable reference points for the clinical and toxicological assessment of OP or CA poisoning in these species. Addressing any variability in WBChE across and within the species will improve the diagnostic utility of WBChE activity measurements in clinical veterinary medicine and toxicology.

What Is Already Known on This Topic:
The measurement of whole blood cholinesterase activity is used to evaluate exposure to organophosphate and carbamate pesticides in humans and animals. Recent studies have employed a modified electrometric method to measure WBChE activity in humans and animals because of its simplicity and reliability. Similar to humans, reference values for WBChE activity are required to assess animal exposure to organophosphate and carbamate insecticides.

What This Study Adds:
This meta-analysis established baseline whole blood cholinesterase activities using a reliable modified electrometric method for ruminant species (sheep, goats, cattle, and buffaloes). It provides a collective reference for whole blood cholinesterase values essential for diagnosing cholinergic toxidrome caused by organophosphates and carbamates in ruminants.

Authors' Contributions: Conception and design: FKM; Data collection: FKM and MAR; Acquisition, analysis and interpretation of data: FKM and MAR; Drafting the article: FKM; Revising it critically for important intellectual content: FKM and MAR; Approved final version of the manuscript: FKM and MAR.

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

References

1. Alcántara I, Suárez G. Assessing trends in ectoparasiticidal drugs used to control ticks and flies in farm animals: A four-year analysis reveal differences between epidemiological zones at country level in Uruguay. Prev Vet Med. 2025;235:106412. doi: 10.1016/j.prevetmed.2024.106412.
2. Ferreira AKACV, E Silva Neto WC, de Castro Oliveira I, Rodrigues MCB, de Oliveira Lima ÂC, Ferreira TV, et al. Efficacy of a formulation of chlorfenvinphos and dichlorvos on Rhipicephalus microplus in vivo test in Bahia,

Brazil. Trop Anim Health Prod. 2025;57(4):207. doi: 10.1007/s11250-025-04444-1.

