Poznan University of Medical Sciences
Poland

JMS Journal of Medical Science

previously Nowiny Lekarskie

Founded in 1889

2025 September
Vol. 94, No. 3

QUARTERLY

Journal Impact Factor 2024:
IF = 1.6

Indexed in:
Web of Science, DOAJ, Crossref,
Google Scholar, Polish Medical Bibliography,
Ministry of Education and Science

www.jms.ump.edu.pl

eISSN 2353-9801
ISSN 2353-9798
doi: 10.20883/ISSN.2353-9798

Publishing Manager: Grażyna Dromirecka

WYDAWNICTWO NAUKOWE
UNIWERSYTETU MEDYCZNEGO
IM. KAROLA MARCINKOWSKIEGO
W POZNANIU

60-812 Poznań, ul. Bukowska 70
tel./fax: +48 618547151
www.wydawnictwo.ump.edu.pl

Ark. wyd. 14,4. Ark. druk. 14,3
Zam. nr 135/25.

SECRETARIAT ADDRESS
27/33 Szpitalna Street, 60-572 Poznań, Poland
phone: +48 618491432, fax: +48 618472685
e-mail: jms@ump.edu.pl
www.jms.ump.edu.pl

DISTRIBUTION AND SUBSCRIPTIONS
70 Bukowska Street, 60-812 Poznań, Poland
phone/fax: +48 618547414
e-mail: sprzedazwydawnictw@ump.edu.pl

PUBLISHER
Poznan University of Medical Sciences
10 Fredry Street, 61-701 Poznań, Poland
phone: +48 618546000, fax: +48 618520455
www.ump.edu.pl

eISSN 2353-9801
ISSN 2353-9798
doi: 10.20883/ISSN.2353-9798

CONTENTS

ORIGINAL PAPERS

REVIEW PAPERS

Ethical guidelines

The Journal of Medical Science applies the ethical principles and procedures recommended by COPE (Committee on Conduct Ethics), contained in the Code of Conduct and Best Practice Guidelines for Journal Editors, Peer Reviewers and Authors available on the COPE website: https://publicationethics.org/resources/guidelines

ORIGINAL PAPER

JMS Journal of Medical Science

In Silico and *In Vitro* Studies of the Antimetastatic and Antiangiogenic Activities of *Cordyceps militaris* Extracts on Breast Cancer Cells (MDA-MB-231)

Alyssa Pei Qi Chang
School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, Subang Jaya, Selangor Darul Ehsan, Malaysia
https://orcid.org/0009-0005-0131-2633

Sabrina Xin Yi Khor
School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, Subang Jaya, Selangor Darul Ehsan, Malaysia
https://orcid.org/0000-0002-4053-5603

Adeline Yoke Yin Chia
School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, Subang Jaya, Selangor Darul Ehsan, Malaysia
Digital Health and Medical Advancement Impact Lab, Taylor's University, Subang Jaya, Selangor Darul Ehsan, Malaysia
https://orcid.org/0000-0002-1856-7211

Sunita Chamyuang
School of Science, Mae Fah Luang University, Chaing Rai, Thailand; Microbial Products and Innovation Research Group, Mae Fah Luang University, Chaing Rai, Thailand
https://orcid.org/0000-0002-3063-742X

Yin-Quan Tang
School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, Subang Jaya, Selangor Darul Ehsan, Malaysia
Digital Health and Medical Advancement Impact Lab, Taylor's University, Subang Jaya, Selangor Darul Ehsan, Malaysia
https://orcid.org/0000-0001-7327-2830

Corresponding author: yinquan.tang@taylors.edu.my

https://doi.org/10.20883/medical.e1255

Keywords: metastasis, angiogenesis, *Cordyceps militaris*, breast cancer

Received 2025-03-18
Accepted 2025-09-29
Published 2025-09-30

How to Cite: xxx. *In Silico* and *In Vitro* Studies of the Antimetastatic and Antiangiogenic Activities of *Cordyceps militaris* Extracts on Breast Cancer Cells (MDA-MB-231). Journal of Medical Science. 2025 September;94(3);e1255. https://doi.org/:10.20883/medical.e1255

ABSTRACT

Introduction. *Cordyceps militaris* (CM), a traditional medicinal fungus in East Asia, has garnered increasing attention due to its potential anticancer properties. Despite extensive use in traditional medicine, the mechanisms underlying its antimetastatic and antiangiogenic effects in breast cancer remain unclear. This study aimed to explore the bioactive components of aqueous extracts derived from CM's mycelium (Aq-CMM) and fruiting body (Aq-CMF), focusing on their potential inhibitory activities on metastasis and angiogenesis in triple-negative breast cancer cells (MDA-MB-231).

Materials and methods. *In silico* molecular docking was conducted to screen for key CM bioactive compounds and evaluate their binding affinities toward metastasis- and angiogenesis-related targets, NF-κB and VEGFR. *In vitro* cytotoxicity was assessed using 2D monolayer and 3D spheroid MDA-MB-231 cultures

treated with Aq-CMM and Aq-CMF. Cell viability (IC_{50}) was determined at 48 hours, and microscopic evaluation of treated spheroids was performed to assess morphological disruption.
Results and conclusions. Docking analyses identified ergothioneine as a primary CM-derived compound with strong binding affinity to NF-κB (–7.83 kcal/mol) and VEGFR (–7.62 kcal/mol), suggesting potent inhibitory effects on metastatic and angiogenic pathways. In vitro assays showed that Aq-CMF exerted greater growth-inhibitory effects (IC_{50} = 54 μg/mL in 2D; 46 μg/mL in 3D) than Aq-CMM at 48 hours. Microscopic observations confirmed notable disruption of spheroid architecture following treatment. These findings highlight the therapeutic potential of ergothioneine-rich CM extracts, particularly from the fruiting body, as promising anticancer agents that warrant further mechanistic and translational studies.

Introduction

Regardless of ongoing efforts to discover novel treatment methods, breast cancer remains one of the most devastating diseases in the world. According to the WHO, 2.3 million women were diagnosed with breast cancer, and 685,000 women died worldwide in 2020. It is predicted that the prevalence of cancer patients will rise to 19 million or more by 2025. Hence, breast cancer is the most common cancer in the world and continues to be the leading cause of cancer-related deaths, presenting a high mortality rate attributed to its high metastatic and angiogenic properties. Current therapeutic compounds and cancer treatments that are available for public use exhibit unwanted effects [1], highlighting the demand for new natural products as treatment options for breast cancer.

Cordyceps militaris (CM), a traditional medicinal fungus widely used in traditional East Asian medicine, has garnered significant scientific interest due to its various health benefits, particularly its potential anticancer properties [2,3]. Several bioactive constituents of CM, including ergothioneine, cordycepin, and adenosine, have demonstrated promising antimetastatic and antiangiogenic properties [1,4–9]. Ergothioneine, in particular, has shown antioxidative and cancer-regulatory effects, including inhibition of tumour growth and modulation of signalling pathways [1]. However, the detailed mechanisms by which compounds act on breast cancer cells remain inadequately understood [10], necessitating further investigation of their molecular interactions and therapeutic potential.

In this study, we compare the anticancer effects of *Cordyceps militaris* fruiting body (CMF) and mycelium (CMM) extracts on breast cancer cells. This comparison is scientifically relevant, as CMF and CMM may differ in the concentration and composition of bioactive compounds due to distinct metabolic processes during their growth stages. Understanding these differences is crucial for identifying the more therapeutically potent component and optimising the use of CM in cancer treatment applications.

Furthermore, to clarify the molecular rationale, we focused on NF-κB and VEGFR as potential targets for analysis. NF-κB is a master regulator of genes involved in inflammation, proliferation, and metastasis, whereas VEGFR is central to angiogenesis and tumour vascularisation. Given that metastasis and angiogenesis are key hallmarks of breast cancer progression, both NF-κB and VEGFR provide suitable targets for in silico docking and in vitro validation of CM-derived compounds.

This study aims to comprehensively evaluate the antimetastatic and antiangiogenic effects of CM extracts, particularly those enriched in ergothioneine, on breast cancer cells. Employing integrated approaches of computational molecular docking studies and advanced *in vitro* models, including 2D monolayer and 3D spheroid cultures, the research seeks to elucidate potential molecular targets and assess the efficacy of CM extracts as promising anticancer agents.

Materials and methods

Preparation of targeted macromolecules and ligands

The ZINC database (https://zinc.docking.org) provides a list of available compounds that can

be used for virtual screening to identify molecular targets. The following ligands were selected for molecular docking based on their reported biological relevance:

- Cordycepin (ZINC1319796) and Adenosine (ZINC2169830): bioactive constituents of CM with reported anticancer activities [11,12].
- Ergothioneine (ZINC1530224): an antioxidant naturally found in CM with potential cancer-protective properties [13].
- Aspirin (ZINC ID: ZINC 53) is used as a positive control for NF-κB docking, as it inhibits IKKβ-mediated NF-κB activation [14].
- Tivozanib (ZINC ID: ZINC1489430): a selective VEGFR tyrosine kinase inhibitor, included as a positive control for VEGFR docking [15].
- Resveratrol (ZINC ID: ZINC6787): a natural polyphenol reported to inhibit angiogenesis via VEGFR signalling [16].
- Lovastatin (ZINC ID: ZINC3812841): a statin with documented NF-κB modulatory and anti-proliferative effects in breast cancer [17].
- GABA (ZINC1532620): Although primarily a neurotransmitter, recent evidence links GABAergic signalling to tumour suppression, inhibition of cancer cell migration, and modulation of angiogenic pathways [18].
- Cisplatin (Tokyo Chemicals, Japan) was used as a positive control in *in vitro* assays, while untreated cells served as the negative control. For *in silico* docking, baseline docking scores without ligands were considered as negative reference values.

Molecular docking simulation

The protein structures of NF-κB (PDB ID: 1SVC) and VEGFR (PDB ID: 4ASE) proteins were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org). All the molecules were prepared, trimmed, and optimised using BIOVIA Discovery Studio Visualizer 4.5. The localisation of bonds was performed, followed by the addition of polar hydrogen atoms, while also excluding water molecules and ligands from the 3D protein structure [19]. The structures of the molecular interactions between the target proteins and the natural compounds were then predicted and better represented using UCSF Chimera (https://www.cgl.ucsf.edu/chimera). The molecular docking result includes a Jmol applet for web browser visualisation of the expected binding modes [20]. The binding affinity between the target protein and the molecular compound was displayed. UCSF Chimaera was launched for additional visual investigations to view the predicted binding modes [20]. In UCSF Chimera, the CSV file from SwissDock was accessed. High-quality images of the docking results of the molecular structures were best simulated and modelled. Finally, both 2D and 3D interactions and bonds at the binding active site were shown in Discovery Studio Visualiser.

Preparation of Cordyceps militaris (CM) extracts and the standard drug.

Lyophilised form of fresh mycelia and fruiting bodies of CM were provided by Mae Fah Luang University (Chiang Rai, Thailand). The master stock was prepared by diluting fresh mycelia (M) and fruiting bodies (F) of CM with distilled water to concentrations of 4 mg/mL and 6 mg/mL, respectively, to obtain master stocks of aqueous (Aq)-CMM and Aq-CMF. The positive control, cisplatin (Tokyo Chemicals, Japan), was prepared at a concentration of 2.53 mg/mL. The master stocks were stored at -20 °C before use.

2D cytotoxicity screening

Briefly, MDA-MD-231 cells were seeded at a concentration of 5000 cells/well into a 96-well plate and incubated overnight for attachment. After 24 hours, cells were treated with different concentrations of Aq-CMM (0–1000 µg/mL), Aq-CMF (0–1000 µg/mL), and cisplatin (0–200 µg/mL), and further incubated for 24, 48, or 72 hours at 37 ºC. At the end of incubation time, 20 µL of MTT solution (5 mg/ml) (Nacalai Tesque Inc, Japan) was added and incubated at 37ºC for 4 hours. Cell viability was estimated by measuring the absorbance at 570 nm using the EPOCH 2 microplate reader (Bio-Tek Instruments, USA). This wavelength corresponds to the detection of formazan crystals produced by the reduction of MTT by mitochondrial succinate dehydrogenase in viable cells, reflecting overall metabolic activity [21]. The absorbance value that was determined for untreated cells was based on 100% viable cells. Each measurement was performed in triplicate.

Generation and treatment of multicellular tumour spheroidal (MCTS) cultures

Briefly, MDA-MB-231 cells were seeded at a concentration of 5,000 cells/well into a 96-well

ultra-low round-bottom plate and incubated for 3 days to allow the formation of compact and homogeneous spheroids. For treatment, the 3-day-old MCTS were treated with different concentrations of Aq-CMM (0–1000 µg/mL), Aq-CMF (0–1000 µg/mL), and cisplatin (0–200 µg/mL), and then further incubated for 24, 48, or 72 hours at 37 ºC. At the end of the incubation time, 20 µL of MTT solution (5 mg/mL) (Nacalai Tesque Inc., Japan) was added and incubated at 37 °C for 4 hours. Cell viability was estimated by measuring absorbance at 570 nm using the EPOCH 2 microplate reader (Bio-Tek Instruments, USA). The absorbance value that was determined for untreated cells was based on 100% viable cells. Each measurement was performed in triplicate.

Light microscopic assessment

The morphological appearances of the MCTS cultures and their structural changes after treatment with Aq-CMM, Aq-CMF, and cisplatin were observed using a Nikon Diaphot-TMD (Nikon, Japan) inverted light microscope equipped with a Phase contrast-2 ELWD 0.3 phase-contrast condenser. The images were captured using the Digital Sight DS-L2 camera (Nikon, Japan).

Statistical Analysis

All experiments were performed in triplicate. The results were analysed using IBM SPSS Statistics 25.0 for Macintosh (SPSS Inc., USA). All results were expressed as mean ± standard deviation (S.D.). Each value is the mean of at least three separate experiments with triplicate. The comparison of cell viability between the untreated and treated groups was performed using one-way ANOVA. The results were considered statistically significant when $P < 0.05$.

Results

In Silico antimetastatic effects of *CM* compounds targeting NF-κB

Aspirin was selected as the positive control for the *in silico* study, targeting NF-κB. According to Li *et al*. [22], aspirin inhibits the proliferation and stimulates the apoptosis of cancer cells. Therefore, the study found that aspirin may be a promising candidate for combination therapy in breast cancer [22]. Based on **Table 1**, ergothioneine (-7.83 kcal/mol) displayed the highest binding affinity scores for the target protein NF-κB compared to aspirin (-7.24 kcal/mol). This strongly suggests that the ergothioneine compound of CM can target the NF-κB pathway more successfully than a well-known synthetic drug. A more negative score denotes a stronger binding affinity, suggesting a greater likelihood of stable interaction [23]. In general, binding affinity values more negative than –6.0 kcal/mol are considered indicative of strong binding affinity in molecular docking studies [24]. This threshold has been widely referenced in computational drug discovery literature to differentiate between weak, moderate, and potent ligand–receptor interactions.

The molecular docking results presented in **Figure 1** offer valuable insights into the potential inhibitory interactions of ergothioneine and GABA with the NF-κB protein. In the 3D and 2D interaction visualisations, both ligands demonstrated favourable binding within the active site of NF-κB, suggesting possible interference with its functional activity. Specifically, ergothioneine exhibited strong binding affinity through multiple hydrogen bonds with critical residues, including Ser281, Glu285, and Tyr227. These residues are located within the DNA-binding domain of NF-κB,

Table 1. Binding affinity prediction of each ligand toward the target protein, NF-κB (PDB ID: 1SVC).

No	Ligand	Target Protein	Binding Affinity
1	Ergothioneine	NF-κB	-7.83 kcal/mol
2	Aspirin (positive control)	NF-κB	-7.24 kcal/mol
3	γ-aminobutyric Acid (GABA)	NF-κB	-7.01 kcal/mol
4	Lovastatin	NF-κB	-6.91 kcal/mol
5	Adenosine	NF-κB	-6.75 kcal/mol
6	Cordycepin	NF-κB	-6.41 kcal/mol

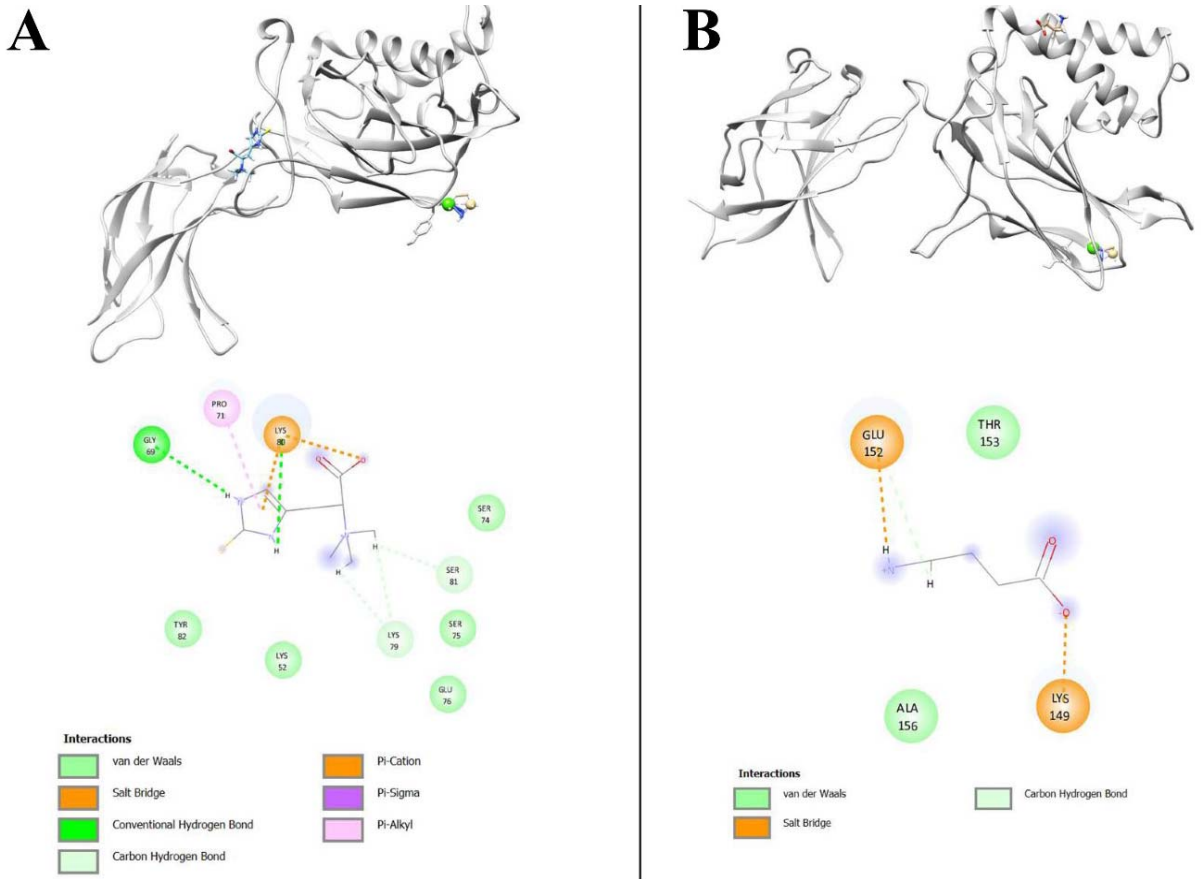


Figure 1. 3D interaction (top) and 2D interaction (bottom) visualisation of ligand-receptor complex interaction. (A) Interaction prediction of ergothioneine with the NF-κB protein. (B) Interaction prediction of GABA with the NF-κB protein.

which plays a central role in its transcriptional regulatory functions. Additionally, ergothioneine established hydrophobic contacts with Phe239 and Ile235, further potentially stabilising the ligand within the binding pocket. These interactions indicate that ergothioneine may potentially inhibit NF-κB activity by sterically hindering its ability to bind to DNA or disrupting conformational integrity required for transcriptional activation.

In contrast, GABA formed hydrogen bonds with Lys221 and Asp245, and a π–π interaction with Tyr227. Although the number of interactions was fewer compared to ergothioneine, their strategic placement near the DNA-binding interface suggests that GABA may also exert modulatory effects on NF-κB signalling, albeit with potentially lower binding stability. The π–π stacking with Tyr227 is particularly notable, as this residue is frequently implicated in ligand-mediated modulation of NF-κB function.

Taken together, these docking observations support the hypothesis that both compounds could act as NF-κB inhibitors through direct binding, which may contribute to anti-inflammatory or anti-angiogenic effects. These findings are consistent with previous reports highlighting NF-κB as a therapeutic target in cancer and chronic inflammatory conditions.

In Silico antiangiogenics effects of *CM* compounds targeting VEGFR

Two well-known VEGFR inhibitors, tivozanib (a synthetic drug) and resveratrol (a natural polyphenolic compound), are used as positive controls in this study. Based on **Table 2**, tivozanib displayed the most decisive inhibitory action on VEGFR (-11.24 kcal/mol), followed by resveratrol and ergothioneine. It is worth noting that ergothioneine is present in higher quantities in both the mycelium and fruiting bodies of CM compared to GABA, lovastatin, and cordycepin [25,26].

Figure 2 illustrates the molecular docking results of ergothioneine and adenosine with the VEGFR, a critical regulator of angiogenesis in

Table 2. Binding affinity prediction of each ligand toward the target protein, VEGFR (PDB ID: 4ASE).

No	Ligand	Target Protein	Binding Affinity
1	Tivozanib (synthetic drug/ positive control)	VEGFR	-11.24 kcal/mol
2	Resveratrol (natural polyphenol compound/ positive control)	VEGFR	-7.84 kcal/mol
3	Ergothioneine	VEGFR	-7.62 kcal/mol
4	Adenosine	VEGFR	-7.33 kcal/mol
5	Cordycepin	VEGFR	-7.29 kcal/mol
6	Lovastatin	VEGFR	-7.15 kcal/mol
7	γ-aminobutyric Acid (GABA)	VEGFR	-7.11 kcal/mol

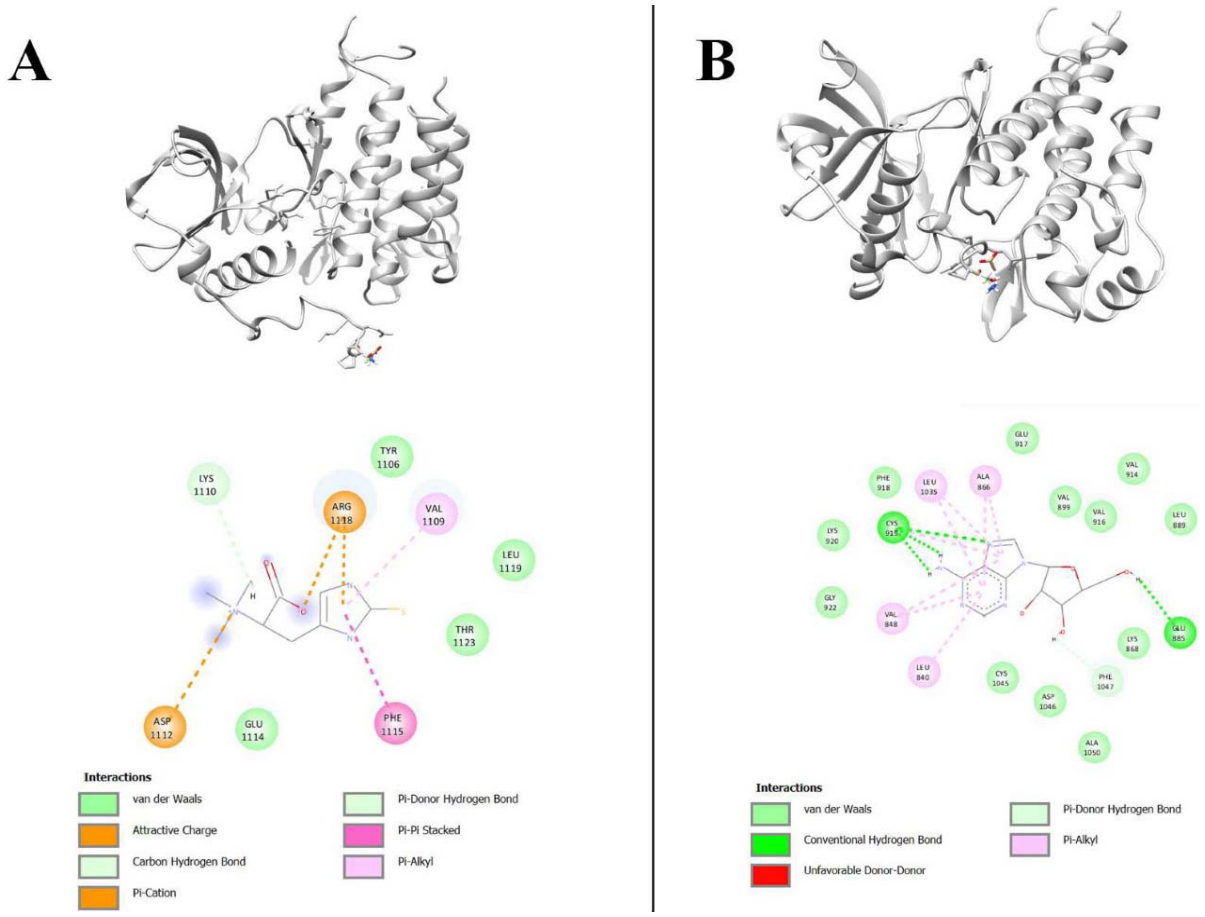


Figure 2. 3D interaction (top) and 2D interaction (bottom) visualisation of ligand-receptor complex interaction. (A) Interaction prediction of ergothioneine with the VEGFR protein. (B) Interaction prediction of adenosine with the VEGFR protein.

cancer and other pathological conditions. The 3D and 2D visualisations provide detailed interaction profiles that suggest both ligands possess the ability to occupy the VEGFR binding pocket and potentially interfere with its activation.

Ergothioneine, as shown in **Figure 2A**, displayed a stable docking pose within the ATP-binding site of VEGFR. It formed hydrogen bonds with key residues, including Glu885 and Cys919, which are known to be crucial for kinase activity [27–29]. Additional interactions were observed with Lys868 and Asp1046 [29], suggesting a potential to inhibit autophosphorylation and downstream VEGF signalling. The presence of both polar and non-polar interactions indicates a well-balanced binding profile, supporting the compound's potential to function as a VEGFR inhibitor.

In **Figure 2B**, adenosine also demonstrated favourable binding within the VEGFR active site. It formed hydrogen bonds with residues Asp1046, Glu885, and Thr916, which are highly conserved in the kinase domain and essential for catalytic

function [27–29]. The π-cation interaction with Lys868 further enhanced the binding stability. Although adenosine is an endogenous purine nucleoside, its docking pose overlaps with that of known VEGFR inhibitors, suggesting that structural analogues of adenosine might be developed into targeted anti-angiogenic agents.

Collectively, these results suggest that both ergothioneine and adenosine can interact with critical residues within the VEGFR active site, potentially modulating receptor function. This supports the hypothesis that these ligands may exert anti-angiogenic effects by inhibiting VEGFR signalling, making them promising candidates for further validation in cancer or vascular disease models.

Cytotoxic screening

Figure 3 illustrates the time-dependent effects of Aq-CMM and Aq-CMF treatments on cell viability in both 2D monolayer and 3D spheroidal culture systems at 24, 48, and 72 hours. In the 2D monolayer culture, both Aq-CMM and Aq-CMF exhibited a progressive cytotoxic effect over time. After 24 hours, a mild reduction in viability was observed for both extracts, with no statistically significant differences compared to the control. However, by 48 hours, Aq-CMF showed a more pronounced inhibitory effect. By 72 hours, both Aq-CMM and Aq-CMF significantly reduced cell viability compared to the untreated control ($p < 0.05$), with Aq-CMF displaying a slightly more potent effect.