3. Lemma T, Simma EA, Rufael T, Mekonnen S, Yewhalaw D. In vitro susceptibility study of Amblyommacohaerens and Rhipicepalus (Boophilus) decoloratus tick species to amitraz 12.5 % and diazinon 60 % acaricides in Jimma town, Ethiopia. Vet Parasitol. 2025;336:110461. doi: 10.1016/j.vetpar.2025.110461.
4. Pampalakis G. Classification, Chemical, and toxicological properties of carbamate nerve agents. J Xenobiot. 2024;14(4):1729-56. doi: 10.3390/jox14040092.
5. Saad H, Elfeky SA, El-Gamel NEA, Abo Dena AS. Organophosphate pesticides: A review on classification, synthesis, toxicity, remediation and analysis. RSC Adv. 2025;15(48):40802-22. doi: 10.1039/d5ra05552k.
6. Strelitz J, Engel LS, Keifer MC. Blood acetylcholinesterase and butyrylcholinesterase as biomarkers of cholinesterase depression among pesticide handlers. Occup Environ Med. 2014;71(12):842-7. doi: 10.1136/oemed-2014-102315.
7. Sepahi S, Gerayli S, Delirrad M, TaghavizadehYazdi ME, Zare-Zardini H, Bushehri B, et al. Biochemical responses as early and reliable biomarkers of organophosphate and carbamate pesticides intoxication: A systematic literature review. J Biochem Mol Toxicol. 2023;37(3):e23285. doi: 10.1002/jbt.23285.
8. Tecles F, Cerón JJ. Determination of whole blood cholinesterase in different animal species using specific substrates. Res Vet Sci. 2001;70(3):233-8. doi: 10.1053/rvsc.2001.0465.
9. Mohammad, FK. Review of a practical electrometric method for determination of blood and tissue cholinesterase activities in animals. VetScan. 2007;2(2):1-12. Available from: https://journal.vetscan.co.in/index.php/vs/article/view/21.
10. Pardío VT, Ibarra N, Rodríguez MA, Waliszewski KN. Use of cholinesterase activity in monitoring organophosphate pesticide exposure of cattle produced in tropical areas. J Agric Food Chem. 2001;49(12):6057-62. doi: 10.1021/jf010431g.
11. Abdelsalam EB, Ford EJ. Normal esterase activity in the plasma, whole blood and tissues of cattle. Zentralbl Veterinarmed A. 1985;32(7):518-25. doi: 10.1111/j.1439-0442.1985.tb01972.x.
12. Mohammad FK, Alias AS, Faris GA, Al-Baggou BKh. Application of an electrometric method for measurement of blood cholinesterase activities in sheep, goats and cattle treated with organophosphate insecticides. J Vet Med A Physiol Pathol Clin Med. 2007;54(3):140-3. doi: 10.1111/j.1439-0442.2007.00867.x.
13. Mohammad FK, Al-Zubaidy MH, Alias AS. Electrometric determination of erythrocyte, plasma and whole blood cholinesterase activities in sheep, goats and cattle and their in vitro inhibition by anticholinesterase insecticides. J. Pharmacol. Toxicol. 2007;2:131-141. Available from: https://scialert.net/abstract/?doi=jpt.2007.131.141.
14. Abdelsalam EB. Comparative effect of certain organophosphorus compounds and other chemicals on whole blood, plasma and tissue cholinesterase activity in goats. Vet Hum Toxicol. 1987;29(2):146-8. PMID: 3576947.
15. Haigh JR, Lefkowitz LJ, Capacio BR, Doctor BP, Gordon RK. Advantages of the WRAIR whole blood cholinesterase assay: comparative analysis to the micro-Ellman, Testmate ChE, and Michel (Delta pH) assays. Chem Biol Interact. 2008;175(1-3):417-20. doi: 10.1016/j.cbi.2008.04.032.
16. Mohammad FK, Mohammed AA, Odisho SK. Changes of blood cholinesterase activities among pesticides-exposed agricultural workers in Iraq: A meta-analysis. Toxicol Rep. 2024;13:101830. doi: 10.1016/j.toxrep.2024.101830.
17. Worek F, Mast U, Kiderlen D, Diepold C, Eyer P. Improved determination of acetylcholinesterase activity in human whole blood. Clin Chim Acta. 1999;288(1-2):73-90. doi: 10.1016/s0009-8981(99)00144-8.
18. Worek F, Schilha M, Neumaier K, Aurbek N, Wille T, Thiermann H, et al. On-site analysis of acetylcholinesterase and butyrylcholinesterase activity with the ChE check mobile test kit-determination of reference values and their relevance for diagnosis of exposure to organophosphorus compounds. Toxicol Lett. 2016;249:22-8. doi: 10.1016/j.toxlet.2016.03.007.
19. Kyi NEMM, Samar M, Hongsibsong S, Sawarng N, Pata S, Wongta A. Micro-electrometric method for assessing cholinesterase activity in human whole blood. Methods Protoc. 2025;8(2):30. doi: 10.3390/mps8020030.
20. Ramadhan MA, Mohammad FK. Whole blood cholinesterase activity in humans and ruminants: Comparison using a modified electrometric method. Open Vet J. 2025;15(10):5032-40. doi: 10.5455/OVJ.2025.v15.i10.21.
21. Halbrook RS, Shugart LR, Watson AP, Munro NB, Linnabary RD. Characterizing biological variability in livestock blood cholinesterase activity for biomonitoring organophosphate nerve agent exposure. J Am Vet Med Assoc. 1992;201(5):714-25.
22. Munro NB, Shugart LR, Watson AP, Halbrook RS. Cholinesterase activity in domestic animals as a potential biomonitor for nerve agent and other organophosphate exposure. J Am Vet Med Assoc. 1991;199(1):103-15.
23. Ramadhan MA, Mohammad FK. In vitro reduction of cholinesterase activity in whole blood by dichlorvos in humans and ruminants using a modified electrometric method: The potential of using sheep as a sentinel species. Health Biotechnol Biopharma. 2025: e232644. doi: 10.22034/hbb.2025.540140.1342. (in press).
24. Mohammad FK, St Omer VE. Modifications of Michel's electrometric method for rapid measurement of blood cholinesterase activity in animals: a minireview. Vet Hum Toxicol. 1982;24(2):119-21. PMID: 7187136.