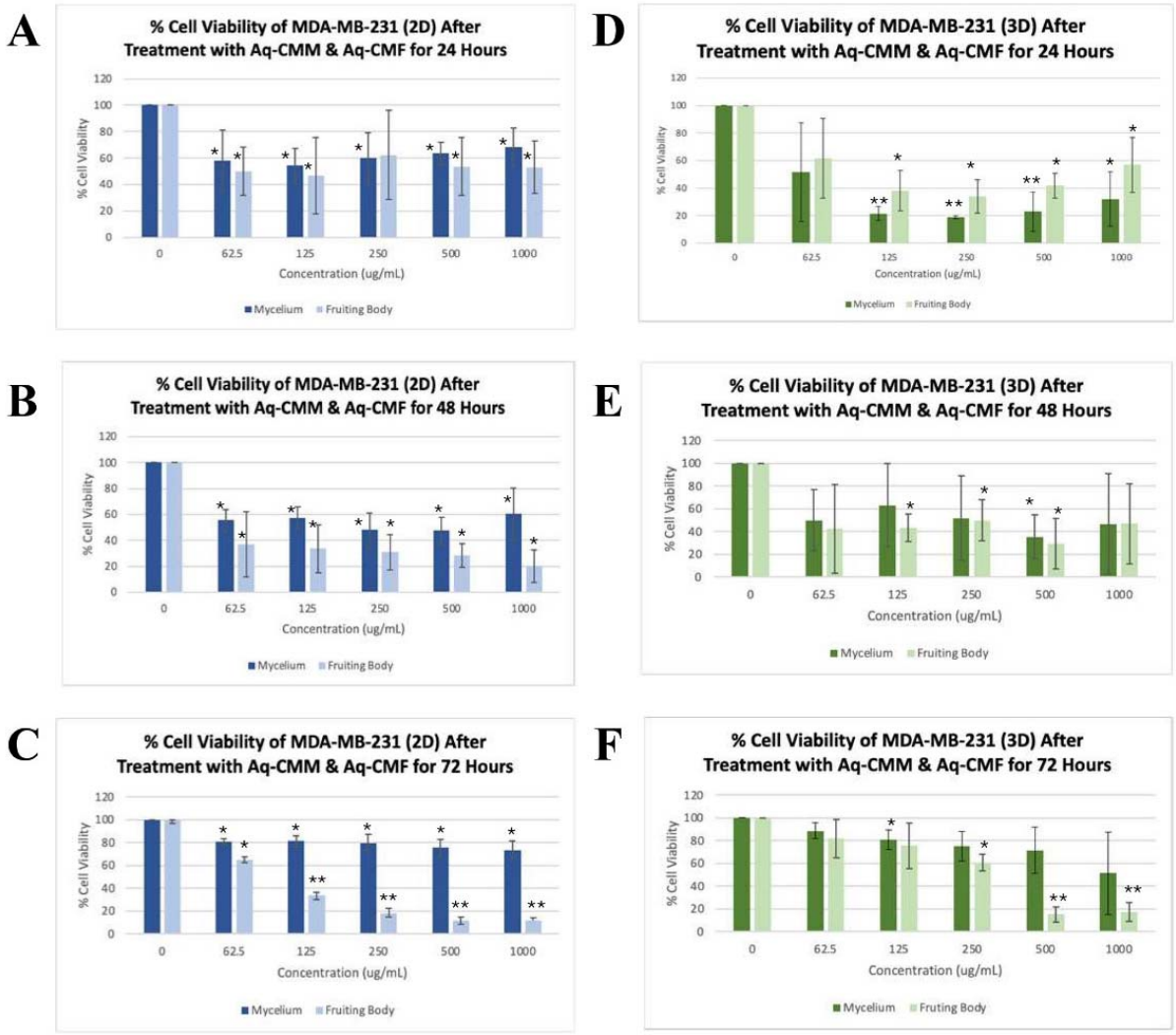


Figure 3. Percentage of viable MDA-MB-231 breast cancer cells after administration with Aq-CMM and Aq-CMF in 2D monolayer culture and 3D spheroidal culture. 2D cell culture treatment for (A) 24 hours, (B) 48 hours, and (C) 72 hours. 3D cell culture treatment for (D) 24 hours, (E) 48 hours, and (F) 72 hours. Data are represented as mean ± S.D., n = 3. Statistical significance was assumed for P-values < 0.05 (P < 0.05, **P < 0.01) compared to the untreated control.

In the 3D spheroidal culture, the response to treatment was more delayed. Minimal changes in viability were noted at 24 hours for both extracts, indicating limited early penetration or activity in the dense spheroid structure. At 48 hours, moderate reductions were observed, with Aq-CMF showing a slightly greater effect than Aq-CMM. By 72 hours, both treatments resulted in a significant decrease in spheroid viability compared to the untreated control ($p < 0.05$). However, the extent of inhibition was slightly lower than that observed in the 2D model, likely due to the enhanced cellular resistance characteristic of 3D cultures.

These findings suggest that while both Aq-CMM and Aq-CMF exert cytotoxic effects in a time-dependent manner, Aq-CMF consistently demonstrated a more potent inhibitory effect in both models. The results also underscore the importance of considering 3D culture systems for better approximation of *in vivo* tumour behaviour, as the delayed response observed highlights the barriers to drug diffusion and resistance mechanisms present in three-dimensional environments.

Additionally, Aq-CMF was able to present a lower number of viable cells after treatment during cell cytotoxicity testing compared to Aq-CMM. It is worth emphasising that the 3D spheroid cells displayed higher levels of viable cells after treatment compared to the 2D monolayer cells (**Table 3**). With treatment of Aq-CMF (250 µg/mL), the percentage of cell viability of MDA-MB-231 decreased to 37.17 ± 26.91 % compared to the untreated control ($p < 0.05$) in the 2D cell culture. In contrast, in the 3D culture, the percentage of cell viability was 48.23 ± 16.33% by administration of the same treatment.

At a concentration of 1000 µg/mL, Aq-CMM exhibits a modest effect, with approximately 64.99 ± 13.91 % of viable cells remaining (**Table 3**). In contrast, Aq-CMF demonstrates a remarkably intensified impact, eliciting a response more than double that of the control. Following treatment with Aq-CMF at the same concentration, the percentage of cell viability of MDA-MB-231 cells was substantially reduced to 32.10 ± 23.34 %. These findings emphasise the potent cytotoxic potential of Aq-CMF and its significant capacity to prevent the survival of MDA-MB-231 cells. Treated cells

Table 3. Comparison of % ± SD cell viability at different concentrations between treatments of CMM on MDA-MB-231 in 2D and 3D culture systems, at 72 hours.

Conc. (µg/mL)	Cell Viability (% ± S.D.)			
	Aq-CMM (2D)	Aq-CMM (3D)	Aq-CMF (2D)	Aq-CMF (3D)
0	100 ± 0[b]	100 ± 0[b]	100 ± 0[b]	100 ± 0[b]
62.5	59.30 ± 23.70[a]	63.40 ± 29.53[a]	50.57 ± 19.72[a]	62.04 ± 30.82[a]
125	61.32 ± 18.10[a]	55.27 ± 32.46[a]	38.24 ± 18.39[a]	52.33 ± 22.28[a]
250	65.70 ± 15.57[a]	48.72 ± 31.49[a]	37.17 ± 26.91[a]	48.23 ± 16.33[a]
500	66.32 ± 10.45[a]	43.40 ± 26.93[a]	31.18 ± 21.76[a]	28.81 ± 16.99[a]
1000	64.99 ± 13.91[a]	39.33 ± 25.34[a]	32.10 ± 23.34[a]	40.55 ± 27.29[a]

Data were expressed as means ± standard deviation of means.
Data were obtained in triplicate from three sets of runs (n = 3 × 3 = 9).
[ab] indicates the values differ significantly within the same column for cell viability ($p < 0.05$).

Table 4. Comparison of IC_{50} ± SD values between treatments of CMM and CMF on MDA-MB-231 in 2D and 3D culture systems, at 72 hours. Statistical significance was assumed for P-values < 0.05 (P < 0.05, **P < 0.01) compared to the untreated control.

Time	IC_{50} (µg/mL)			
	Aq-CMM (2D)	Aq-CMM (3D)	Aq-CMF (2D)	Aq-CMF (3D)
24	N/A	58 ± 2.36*	60 ± 7.29*	97 ± 8.27*
48	N/A	57 ± 1.50*	54 ± 1.38*	46 ± 5.28*
72	N/A	997 ± 5.63*	90 ± 8.38*	280 ± 0.93*

also showed a decrease in cell viability due to cytotoxicity of Aq-CM, relative to untreated cells ($p < 0.05$).

Similarly, in a study conducted by Jenkham et al. [30], similar percentages for cell viability were observed. At an effector-to-target ratio of 20:1, it was found that Aq-CM at 100 µg/mL considerably increased the efficacy of non-adherent cells in killing MDA-MB-231 compared to the group treated with extract at 100 µg/mL and without immune cells. The percentage of cell viability for MDA-MB-231 cells was 70.26 ± 8.29 [30].

The IC_{50} values were determined by analysing the dose-response relationship depicted in **Table 4**. It can be observed that lower concentrations of Aq-CMF were sufficient to achieve 50% inhibition of cell growth or viability compared to Aq-CMM at both 48 and 72 hours (**Figure 3**). This suggests that the fruiting body of CM exhibited higher potency or effectiveness in inhibiting the growth of MDA-MB-231 cells compared to the mycelium. Additionally, Aq-CMF is potentially effective at lower concentrations when compared to Aq-CMM. The estimation of IC_{50} values provides valuable information about the relative potency of various CM components in inhibiting cancer cell growth. These quantifiable values enable researchers to assess their efficacy, determine appropriate dosages for future studies, and explore potential therapeutic applications [31].

In the 3D MCTS cultures at the 48-hour treatment period, both Aq-CMM and Aq-CMF had the lowest IC_{50} of 57 ± 1.50 µg/mL and 46 ± 5.28 µg/mL, respectively. These results suggest that administering treatment for 48 hours may be optimal for 3D cell culture. Additionally, Aq-CMF is potentially effective at lower concentrations when compared to Aq-CMM. The estimation of IC_{50} values provides valuable information about the relative potency of various CM components in inhibiting cancer cell growth. These quantifiable values enable researchers to assess their efficacy, determine appropriate dosages for future studies, and explore potential therapeutic applications [31].

The fruiting body of CM exhibited a higher capacity to induce cell death compared to the mycelium of CM. Microscopic examination of the 3D spheroidal cells revealed more prominent changes in diameter following treatment with Aq-CMF and cisplatin, as demonstrated in **Table 5**. Initially, the matured spheroids displayed uniformity with an average diameter of approximately 385.58 µm. However, post-treatment with

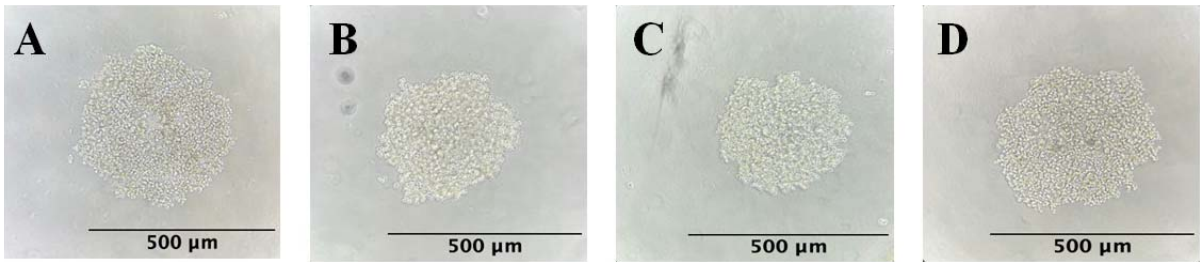


Figure 4. Morphology of breast cancer MDA-MB-231 spheroids (10x magnification). Representative images (A) Pre-treatment (control) and post-treatment of cells with (B) Aq-CMM (250 µg/mL), (C) Aq-CMF (250 µg/mL) and (D) Cisplatin (50 µg/mL) for 72 hours. Images were assembled using ImageJ; scale bar, 500 µm.

Table 5. Comparison of spheroid size between untreated and treated 3D spheroid cancer cells. Treatment with Aq-CMM, Aq-CMF and cisplatin resulted in a significant reduction in spheroid size compared to the untreated control group. Data are presented as mean ± SD from three independent experiments. Statistical significance was assumed at $P < 0.05$ ($P < 0.05$), compared to the untreated control.

MDA-MB-231 Spheroids Treatment	Diameter (µm) ± S.D.
Untreated	385.58 ± 9.54
Aq-CMM (250 µg/mL)	369.23 ± 12.72*
Aq-CMF (250 µg/mL)	336.54 ± 15.41*
Cisplatin (50 µg/mL)	364.71 ± 10.98*

Aq-CMF and Aq-CMM, a reduction in cell diameter was observed, measuring 336.54 µm and 369.23 µm, respectively.

Discussion

This study demonstrates the significant anti-metastatic and antiangiogenic activities of *Cordyceps militaris* (CM) extracts on the MDA-MB-231 breast cancer cell line, utilising both *in silico* molecular docking and *in vitro* 2D and 3D cell culture systems. Ergothioneine, identified as a key bioactive compound in CM, exhibited strong binding affinities toward crucial metastatic and angiogenic pathway targets, NF-κB and VEGFR. These interactions suggest that ergothioneine may play a vital role in modulating pathways associated with cancer cell metastasis and angiogenesis [32].

The docking results highlighted ergothioneine's higher affinity for NF-κB compared to aspirin, a well-established anti-inflammatory agent, indicating a substantial potential for therapeutic intervention in NF-κB-mediated pathways [33]. Similarly, ergothioneine's notable binding affinity for VEGFR suggests efficacy in disrupting angiogenic signalling. These findings align well with previous reports highlighting ergothioneine's antioxidative and anti-inflammatory effects [34].

Ergothioneine, which is found in the fruiting body of CM, exhibits antioxidant activity [34]. One of the primary functions of ergothioneine is to absorb and neutralise free radicals. Free radicals are chemical compounds produced during the oxygen metabolism process, exhibiting a remarkable level of reactivity towards a wide range of chemicals. As both high and low levels of reactive oxygen species (ROS) can induce cell death, the presence of ROS within cells must be carefully managed to sustain cell proliferation. Natural defence mechanisms in the human body work to mitigate the damaging effects of free radicals by slowing down cell proliferation and, in some instances, repairing the damage caused by these radicals. Inflammatory responses can be produced in a tumour microenvironment where ROS levels are increased. To treat cancer, controlling and maintaining an ideal level of free radicals may be an effective strategy. A higher amount of oxidative stress can also make cancer cells more resistant to treatment with pharmaceuticals. Correspondingly, excessive oxidative stress can directly harm cells and induce cell death through apoptosis [35,36]. Hence, ergothioneine, found in CM, emerges as a valuable bioactive compound with demonstrated antioxidative properties, capable of mitigating free radicals.

The comparative cytotoxic analyses of CM extracts showed that the aqueous extract of CM fruiting bodies (Aq-CMF) demonstrated significantly higher anticancer potency compared to the mycelium extract (Aq-CMM). Notably, the fruiting body extract displayed lower IC_{50} values, indicating higher potency at lower concentrations, particularly evident in 3D cultures, which more closely mimic the *in vivo* tumour microenvironment. This aligns with previous studies reporting higher ergothioneine and bioactive metabolites concentrations in the fruiting body compared to the mycelium, enhancing its therapeutic potential [37].

It is essential to recognise that the use of aqueous extraction in this study may impact the spectrum of bioactive compounds present in the CMF and CMM extracts. Aqueous solvents are effective at extracting water-soluble constituents such as cordycepin, adenosine, and ergothioneine, which have been reported to exhibit potent antimetastatic and antiangiogenic effects [12,38]. However, hydrophobic or lipophilic compounds, which may also contribute to the therapeutic potential of Cordyceps militaris, are likely underrepresented due to their poor solubility in water. Therefore, the observed biological effects in this study may predominantly reflect the activity of water-soluble compounds. Further work utilising organic or mixed-solvent extraction methods, followed by compositional profiling and comparative bioactivity assays, is warranted to comprehensively evaluate the contribution of hydrophobic constituents to the overall anticancer activity of CM extracts.

Interestingly, the 3D spheroid models demonstrated higher cell viability post-treatment compared to the 2D monolayer cultures. This difference likely arises from nutrient and oxygen gradients, cellular heterogeneity, and the limited penetration of treatments into the spheroids, which closely resemble the in vivo conditions *of solid tumours* [39]. This observation highlights the importance of employing 3D culture systems for

an accurate assessment of drug efficacy in cancer research, as monolayer cultures may overestimate treatment effectiveness [40].

Besides ergothioneine, multiple bioactive compounds in CM demonstrate antimetastatic and antiangiogenic characteristics, including GABA, adenosine, lovastatin, and cordycepin (**Table 1** and **Table 2**). The binding affinity values of all bioactive compounds were below -6.00 kcal/mol. Therefore, the agents demonstrated potential abilities to inhibit the target proteins of NF-κB and VEGFR.

Furthermore, adenosine, another significant bioactive component from CM, potentially contributes to anticancer activities through inhibition of the phospho-AMPK1α signalling pathway, consequently decreasing proliferation and invasiveness of breast cancer cells [41,42]. Additionally, γ-aminobutyric acid (GABA) displayed notable anticancer effects by regulating ERK1/2 phosphorylation and matrix metalloproteinases (MMPs). These findings align with previous studies emphasising GABA's capability to modulate pathways closely regulated by NF-κB, suggesting its potential role in hindering cancer cell migration and invasion [43–45].

Lovastatin, another bioactive compound from CM, also exhibits vigorous anticancer activity through the effective inhibition of the NF-κB and VEGFR signalling pathways. Previous studies highlighted its capability to modulate cancer cell growth and apoptosis by affecting the PI3K/AKT/mTOR signalling axis [46]. Interestingly, lovastatin can reverse receptor-negative phenotypes in triple-negative breast cancer cells, sensitising these cells to targeted therapies by re-expressing human epidermal growth factor receptor 2 (HER2) [47]. Apart from that, lovastatin and other chemotherapeutic medications may work together to minimise the drug resistance of cancer cells, which would significantly enhance the therapeutic efficacy of these medications [48]. These observations highlight the potential use of lovastatin in combination therapies to improve treatment efficacy and overcome drug resistance.

Cordycepin, widely researched for its anticancer properties, showed pronounced inhibitory effects on metastasis and angiogenesis by targeting critical signalling pathways, including NF-κB, VEGFR, mTOR, and the Hedgehog pathway [49,50]. Cordycepin further induced oxidative stress (ROS production), promoted apoptosis, and disrupted cellular metabolism by activating the AMPK pathway [41,51–53]. These multifaceted mechanisms underscore cordycepin's broad-spectrum therapeutic potential against breast cancer, reinforcing the importance of further mechanistic studies.

In the comparison of culture models, the higher resistance observed in 3D spheroid cultures compared to 2D monolayers is consistent with the biological characteristics of solid tumours. Factors such as nutrient gradients, hypoxic conditions, cellular heterogeneity, and limited treatment penetration likely contributed to the observed reduced drug sensitivity in 3D cultures. Consequently, this underscores the necessity of using 3D culture systems for more accurate predictions of *in vivo* drug responses and highlights the limitations inherent in traditional monolayer assays for anticancer drug screening [39,40]. The variability in IC_{50} data and the. Nonlinear responses in cytotoxic assays highlight common challenges associated with in vitro assays, including interference from assay components, variations in cell density, and random fluctuations in experimental conditions. These factors emphasise the importance of rigorous protocol optimisation and standardisation, ensuring consistent, reproducible, and accurate data interpretation, particularly when evaluating cytotoxicity.

In addition to limited drug penetration, the 3D spheroidal culture system itself contributes to reduced treatment efficacy due to its ability to better recapitulate the complexity of the *in vivo* tumour microenvironment. Unlike 2D monolayer cultures, 3D models enable cell–cell and cell–matrix interactions, the development of oxygen and nutrient gradients, and the establishment of hypoxic cores, which are hallmarks of solid tumours [54,55]. These physiological features contribute to enhanced cancer cell survival, progression, and drug resistance, making 3D cultures a more predictive platform for evaluating therapeutic responses.

The increased resistance observed in the 3D model in this study likely reflects these microenvironmental barriers, which more closely resemble actual tumour conditions. Consequently, the differential responses between 2D and 3D cultures underscore the importance of incorporating 3D

models in preclinical evaluations, as they provide a more realistic context for screening the efficacy of natural product-based treatments, such as Cordyceps militaris extracts. This approach aligns with current trends in cancer research, which emphasise the use of physiologically relevant *in vitro* models to bridge the gap between *in vitro* findings and *in vivo* outcomes [56].

While this study provides important insights into the potential anticancer properties of Cordyceps militaris extracts, several limitations should be acknowledged. Firstly, the conclusions regarding anti-metastatic and anti-angiogenic effects are based primarily on molecular docking predictions and general cytotoxicity assays in 2D and 3D culture models. More targeted *in vitro* experiments—such as wound healing assays, migration and invasion assays, and endothelial tube formation—would be necessary to confirm the anti-metastatic and anti-angiogenic mechanisms directly. Additionally, although aqueous extracts were analysed, the absence of detailed compositional profiling, such as LC-MS quantification of ergothioneine and other bioactive compounds, limits the precision of structure–activity correlations. These aspects are the focus of ongoing work and will be addressed in future studies to support a better understanding and validate the therapeutic potential of CM extracts.

Further research could explore the potential synergistic effects of CM extracts when combined with conventional chemotherapy drugs. Additionally, conducting *in vivo* studies would provide deeper insights into the pharmacodynamics, bioavailability, and systemic effects of these bioactive compounds, particularly ergothioneine and cordycepin, in preclinical models of breast cancer. Such studies would be crucial in evaluating CM's suitability and efficacy as an adjunct or alternative therapeutic strategy in clinical settings. Moreover, understanding the precise molecular mechanisms through advanced molecular techniques, such as transcriptomics and proteomics analyses, would significantly contribute to elucidating the mode of action of CM. Investigating these underlying mechanisms can facilitate the targeted application of CM-derived compounds and enhance therapeutic precision, ultimately enabling more effective and personalised treatment approaches for breast cancer.

Conclusions

This study demonstrates that *Cordyceps militaris* (CM) extracts, particularly those derived from the fruiting body, exhibit potential antimetastatic and antiangiogenic activities against the MDA-MB-231 breast cancer cell line, as supported by molecular docking and time-dependent cytotoxicity analyses. Ergothioneine and other bioactive compounds such as adenosine, GABA, lovastatin, and cordycepin were predicted to interact with key targets involved in cancer metastasis and angiogenesis, notably NF-κB and VEGFR. The higher potency observed in fruiting body extracts compared to mycelium extracts highlights the importance of bioactive compound concentration in therapeutic efficacy. Additionally, the utilisation of 3D cell culture systems provided more realistic insights into the tumour microenvironment and revealed essential differences in drug sensitivity compared to traditional 2D cultures. These findings underscore the potential of CM, especially its fruiting body extracts, as promising natural sources for developing adjunct or alternative therapies for breast cancer.

Further comprehensive preclinical and clinical studies are warranted to validate these promising results, elucidate detailed mechanisms of action, and explore the practical applicability of CM extracts in cancer therapy. Ultimately, this research contributes valuable knowledge toward developing safer and more effective anticancer therapeutic strategies derived from natural products.

Disclosures

Author Contributions

YQT and SC conceived the study idea. APQC performed the *in silico* analyses and carried out the *in vitro* cytotoxicity and spheroid assays. APQC and SXYK analysed the data and prepared the figures. YQT and AYYC reviewed and supervised the work. The manuscript was critically reviewed and edited by YQT, SC and AYYC. All authors contributed to the article, and the final version of the manuscript was reviewed and approved by all the authors.

Conflict of interest statement

The authors declare no conflict of interest.

Funding sources

This study was supported by Taylor's University, Malaysia and Mae Fah Luang University, Thailand.

References

1. Jin Y, Meng X, Qiu Z, Su Y, Yu P, Qu P. Anti-tumor and anti-metastatic roles of cordycepin, one bioactive compound of Cordyceps militaris. Saudi Journal of Biological Sciences 2018;25:991–5. https://doi.org/10.1016/j.sjbs.2018.05.016.
2. Tuli HS, Sandhu SS, Sharma AK. Pharmacological and therapeutic potential of Cordyceps with special reference to Cordycepin. 3 Biotech 2014;4:1–12. https://doi.org/10.1007/s13205-013-0121-9.
3. Thoe ES, Chia YY, Tang YQ, Chamyuang S. Implementation of Omics Approaches in Unravelling the Potency of Cordyceps militaris in Drug Discovery. NPJ 2025;15:e030424228575. https://doi.org/10.2174/0122103155294164240323134513.
4. Quan X, Kwak BS, Lee J-Y, Park JH, Lee A, Kim TH, Park S. Cordyceps militaris Induces Immunogenic Cell Death and Enhances Antitumor Immunogenic Response in Breast Cancer. Evidence-Based Complementary and Alternative Medicine 2020;2020:9053274. https://doi.org/10.1155/2020/9053274.
5. Lee HH, Lee S, Lee K, Shin YS, Kang H, Cho H. Anticancer effect of Cordyceps militaris in human colorectal carcinoma RKO cells via cell cycle arrest and mitochondrial apoptosis. DARU J Pharm Sci 2015;23:35. https://doi.org/10.1186/s40199-015-0117-6.
6. Yang C-H, Kao Y-H, Huang K-S, Wang C-Y, Lin L-W. Cordyceps militaris and mycelial fermentation induced apoptosis and autophagy of human glioblastoma cells. Cell Death Dis 2012;3:e431–e431. https://doi.org/10.1038/cddis.2012.172.
7. Park SE, Kim J, Lee Y-W, Yoo H-S, Cho C-K. Antitumor Activity of Water Extracts From Cordyceps Militaris in NCI-H460 Cell Xenografted Nude Mice. Journal of Acupuncture and Meridian Studies 2009;2:294–300. https://doi.org/10.1016/S2005-2901(09)60071-6.
8. Park SE, Yoo HS, Jin C-Y, Hong SH, Lee Y-W, Kim BW, Lee SH, Kim W-J, Cho CK, Choi YH. Induction of apoptosis and inhibition of telomerase activity in human lung carcinoma cells by the water extract of Cordyceps militaris. Food and Chemical Toxicology 2009;47:1667–75. https://doi.org/10.1016/j.fct.2009.04.014.
9. Chamyuang S, Owatworakit A, Honda Y. New insights into cordycepin production in Cordyceps militaris and applications. Ann Transl Med 2019;7:S78–S78. https://doi.org/10.21037/atm.2019.04.12.
10. Kapałczyńska M, Kolenda T, Przybyła W, Zajączkowska M, Teresiak A, Filas V, Ibbs M, Bliźniak R, Łuczewski Ł, Lamperska K. 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. Aoms 2016. https://doi.org/10.5114/aoms.2016.63743.
11. Liu Y, Guo Z-J, Zhou X-W. Chinese Cordyceps: Bioactive Components, Antitumor Effects and Underlying Mechanism—A Review. Molecules 2022;27:6576. https://doi.org/10.3390/molecules27196576.
12. Tuli HS, Sharma AK, Sandhu SS, Kashyap D. Cordycepin: A bioactive metabolite with therapeutic potential. Life Sciences 2013;93:863–9. https://doi.org/10.1016/j.lfs.2013.09.030.
13. Chen L, Zhang L, Ye X, Deng Z, Zhao C. Ergothioneine and its congeners: anti-ageing mechanisms and pharmacophore biosynthesis. Protein & Cell 2024;15:191–206. https://doi.org/10.1093/procel/pwad048.
14. Shi C, Zhang N, Feng Y, Cao J, Chen X, Liu B. Aspirin Inhibits IKK-β-mediated Prostate Cancer Cell Invasion by Targeting Matrix Metalloproteinase-9 and Urokinase-Type Plasminogen Activator. Cell Physiol Biochem 2017;41:1313–24. https://doi.org/10.1159/000464434.
15. Al-Sanea MM, Chilingaryan G, Abelyan N, Sargsyan A, Hovhannisyan S, Gasparyan H, Gevorgyan S, Albogami S, Ghoneim MM, Farag AK, Mohamed AAB, El-Damasy AK. Identification of Novel Potential VEGFR-2 Inhibitors Using a Combination of Computational Methods for Drug Discovery. Life 2021;11:1070. https://doi.org/10.3390/life11101070.
16. Hu W-H, Duan R, Xia Y-T, Xiong Q-P, Wang H-Y, Chan GK-L, Liu S-Y, Dong TT-X, Qin Q-W, Tsim KW-K. Binding of Resveratrol to Vascular Endothelial Growth Factor Suppresses Angiogenesis by Inhibiting the Receptor Signaling. J Agric Food Chem 2019;67:1127–37. https://doi.org/10.1021/acs.jafc.8b05977.
17. Yang T, Yao H, He G, Song L, Liu N, Wang Y, Yang Y, Keller ET, Deng X. Effects of Lovastatin on MDA-MB-231 Breast Cancer Cells: An Antibody Microarray Analysis. J Cancer 2016;7:192–9. https://doi.org/10.7150/jca.13414.
18. Yang Y, Ren L, Li W, Zhang Y, Zhang S, Ge B, Yang H, Du G, Tang B, Wang H, Wang J. GABAergic signaling as a potential therapeutic target in cancers. Biomedicine & Pharmacotherapy 2023;161:114410. https://doi.org/10.1016/j.biopha.2023.114410.
19. Lim J, Gew L, Tang Y-Q. Biocomputational-mediated screening and molecular docking platforms for discovery of coumarin-derived antimelanogenesis agents. Dermatol Sin 2023;41:8. https://doi.org/10.4103/ds.DS-D-22-00087.
20. Grosdidier A, Zoete V, Michielin O. SwissDock, a protein-small molecule docking web service based on EADock DSS. Nucleic Acids Research 2011;39:W270–7. https://doi.org/10.1093/nar/gkr366.
21. Ghasemi M, Turnbull T, Sebastian S, Kempson I. The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. IJMS 2021;22:12827. https://doi.org/10.3390/ijms222312827.
22. Li L, Hu M, Wang T, Chen H, Xu L. Repositioning Aspirin to Treat Lung and Breast Cancers and Overcome Acquired Resistance to Targeted Therapy. Front Oncol 2020;9:1503. https://doi.org/10.3389/fonc.2019.01503.
23. Putra WE, Salma, WO, Widiastuti, D. The Potency of Boesenbergin A and Boesenbergin B Compounds from Kaempferia pandurata as Anti-metastasis Agent: in silico study. Malaysian Journal of Biochemistry & Molecular Biology (MJBMB). 2020;23(3):128-32.