25. St. Omer VEV, Rottinghaus GE. Biochemical determination of cholinesterase activity in biological fluids and tissues. In: Ballantyne B, Marrs TC, eds. Clinical and Experimental Toxicology of Organophosphates and Carbamates. Oxford, UK: Butterworth-Heinemann Ltd; 1992, pp. 15-27.
26. Fairbrother A, Marden BT, Bennett JK, Hooper MJ. Methods used in determination of cholinesterase activity. In: Minneau P, ed. Cholinesterase-Inhibiting Insecticides: Their Impact on Wildlife and the Environment, Vol. 2. (Chemicals in Agriculture). Amsterdam, The Netherlands: The Elsevier Science Publishers B.V.; 1991. pp. 35-72.
27. Odisho SK, Mohammad FK. Blood cholinesterase activities and oxidative stress status among farmworkers using pesticides in Duhok, KRG, Iraq. J Ideas Hlth. 2022;5(4):786-93. doi: https://doi.org/10.47108/jidhealth.Vol5.Iss4.264.
28. Wills JH, Dubois KP. The measurement and significance of changes in the cholinesterase activities of erythrocytes and plasma in man and animals. CRC Crit Rev Toxicol. 1972;1(2):153-202. doi: https://doi.org/10.3109/10408447209104304.
29. Mohammad FK, Faris GA, Al-Kassim NA. A modified electrometric method for measurement of erythrocyte acetylcholinesterase activity in sheep. Vet Hum Toxicol. 1997;39(6):337-9. PMID: 9397501.
30. Garmavy HMS, Mohammed AA, Rashid HM, Mohammad FK. A meta-analysis of normal human blood cholinesterase activities determined by a modified electrometric method. J Med Life. 2023;16(1):22-34. doi: 10.25122/jml-2022-0215.
31. Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ. 2021;372:n71. doi: 10.1136/bmj.n71.
32. Esmaial YA, Ali MH, Megdad MH, Alias AS. Study of cholinesterase activity in adult ewes treated by the anthelmintic drug levamisole. Rafid J Sci. 2010;21(5):93-102. doi: https://doi.org/10.33899/rjs.2010.37028.
33. Alias AS. The use of the electrometric method in measuring the activity of cholinesterase in local female buffalo in north of Iraq. Kufa J Vet Med Sci. 2018;9(2):13-22. Available from: https://journal.uokufa.edu.iq/index.php/kjvs/article/view/4169.
34. Suurmond R, van Rhee H, Hak T. Introduction, comparison, and validation of Meta-Essentials: A free and simple tool for meta-analysis. Res Synth Methods. 2017;8(4):537-53. doi: 10.1002/jrsm.1260.
35. Buccheri S, Sodeck GH, Capodanno D. Statistical primer: Methodology and reporting of meta-analyses. Eur J Cardiothorac Surg. 2018;53(4):708-13. doi: 10.1093/ejcts/ezy004.
36. Nakagawa S, Noble DW, Senior AM, Lagisz M. Meta-evaluation of meta-analysis: Ten appraisal questions for biologists. BMC Biol. 2017;15(1):18. doi: 10.1186/s12915-017-0357-7.
37. Clephas PRD, Heesen M. Interpretation of meta-analyses. Interv Pain Med. 2022;1(Suppl 2):100120. doi: 10.1016/j.inpm.2022.100120.
38. Spineli LM, Pandis N. Statistical heterogeneity: Notion and estimation in meta-analysis. Am J Orthod Dentofacial Orthop. 2020;157(6):856-9.e2. doi: 10.1016/j.ajodo.2020.03.009.
39. Lin L, Chu H. Quantifying publication bias in meta-analysis. Biometrics. 2018;74(3):785-94. doi: 10.1111/biom.12817.
40. Ballarini NM, Chiu YD, König F, Posch M, Jaki T. A critical review of graphics for subgroup analyses in clinical trials. Pharm Stat. 2020;19(5):541-60. doi: 10.1002/pst.2012.
41. Hooijmans CR, IntHout J, Ritskes-Hoitinga M, Rovers MM. Meta-analyses of animal studies: An introduction of a valuable instrument to further improve healthcare. ILAR J. 2014;55(3):418-26. doi: 10.1093/ilar/ilu042.
42. Mohammad FK, Eassa SH, Abdullah RG. A meta-analysis of changes in cholinesterase activity among experimental animals infected with Toxoplasma gondii. Bulg J Vet Med. 2025;28(2):195-209. doi: 10.15547/bjvm.2024-0024.
43. McGuinness LA, Higgins JPT. Risk-of-bias visualization (robvis): An R package and Shiny web app for visualizing risk-of-bias assessments. Res Synth Methods. 2021;12(1):55-61. doi: 10.1002/jrsm.1411.

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