24. Ferreira L, Dos Santos R, Oliva G, Andricopulo A. Molecular Docking and Structure-Based Drug Design Strategies. Molecules 2015;20:13384–421. https://doi.org/10.3390/molecules200713384.
25. Chen S-Y, Ho K-J, Hsieh Y-J, Wang L-T, Mau J-L. Contents of lovastatin, γ-aminobutyric acid and ergothioneine in mushroom fruiting bodies and mycelia. LWT 2012;47:274–8. https://doi.org/10.1016/j.lwt.2012.01.019.
26. Lin S-Y, Chen Y-K, Yu H-T, Barseghyan GS, Asatiani MD, Wasser SP, Mau J-L. Comparative Study of Contents of Several Bioactive Components in Fruiting Bodies and Mycelia of Culinary-Medicinal Mushrooms. Int J Med Mushr 2013;15:315–23. https://doi.org/10.1615/IntJMedMushr.v15.i3.80.
27. Kajal K, Panda AK, Bhat J, Chakraborty D, Bose S, Bhattacharjee P, Sarkar T, Chatterjee S, Kar SK, Sa G. Andrographolide binds to ATP-binding pocket of VEGFR2 to impede VEGFA-mediated tumor-angiogenesis. Sci Rep 2019;9:4073. https://doi.org/10.1038/s41598-019-40626-2.
28. Bhuiyan MR, Ahmed KS, Reza MS, Hossain H, Siam SMM, Nayan S, Jafrin S, Shuvo SR, Ud Daula AFMS. Prediction of angiogenesis suppression by myricetin from Aeginetia indica via inhibiting VEGFR2 signaling pathway using computer-aided analysis. Heliyon 2025;11:e41749. https://doi.org/10.1016/j.heliyon.2025.e41749.
29. Ghith A, Youssef KM, Ismail NSM, Abouzid KAM. Design, synthesis and molecular modeling study of certain VEGFR-2 inhibitors based on thienopyrimidine scaffold as cancer targeting agents. Bioorganic Chemistry 2019;83:111–28. https://doi.org/10.1016/j.bioorg.2018.10.008.
30. Jenkham P, Wibunkul K, Jantakee K, Katasai A, Thephinlap C, Khoothiam K, Suwannasom N, Panya A, Thepmalee C. Effect of aqueous extract from cordyceps militaris on the immune cell activation to kill breast cancer and hepatocellular carcinoma cell lines. Health Science, Science and Technology Reviews 2023;16:30–45.
31. Berrouet C, Dorilas N, Rejniak KA, Tuncer N. Comparison of Drug Inhibitory Effects ($$\hbox {IC}_{50}$$) in Monolayer and Spheroid Cultures. Bull Math Biol 2020;82:68. https://doi.org/10.1007/s11538-020-00746-7.
32. Jędrejko KJ, Lazur J, Muszyńska B. Cordyceps militaris: An Overview of Its Chemical Constituents in Relation to Biological Activity. Foods 2021;10:2634. https://doi.org/10.3390/foods10112634.
33. Yow HY, Tang YQ, Lee JS. Antiproliferative effect of aspirin and diclofenac on MDA-MD-231 triple-negative breast cancer cells. Malaysian Journal of Biochemistry & Molecular Biology (MJBMB). 2022;25:88-92.
34. Chen B-X, Xue L-N, Wei T, Ye Z-W, Li X-H, Guo L-Q, Lin J-F. Enhancement of ergothioneine production by discovering and regulating its metabolic pathway in Cordyceps militaris. Microb Cell Fact 2022;21:169. https://doi.org/10.1186/s12934-022-01891-5.
35. Lan Y-H, Lu Y-S, Wu J-Y, Lee H-T, Srinophakun P, Canko GN, Chiu C-C, Wang H-MD. Cordyceps militaris Reduces Oxidative Stress and Regulates Immune T Cells to Inhibit Metastatic Melanoma Invasion. Antioxidants 2022;11:1502. https://doi.org/10.3390/antiox11081502.
36. Yi Leong HJ. Identification of Potentially Therapeutic Target Genes in Metastatic Breast Cancer via Integrative Network Analysis. EJMO 2023:371–87. https://doi.org/10.14744/ejmo.2023.80452.
37. Chan JSL, Barseghyan GS, Asatiani MD, Wasser SP. Chemical Composition and Medicinal Value of Fruiting Bodies and Submerged Cultured Mycelia of Caterpillar Medicinal Fungus Cordyceps militaris CBS-132098 (Ascomycetes). Int J Med Mushrooms 2015;17:649–59. https://doi.org/10.1615/IntJMedMushrooms.v17.i7.50.
38. Wiley JL, Razdan RK, Martin BR. Evaluation of the role of the arachidonic acid cascade in anandamide's in vivo effects in mice. Life Sciences 2006;80:24–35. https://doi.org/10.1016/j.lfs.2006.08.017.
39. Saydé T, El Hamoui O, Alies B, Gaudin K, Lespes G, Battu S. Biomaterials for Three-Dimensional Cell Culture: From Applications in Oncology to Nanotechnology. Nanomaterials 2021;11:481. https://doi.org/10.3390/nano11020481.
40. Rolver MG, Elingaard-Larsen LO, Pedersen SF. Assessing Cell Viability and Death in 3D Spheroid Cultures of Cancer Cells. JoVE 2019:59714. https://doi.org/10.3791/59714.
41. Aggarwal V, Tuli H, Varol A, Thakral F, Yerer M, Sak K, Varol M, Jain A, Khan Md, Sethi G. Role of Reactive Oxygen Species in Cancer Progression: Molecular Mechanisms and Recent Advancements. Biomolecules 2019;9:735. https://doi.org/10.3390/biom9110735.
42. Virtanen SS, Kukkonen-Macchi A, Vainio M, Elima K, Härkönen PL, Jalkanen S, Yegutkin GG. Adenosine Inhibits Tumor Cell Invasion via Receptor-Independent Mechanisms. Molecular Cancer Research 2014;12:1863–74. https://doi.org/10.1158/1541-7786.MCR-14-0302-T.
43. Kanbara K, Otsuki Y, Watanabe M, Yokoe S, Mori Y, Asahi M, Neo M. GABAB receptor regulates proliferation in the high-grade chondrosarcoma cell line OUMS-27 via apoptotic pathways. BMC Cancer 2018;18:263. https://doi.org/10.1186/s12885-018-4149-4.
44. Zhang D, Li X, Yao Z, Wei C, Ning N, Li J. GABAergic signaling facilitates breast cancer metastasis by promoting ERK1/2-dependent phosphorylation. Cancer Letters 2014;348:100–8. https://doi.org/10.1016/j.canlet.2014.03.006.
45. Wang W, Nag SA, Zhang R. Targeting the NFκB signaling pathways for breast cancer prevention and therapy. Curr Med Chem. 2015;22(2):264-89. doi: 10.2174/0929867321666141106124315. n.d.
46. Choi H-W, Shin P-G, Lee J-H, Choi W-S, Kang M-J, Kong W-S, Oh M-J, Seo Y-B, Kim G-D. Anti-inflammatory effect of lovastatin is mediated via the modulation of NF-κB and inhibition of HDAC1 and the PI3K/Akt/mTOR pathway in RAW264.7 macrophages. Int J Mol Med 2017. https://doi.org/10.3892/ijmm.2017.3309.

47. Xie L, Zhu G, Shang J, Chen X, Zhang C, Ji X, Zhang Q, Wei Y. An overview on the biological activity and anti-cancer mechanism of lovastatin. Cellular Signalling 2021;87:110122. https://doi.org/10.1016/j.cellsig.2021.110122.
48. Khandelwal Gilman KA, Han S, Won Y-W, Putnam CW. Complex interactions of lovastatin with 10 chemotherapeutic drugs: a rigorous evaluation of synergism and antagonism. BMC Cancer 2021;21:356. https://doi.org/10.1186/s12885-021-07963-w.
49. Wong YY, Moon A, Duffin R, Barthet-Barateig A, Meijer HA, Clemens MJ, De Moor CH. Cordycepin Inhibits Protein Synthesis and Cell Adhesion through Effects on Signal Transduction. Journal of Biological Chemistry 2010;285:2610–21. https://doi.org/10.1074/jbc.M109.071159.
50. Li J, Cai H, Sun H, Qu J, Zhao B, Hu X, Li W, Qian Z, Yu X, Kang F, Wang W, Zou Z, Gu B, Xu K. Extracts of Cordyceps sinensis inhibit breast cancer growth through promoting M1 macrophage polarization via NF-κB pathway activation. Journal of Ethnopharmacology 2020;260:112969. https://doi.org/10.1016/j.jep.2020.112969.
51. Bjornsti M-A, Houghton PJ. The tor pathway: a target for cancer therapy. Nat Rev Cancer 2004;4:335–48. https://doi.org/10.1038/nrc1362.
52. Feng C, Chen R, Fang W, Gao X, Ying H, Zheng X, Chen L, Jiang J. Synergistic effect of CD47 blockade in combination with cordycepin treatment against cancer. Front Pharmacol 2023;14:1144330. https://doi.org/10.3389/fphar.2023.1144330.
53. Wang D, Zhang Y, Lu J, Wang Y, Wang J, Meng Q, Lee RJ, Wang D, Teng L. Cordycepin, a Natural Antineoplastic Agent, Induces Apoptosis of Breast Cancer Cells via Caspase-dependent Pathways. Nat Prod Commun. 2016 Jan;11(1):63-8. PMID: 26996021.
54. Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. ASSAY and Drug Development Technologies 2014;12:207–18. https://doi.org/10.1089/adt.2014.573.
55. Ravi M, Paramesh V, Kaviya SR, Anuradha E, Solomon FDP. 3D Cell Culture Systems: Advantages and Applications. Journal Cellular Physiology 2015;230:16–26. https://doi.org/10.1002/jcp.24683.
56. Lv D, Hu Z, Lu L, Lu H, Xu X. Three-dimensional cell culture: A powerful tool in tumor research and drug discovery (Review). Oncol Lett 2017. https://doi.org/10.3892/ol.2017.7134.

ORIGINAL PAPER

JMS Journal of Medical Science

The natural HLA-Peptidome of Sezary Syndrome: uncovering antigens for T cell-based immunotherapy

Lydon Nyambura

Department of Dermatology, Venerology and Allergology, Charité – Universitätsmedizin Berlin corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

https://orcid.org/0000-0003-2197-6048

Corresponding author: nodyl_w@yahoo.com

Kathrin Textoris-Taube

Core Facility – High-Throughput Mass Spectrometry, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Core Facility – High-Throughput Mass Spectrometry, Berlin, Germany

https://orcid.org/0000-0002-1224-8236

Peter Walden

Department of Dermatology, Venerology and Allergology, Charité – Universitätsmedizin Berlin corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

https://orcid.org/0000-0002-8986-3667

https://doi.org/10.20883/medical.e1335

Keywords: Sezary syndrome, HLA-I peptides, LC-MS/MS, immunopeptidome, immunogenicity

Received 2025-07-03
Accepted 2025-09-17
Published 2025-09-30

How to Cite: Nyambura L, Textoris-Taube K, Walden P. The natural HLA-Peptidome of Sezary Syndrome: uncovering antigens for T cell-based immunotherapy. Journal of Medical Science. 2025 September;94(3);e1335. https://doi.org/10.20883/medical.e1335

ABSTRACT

Introduction. Sezary syndrome (SS) is a rare and aggressive form of cutaneous T-cell lymphoma that has a poor prognosis, with a median overall survival time of less than 3 years. Despite advances in its treatment, SS is a challenge to manage, often characterised by high rates of relapse and limited response to therapy in many patients. The main challenge for treatment, including vaccine development, is its heterogeneity in its molecular and genetic characteristics, clinical presentation, disease progression, and treatment response. Understanding the SS heterogeneity at the omics level is vital in developing T-cell-mediated immunotherapeutic.

Material and methods. In this study, naturally presented human leukocyte antigen class I (HLA-I) peptides were isolated from leukapheresis samples of SS patients and analysed using high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The source proteins were evaluated for potential immunotherapy applications.

Results. The total number of HLA-I–restricted peptides and source proteins identified in SS leukapheresis patient samples was approximately the same, and they were heterogeneous and individualised. Only a small fraction of HLA-I peptides and source proteins was found to be shared between and among the patients. Peptide lengths were dominated by nanopeptides, with a preference for processing by chymotrypsin. The source proteins were predominantly from the cytoplasm and were primarily involved in biosynthesis and regulation. Furthermore, the HLA-I peptides were presented from proteins of the top 20 genes with somatic mutations in SS, which include *NCOR1, TRRAP, JAK3, PLCG1, TP53, and STAT3* (SS-associated antigens – SAAs). These SAAs had varying mutation types and frequencies, dominated by missense variants, with

allele-dependent immunogenicity being highest in HLA-A*11:01 and HLA-A*02:01, lowest for HLA-A*01:01. TRRAP showed high-affinity peptides and low gene expression levels in normal tissue (except *for STAT3*), as well as a significant protein interaction network, including JAK3 and STAT3 at the primary level.
Conclusions. This study's findings contribute to the overall understanding of the SS HLA-I peptidome landscape and highlight potential T-cell-mediated immunotherapeutic targets.

Introduction

Sezary syndrome (SS) is a rare and aggressive form of cutaneous T-cell lymphoma, characterised by the proliferation and accumulation of malignant T cells in the skin [1,2]. SS typically presents with generalised erythroderma, intense pruritus, and the presence of atypical Sezary cells in the peripheral blood [2]. The disease primarily affects older individuals, with a higher incidence in males [3]. Unfortunately, SS has a poor prognosis, with a median overall survival time of less than 3 years [3,4]. Therapeutic management of SS involves a multimodal approach, combining skin-directed therapies (topical corticosteroids, phototherapy, and local radiotherapy), extracorporeal photopheresis, systemic treatments such as retinoids, chemotherapy, and targeted agents [5–7]. Immunotherapies, including immune checkpoint inhibitors and adoptive T-cell therapy, show promise in enhancing anti-tumour immune responses [8]. Despite these advances in treatment, SS remains challenging to manage, often characterised by high rates of relapse and limited response to therapy in many patients [9].

The main challenge for SS treatment, including vaccine development, is its heterogeneity [7,10–12] in clinical presentation, disease progression, and treatment response, as well as molecular and genetic characteristics among patients, which leads to distinct patterns of gene expression and signalling pathways. Understanding the SS heterogeneity at the omics level is vital in developing T-cell-mediated immunotherapeutic approaches, such as peptide-based vaccines.

HLA peptidomics refers to the study of the peptide repertoire presented by human leukocyte antigen (HLA) molecules [13–18]. These peptides are processed from intracellular proteins primarily by the proteasome and presented to the cell surface by HLA for T-cell recognition. HLA peptidomics enables the understanding of the mechanism of antigen processing and presentation in cancer cells. Including identification and characterisation of peptides from tumour-associated antigens (TAAs) and neoepitopes presented by HLA molecules on cancer cells. Overall, HLA peptidomics in cancer represents a powerful tool for understanding the mechanisms of antigen processing and presentation, highlighting potential tumour targets for T-cell-mediated immunotherapies.

HLA peptidomics studies for SS are warranted to determine how this SS heterogeneity impacts antigen processing and presentation, and to identify potential targets for SS T-cell-mediated immunotherapeutic vaccines. In this study, immunoaffinity purification of HLA-I peptide complexes from samples of SS clinical leukophoresis patients was carried out and analysed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). These HLA peptidomes were studied and compared in reference to presentation, lengths, subcellular locations, molecular/biological functions, and the top 20 genes with somatic mutation in SS as per the COSMIC database and whose protein HLA-I peptides were presented by all the four patients or at least 3 of the four patients (Rom, IrK, FrA and Seo); NCOR1, TRRAP, JAK3, PLCG1, TP53 and STAT3 (SAAs) analysed according to their immunogenicity, gene expression profiles, and protein interaction partners.

Materials and methods

Sezary Syndrome patients

The clinical leukapheresis material from 4 SS patients was used, with approval from the Charité ethics committee (Approval Nos. EA1/222/14 and EA1/026/14, dated 10/09/2014 and 28/02/2014, respectively) and written informed consent from

the volunteer donors. HLA type was determined by Charité – Universitätsmedizin Berlin, HLA typing laboratory.

Isolation and purification of MHC I-presented peptides

MHC class I molecules were isolated as described in detail elsewhere [19]. Briefly, cells were lysed in 20 mM Tris-HCl buffer, pH 7.4, containing 0.3% CHAPS, 0.2% NP-40, 145 mM NaCl, 1 mM EDTA, and 1 mM Pefabloc. Lysates were ultracentrifuged for 1h at 100,000 x g. The supernates were passed through a column with a monoclonal antibody of irrelevant specificity, followed by a column with the HLA-I anti-human monoclonal antibody (W6/32), both coupled to activated CH Sepharose as per the manufacturer's protocol (Amersham Biosciences AB, Uppsala, Sweden). After adsorption of the proteins, the anti-human HLA-I column was washed with the following in descending order: 20 mM Tris, 145 mM NaCl, pH 7.4 (TBS), 0.3% CHAPS in TBS, TBS, 0.3% ß-octylglycoside in TBS, TBS, and lastly with ultrapure H_2O. HLA-peptide complexes were eluted from the column with 0.7 % TFA in ultrapure H_2O. High-molecular-weight components were separated from peptides by centrifugal ultrafiltration using a molecular weight cutoff of 3 kDa (Centricon, Millipore, Schwalbach, Germany). The filtrates were fractionated on a Smart HPLC system (Amersham Biosciences, Freiburg, Germany) using a reverse phase column µRPC C2/C18, SC2.1/10 (Amersham Biosciences) and an acetonitrile gradient of 5–90% of B (solvent B: 0.1% TFA, 90% of acetonitrile; solvent A: 0.1% TFA in ultrapure H_2O). The fractions obtained were lyophilised and then re-dissolved in 0.1% TFA and 2% acetonitrile for LC-MS/MS analysis.

LC-MSMS analysis of HLA ligands

The peptide fractions were analysed by reversed-phase LC (Ultimate 3000 RSLCnano) coupled online with Q Exactive Plus MS (both, Thermo Fisher Scientific). Fractionated peptides were trapped on a C18 precolumn at 20 µL/min (2% acetonitrile, 0.1% TFA) for 4 min. Subsequently, peptides were separated at a flow rate of 300nl/min onto a 75-µm × 25 cm PepMap nano-HPLC column with a gradient of 3–30% of 80% acetonitrile and 0.1% FA acid in ultrapure H_2O over 90 min. Eluted peptides were nanospray-ionised and fragmented based on the ten most intense precursor ion signals, with a 20 sec dynamic exclusion time to avoid repeated fragmentation.

Data processing and analysis

The MS and MS/MS spectra were processed via Data Analysis (vers. 3.4) and Bio-tools (vers. 3.1) software (Bruker Daltonics). The local MASCOT server (vers. 2.2), utilising the Swissprot databank (vers. 56.3) for human proteins (20,408 reviewed entries), was used to identify the peptides. The precursor mass tolerance was 5 ppm for MS and 10 ppm for MS/MS, with methionine oxidation as a possible variable modification. For each peptide-spectrum match, candidate sequences were validated using a statistical evaluation -10logP, where logP is the logarithm to the base 10 of P ($P < 0.05$) as the absolute probability. Further validation of the identified peptides by *de novo* sequencing was performed using the Sequit software [20] and by manual inspection of the peptide-spectrum matches. The protein sequence, protein ID, and gene symbol for proteomic data analyses were extracted from the UniProt database [21]. The Human Protein Database [22] was used to classify proteins according to their subcellular location and biological function.

Somatic mutations.

To identify the top 20 genes with somatic mutations in SS, the Catalogue of Somatic Mutations in Cancer (COSMIC) was utilised https://cancer.sanger.ac.uk/cosmic [23]. The main search was set to SS, using the following browser filters: Tissue selection (Hematopoietic and lymphoid), Sub-tissue selection (All), Histology selection (lymphoid neoplasm) and Sub-histology selection (mycosis fungoides-sezary syndrome). In addition, the type of somatic mutation of the SAAs (The top 20 genes with somatic mutation in SS as per the COSMIC database and those whose HLA-I peptides were presented by all four patients or at least 3 of the four patients (Rom, IrK, FrA and Seo)) was also determined by COSMIC.

Sezary syndrome-associated antigens (SAAs) and their immunogenicity

To determine the immunogenicity of the SAAs, NetMHCpan 4.1 was used in the IEDB (http://www.iedb.org) [24]. The immunogenicity was determined for HLA-A*01:01, HLA-A*02:01,

HLA-A*11:01, HLA-A*24:02, HLA-C*06:02, HLA-C*07:01 and HLA-C*07:02, individually and all combined. These alleles are expressed together in 90% of the population [25]. IC50(500) nM binding affinity threshold was used as a threshold for immunogenicity, and immunogenicity scores were presented as 1/IC50(500) nM.

SAAs gene expression in primary normal human tissues, and protein interaction partners

To determine the gene expression profiles of the SAAs in normal major human tissues, the GeneCards database [26] was used, with a low gene expression intensity cut-off of 10%. The SAAs' protein interaction partners were determined using STRING version 11.5, a database of known and predicted protein-protein interactions [27]. STRING was used to determine known SAAs protein interaction partners, experimentally determined from various biochemical, biophysical and genetic techniques. A medium interaction score of 0.400 was applied, with a cut-off of 10 interaction partners at the primary level, against *Homo sapiens*.

Results

Naturally presented HLA I ligands of Sezary syndrome patients

The SS leukophoresis patient samples (Rom, IrK, FrA, and Seo) were lysed, and MHC class I molecules were isolated by affinity chromatography. LC-MS/MS then analysed peptides extracted from the MHC molecules. The sequences of a total of 7668, 8492, 8123, and 4854 HLA class I-bound peptides were identified from 3003, 4027, 3865, and 2390 source proteins in Rom, IrK, FrA, and Seo, respectively (see **Figure 1A**). 6086, 7512, 6905, and 3441 HLA class I-bound peptides were unique in Rom, IrK, FrA, and Seo, respectively (see **Figure 1A**). The shared peptides between patients ranged from 146 (0.6%) to 748 (2.8%), and were

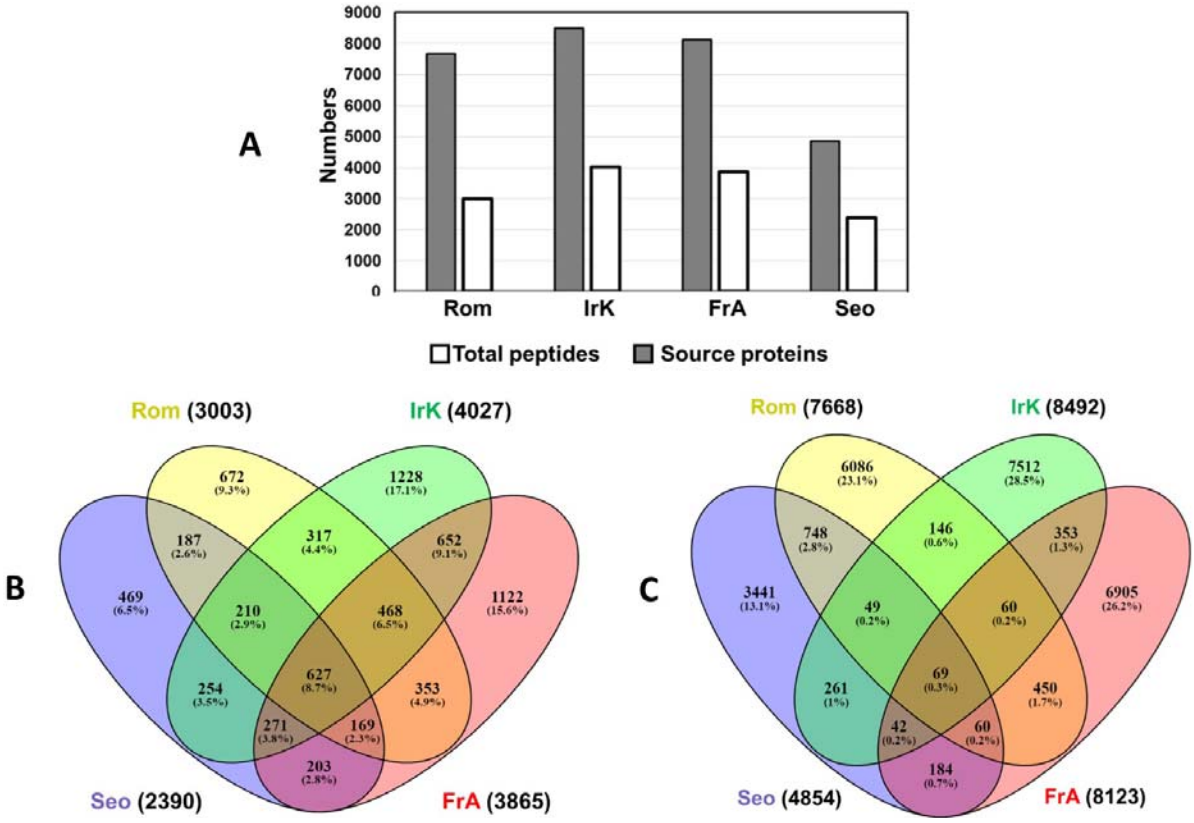


Figure 1. Naturally presented HLA class I ligands and Source proteins of Sezary syndrome leukapharesis patients' samples. The leukapharesis samples were lysed, MHC I molecules were isolated by affinity chromatography, and LC-MS/MS analysed peptides extracted from the MHC molecules. A) Number of naturally presented HLA class I ligands and Source proteins. B) Shared-Naturally presented HLA class I ligands. C) Shared- Naturally presented HLA class I ligand source proteins.

well below 1% among the patients. Only 69 (0.3%) HLA I peptide sequences were found to be shared among all four patients based only on peptide sequences. Precursor peptide mass signals and retention time, respectively (see **Figure 1B**). The HLA expression was HLA-A2, HLA-A24, HLA-B7 and HLA-B8, HLA-C6 and HLA-C7 in Seo, HLA-A1, HLA-A33, HLA-B8, HLA-B14, HLA-C7 and HLA-C8 in Rom, while the HLA expression in IrK and FrA was undetermined. The shared proteins between the patients ranged from 187 (2.6%) to 652 (9.1%), with only 627 (8.7%) shared among the four patients (see **Figure 1C**).

MHC I-bound peptide lengths and C-Terminus processing

The MHC I-bound peptide lengths in all patients were dominated by nanopeptides, constituting 29.0% to 55.1% of all identified peptides. At the same time, decapeptides were the second dominant, constituting 8.0% – 14.8%. Undecapeptides and above, on the other hand, were less than 11% in all patients (see **Figure 2**). The C-terminus peptide processing by the proteasome, based on peptide numbers and percentages, was Chymotrypsin > Trypsin > Caspase in all patients (see **Figure 3).**

Sub-cellular locations and molecular functions of source proteins

The human protein reference database was used to assign the subcellular location of the source proteins of the HLA I-bound peptides from Rom, IrK, FrA and Seo. The cytoplasm, intracellular membrane-bounded organelles, and cytosol were the dominant subcellular locations of the source

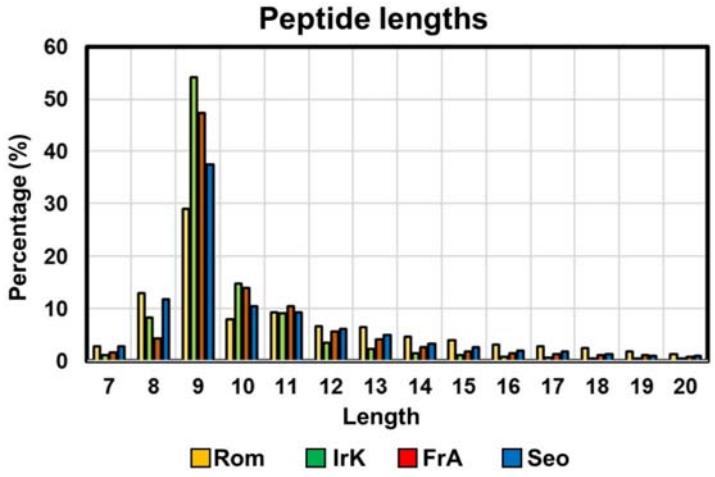


Figure 2. MHC Class I -peptide lengths in Sezary syndrome Leukapheresis patients' samples.

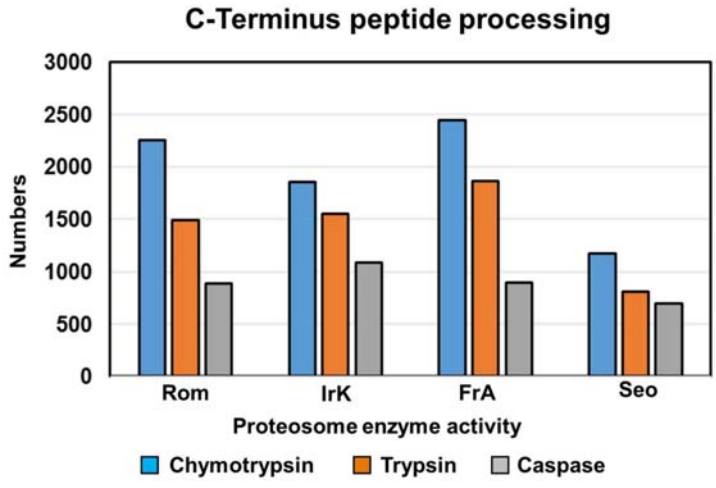


Figure 3. MHC Class I –peptides C-Terminus processing by the proteasome.

proteins (see **Figure 4**). Together, they accounted for 29.6%, 28.8%, 27.1%, and 24.7% of all the source proteins in Rom, IrK, FrA, and Seo, respectively. Approximately half of this percentage was solely from the cytoplasmic part, which accounted for more than 14% of all the source proteins. Source proteins from the cell junction were 10.3% in Seo, considerably higher compared to those in Rom, Irk, and FrA, which were 3.9%, 3.9%, and 3.3%, respectively. Source proteins' subcellular locations were lowest in the condensed chromosome, endoplasmic reticulum lumen, and intracellular organelle lumen in all patients, with values ranging from 0% to 0.32% (data not shown). The source proteins originated from diverse subcellular locations in all patients (see **Figure 4**). These proteins were further evaluated for their biological/molecular functions, using the human protein reference database [22]. Although source proteins possessed multiple biological/molecular functions, a vast majority were involved in biosynthetic process (14.7%, 13.4%, 12.0%, 11.6%), biological regulation (8.9%, 10.0%, 10.5%, 11.1%), catabolic process (8.1%, 5.3%, 4.8%, 5.6%) and anatomical structure development (5.3%, 4.1%, 4.6%, 4.4%) in Rom, IrK, FrA and Seo respectively (see **Figure 5**). They were also those involved in immune response, cell death and apoptosis, but were below 2% in all patients.

Somatic mutations in cosmic

To determine the top 20 genes with somatic mutations in SS, the Catalogue of Somatic Mutations in Cancer (COSMIC) was used. The top 20 genes with somatic mutation as per COSMIC, search filtered as follows; tissue (hematopoietic and lymphoid), sub-tissue (All), histology (lymphoid neoplasm) and sub-histology (mycosis fungoides-sezary syndrome) were Fat Atypical Cadherin 4 (*FAT4*), Fat Atypical Cadherin 1 *(FAT1*), Caspase Recruitment Domain Family Member 11 (*CARD11*), Nuclear Receptor Corepressor 1 (*NCOR1*), Phospholipase C Gamma 1 (*PLCG1*), Tumor Protein P53 (*TP53*), Glutamate Ionotropic Receptor NMDA Type Subunit 2A (*GRIN2A*), AT-Rich Interaction Domain 1A (*ARID1A*), LDL Receptor Related Protein 1B (*LRP1B*), phosphatidylinositol-3,4,5-trisphosphate dependent rac exchange factor 2 (*PREX2*),

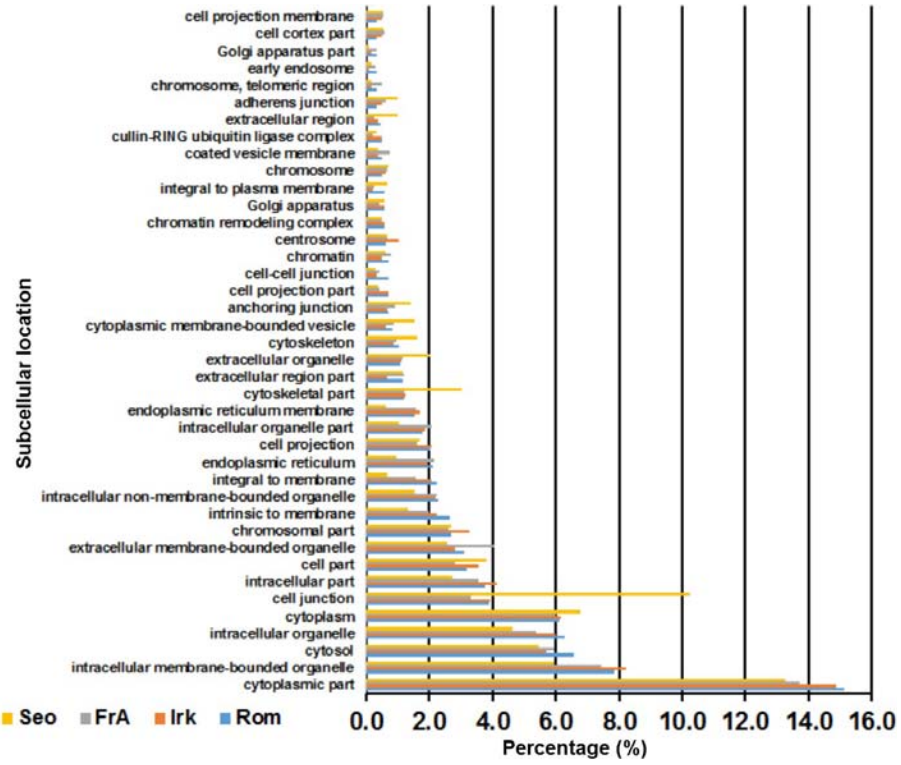


Figure 4. Subcellular location of the source proteins. The subcellular locations of the source proteins of the HLA class I-bound peptides from Sezary syndrome leukapheresis patients' samples identified by mass spectrometry were assigned using the Human Protein Reference Database.

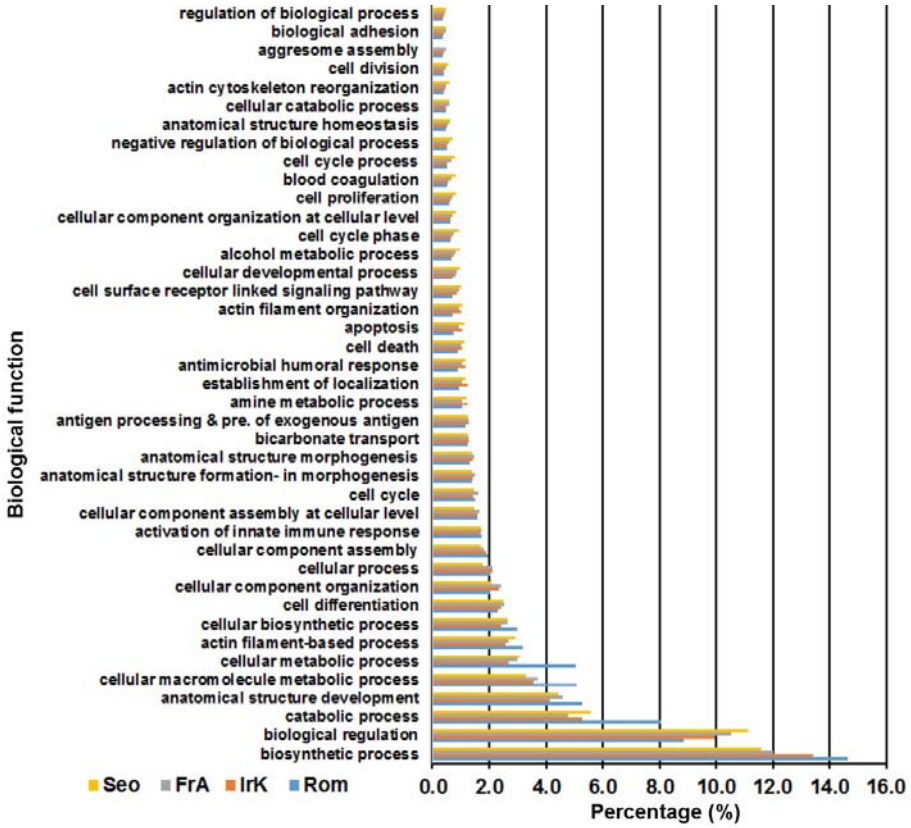
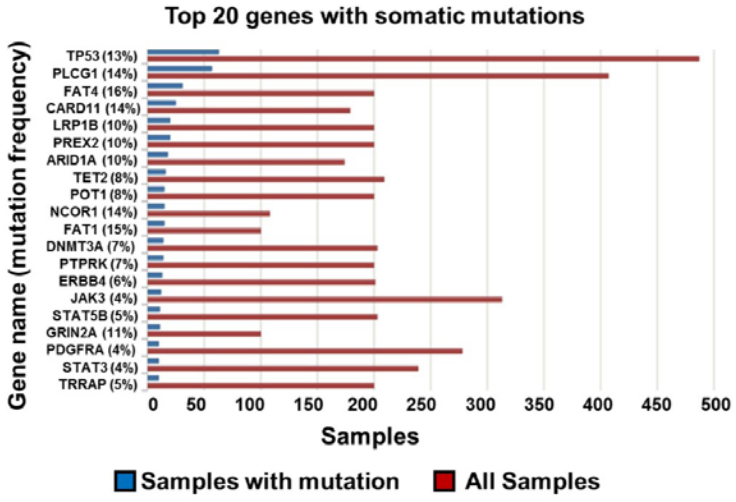


Figure 5. Biological/molecular functions of the source proteins. The biological/molecular functions of the source proteins of the HLA class I-bound peptides from Sezary syndrome Leukapheresis patients' samples were assigned using the Human Protein Reference Database.

Protection of Telomeres 1 (*POT1*), Tet Methylcytosine Dioxygenase 2 (*TET2*), DNA Methyltransferase 3 Alpha (*DNMT3A*), Protein Tyrosine Phosphatase Receptor Type K (*PTPRK*), Erb-B2 Receptor Tyrosine Kinase 4 (*ERBB4*), Signal Transducer and Activator of Transcription 5B (*STAT5B*), Transformation/Transcription Domain Associated Protein (*TRRAP*), Janus Kinase 3 (*JAK3*), Platelet-Derived Growth Factor Receptor Alpha (*PDGFRA*) and Signal Transducer and Activator of Transcription 3 (*STAT3*) with mutation frequencies of 16%, 15%, 14%, 14%, 14%, 13%, 11%, 10%, 10%, 10%, 8%, 8%,7%,7%, 6%, 5%, 5%, 4%, 4%, and 4% respectively, from the all the tumors samples (see **Figure 6A**). HLA-I peptides from NCOR1, TRRAP and JAK3 were presented by all the four patients Rom, IrK, FrA and Seo. PLCG1, TP53, and STAT3 were altered in at least three of the four patients, while FAT1 and LRP1B were changed in at least two of the four patients, and FAT4, TET2, and PTPRK were altered in at least one of the four patients. No HLA-I peptides was presented from FAT4, CARD11, GRIN2A, ARID1A, PREX2, POT1, DNMT3A, ERBB4, STAT5B and PDGFRA (see **Figure 6B**). The somatic in the SAAs (the top 20 genes with somatic mutation in SS as per the COSMIC database and those whose HLA-I peptides were presented by all the four patients or at least 3 of the four patients (Rom, IrK, FrA and Seo), that include *NCOR1, TRRAP, JAK3, PLCG1, TP53* and *STAT3*), were predominantly missense variant (a type of substitution in which the nucleotide change results in the replacement of one amino acid with another, a replacement that may alter the function of the protein). With mutation frequencies of 46.7%, 60.0%, 100%, 94.74%, 52.4% and 70% respectively. Nonsense mutations (that occur due to the substitution of a single base pair in a triplet codon), leading to one of three stop codons (UAG, UAA, and UGA). The triplet codon coding for an amino acid is therefore altered to one that prematurely stops mRNA translation and results in a truncated protein. They were found only in *NCOR1* and *TP53*, with frequencies of 20% and 23%, respectively (data not shown).



A

Gene	Mutation frequency	Patient			
		Rom	IrK	Seo	FrA
NCOR1	14%	x	x	x	x
TRRAP	5%	x	x	x	x
JAK3	4%	x	x	x	x
PLCG1	14%	x	x		x
TP53	13%	x	x	x	
STAT3	4%	x		x	x
FAT1	15%	x			x
LRP1B	10%		x		x
FAT4	16%		x		
TET2	8%		x		
PTPRK	7%	x			
CARD11	14%				
GRIN2A	11%				
ARID1A	10%				
PREX2	10%				
POT1	8%				
DNMT3A	7%				
ERBB4	6%				
STAT5B	5%				
PDGFRA	4%				

B

Figure 6. Top 20 genes with somatic mutation in Sezary syndrome as per catalogue of Somatic mutations in Cancer (COSMIC). A) COSMIC search set to Sezary syndrome, and filtered by tissue selection (hematopoietic and lymphoid), Sub-tissue selection (All), Histology selection (lymphoid neoplasm) and Sub-histology selection (mycosis fungoides-sezary syndrome). B) Frequency of somatic mutation of the SAAs (the top 20 genes with somatic mutation in Sezary syndrome as per the COSMIC database and HLA-I peptides presentation from the SAAs by the patients (Rom, IrK, FrA and Seo).

Immunogenicity of the LAAs

The immunogenicity of the SAAs (the top 20 genes with somatic mutation in SS as per the COSMIC database and whose protein HLA-I peptides were presented by all four patients or at least 3 of the four patients (Rom, IrK, FrA and Seo); NCOR1, TRRAP, JAK3, PLCG1, TP53 and STAT3) was determined by reverse immunology. NetMHCpan 4.1 BA in the IEDB was used with a binding affinity threshold of IC_{50} (500 nM), for the most frequent HLA alleles HLA-A*01:01, HLA-A*02:01, HLA-A*11:01, HLA-A*24:02, HLA-C*06:02, HLA-C*07:01 and HLA-C*07:02 that together represent ~90% of the human population. The immunogenicity score is represented as $1/IC_{50}$ (500 nM) and ranges from 0 to 1, indicating low to high immunogenicity. The immunogenicity of the SAAs varied depending the HLA allele (see **Figure 7**). It was generally highest for HLA-A*11:01 and HLA-A*02:01 and lowest for HLA-A*01:01. The immunogenicity of SAAs for all the alleles was comparable, with median score of less than 0.2 for HLA-A*11:01 and HLA-A*02:01 alleles, and less than 0.4 for all the other alleles, except HLA-A*01:01 which ranged between 0.1 and 0.6. TRRAP had the highest number of peptides within this binding affinity threshold of IC50 (500 nM).

SAA gene expression in major normal tissues

The gene expression profiles of the SAAs in primary normal human tissues were compared using the GeneCards database, as detailed in the Materials and Methods section. All the SAAs were expressed at low levels in all primary normal human tissues based on a 10% gene expression intensity cutoff. Only *STAT3* were expressed beyond the 10% cutoff. Beyond this cutoff, *STAT3* was expressed in whole blood, thymus, adipocyte, lung and prostate (see **Figure 8**).

Protein interaction partners of the LAAs

High numbers of protein interaction partners and the interaction of SAAs (NCOR1, TRRAP, JAK3, PLCG1, TP53 and STAT3) between and among them may indicate vital roles in SS. The STRING database was used to determine the protein interaction partners of the SAAs, which had been initially identified from experimental data obtained using a variety of biochemical, biophysical, and genetic techniques, as detailed in the Materials and Methods section. All SAAs (NCOR1, TRRAP, JAK3, PLCG1, TP53 and STAT3) had ten known interaction partners (see **Figure 9**). None of the SAAs were found to interact with each other, both

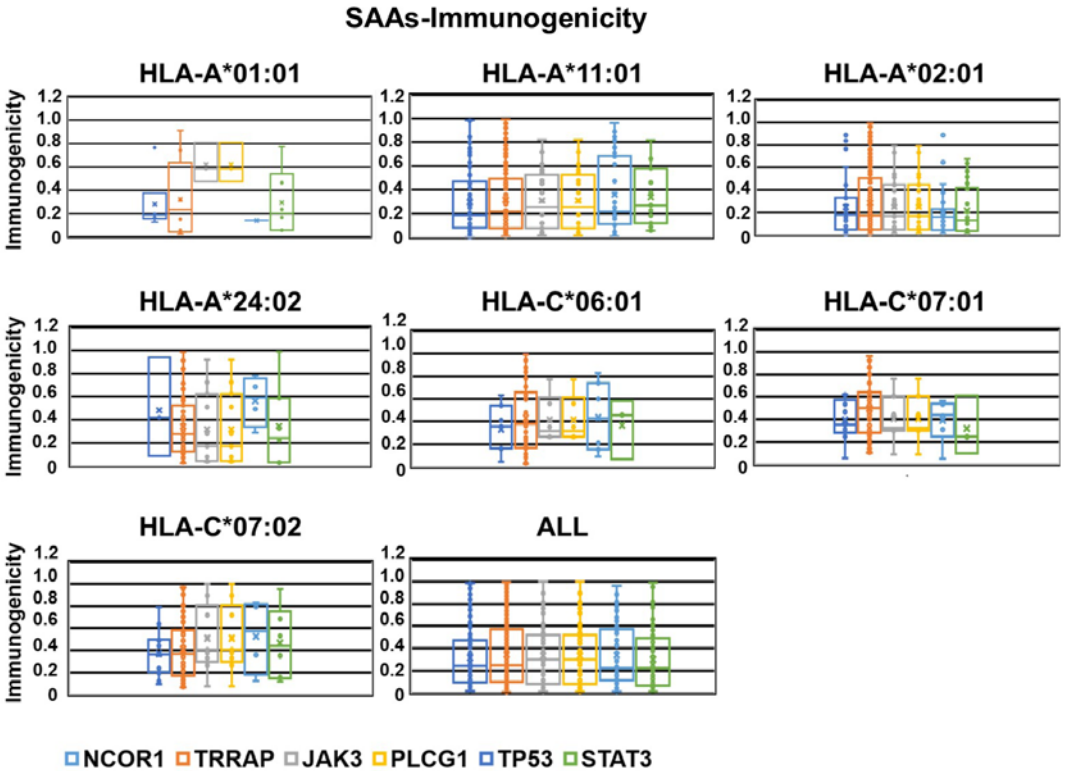


Figure 7. Immunogenicity of the SAAs for the most frequent HLA alleles, HLA-A*01:01, HLA-A*02:01, HLA-A*11:01, HLA-A*24:02, HLA-C*06:02, HLA-C*07:01, and HLA-C*07:02, which together represent more than 90% of the human population. The immunogenicity was determined using NetMHCpan 4.0 in IEDB [64], with a binding affinity threshold of IC_{50} (500 nM). The immunogenicity scores are presented as $1/IC_{50}$ (500 nM), with values ranging from 0 to 1, indicating low to high immunogenicity.

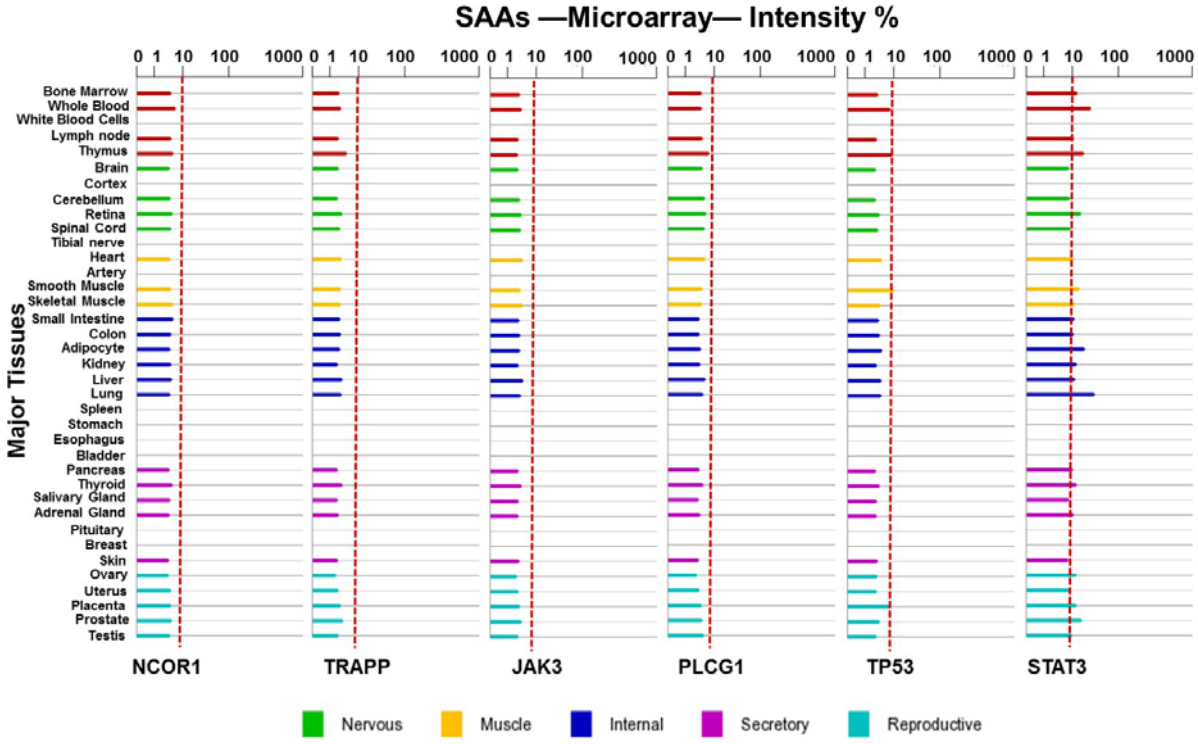


Figure 8. Gene expression profiles of the SAAs in normal human tissue were analysed and visualised using BioGPS (http://biogps.org). A low gene expression intensity cutoff of 10% indicated by the dotted line.

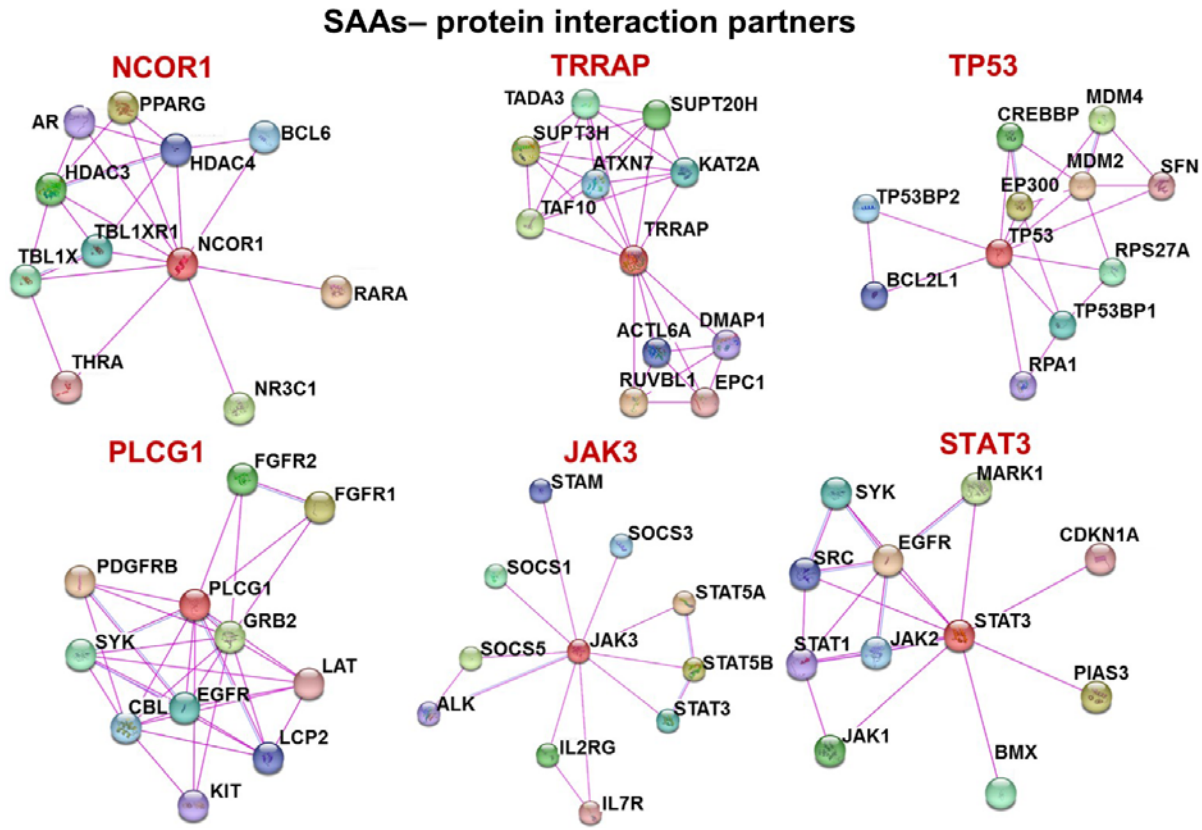


Figure 9. Known protein interaction partners of the SAAs were determined using STRING version 11.0 [66] for *Homo sapiens* with a medium score of 0.400 and a cutoff of 10 interaction partners.

at primary (directly via the first shell) (see **Figure 9**) and at secondary level (indirectly via the second shell) (data not shown), except JAK3 and STAT3, which interacted at the primary level.

Discussion

The total number of HLA-I–restricted peptides and source proteins identified in SS leukophoresis patient samples was about the same, and they were heterogeneous and individualised (see **Figure 1A**, **B** and **C**). Only a small fraction of HLA-I peptides and source proteins was found to be shared between and among the patients (see **Figure 1B** and **C**). The HLA-I–bound peptides were more heterogeneous and individualised than the source proteins, as the number of shared HLA-I peptides was slightly lower compared with that of the source proteins. This has also been observed in other human cell lines and tumour samples, such as melanoma, and depicts differences in the antigen processing and presentation in SS. In this case, the HLA-I peptides may also be attributed to the difference in the patient's HLA type, although this has not been fully confirmed.

Despite the fact that the HLA-I peptides and the source proteins were heterogeneous and individualised, a number of similarities were observed. First, in all SS leukophoresis patients' samples, nanopeptides were the most dominant and constituted between 29.0% to 55.1% of all the peptides identified, followed by decapeptides with 8.0% to 14.8% (see **Figure 2**). This dominance of nanopeptides has also been observed in other patient tumour samples and cell lines [19,28–31]. It indicates that the optimum length for MHC I-binding peptides in SS is nine amino acids. Secondly, the proteasome activity, in reference to C-terminus peptide processing, was found to be decreasing in activity with chymotrypsin, trypsin, and, lastly, caspase in all patients (see **Figure 3**).

Similarities were also observed in the HLA-I peptides of SS patients, their source proteins, subcellular locations, and molecular functions. The cytoplasm part, intracellular membrane-bounded organelle and cytosol were the

dominant subcellular locations of the source proteins (see **Figure 4**). Together, they accounted for 24.7% to 29.6 %, with the cytoplasmic part accounting for more than 14% of all the source proteins. This is different to the dominant subcellular locations of the HLA-I peptide source proteins of other human tumor samples and cell lines such as melanoma, multiple sclerosis autopsy samples, B lymphoblastic cell line, tipple- negative breast cancer cell line, leukemia tumuor samples and cell lines; where the dominant subcellular locations differed and varied e.g. in melanoma the dominant subcellular locations were the nucleus and the cytoplasm, multiple sclerosis autopsy samples, where cytoplasm and plasma membrane [19,28–32].

Second, the source proteins were involved in various molecular functions, especially in biosynthetic processes, biological regulation, catabolic processes, and anatomical structure development, with a similar proportion of proteins per molecular function (see **Figure 5**). This is different from other human tumour samples and cell lines. For instance, in leukaemia cell lines MUTZ3 and THP1, the source proteins were involved in cell communication/signal transduction, protein metabolism, and transcription factor activity/regulator activity [19]. In the B lymphoblastic cell line 721.221, the source proteins were predominantly involved in metabolism, cell growth and maintenance, cell communication, and stress response [30]. In multiple sclerosis autopsy samples, they entailed cellular assembly and organisation, nervous system function and development, cellular growth, and proliferation [31]. The similarities in source protein peptide sampling in SS, although unconfirmed, would imply similarities in protein turnover, because protein turnover correlates with source protein peptide sampling [33,34]. The availability of source proteins involved in immune response, apoptosis, and cell death in all patients would indicate an active immune response against the cancer cells by the patients.

Furthermore, the top 20 genes with somatic mutation in SS were *FAT4, FAT1, CARD11, NCOR1, PLCG1, TP53, GRIN2A, ARID1A, LRP1B, PREX2, POT1, TET2, DNMT3A, PTPRK, ERBB4, STAT5B, TRRAP, JAK3, PDGFRA* and *STAT3*, as per COSMIC, with decreasing mutation frequencies of 16% to 4% (see **Figure 6A**). Out of these, only HLA-I peptides from NCOR1, TRRAP and JAK3 were presented by all the four patients, PLCG1, TP53 and STAT33 by three of the four patients, FAT1 and LRP1B by two of the four patients, TET2 and PTPRK by one of the four patients. No HLA-I peptides were presented from FAT4, CARD11, GRIN2A, ARID1A, PREX2, POT1, DNMT3A, ERBB4, STAT5B and PDGFRA (see **Figure 6B**). The missense mutation was the predominant mutation, with mutation frequency ranging between 46.7% to 100% in the top 20 genes sampled by at least three of the four patients (see **Supplementary Figure 1**). Nonsense mutations were found only in *NCOR1* and *TP53*, with mutation frequency of 20% and 23% respectively (see **Supplementary Figure 1**). These mutation finding highlights the genetic heterogeneity of SS and points to *NCOR1, TRRAP, JAK3, PLCG1, TP53* and *STAT3* (the SAAs) as initial potential targets for T-cell based immunotherapeutic intervention, based on their high mutation rate and their protein HLA-I peptide presentation by at least three of the four patients. This is further substantiated by previous studies showing that SAAs are key regulators in various stages of cancer progression and are critical therapeutic targets. NCOR1 promotes cancer progression and therapy resistance by dysregulating transcriptional networks [35–41]. TRAPP promotes cancer development and progression by enhancing cancer stem cell traits, suppressing immune responses, regulating oncogenic transcription factors, and supporting cancer cell proliferationsupport [42–46]. JAK3 promotes the proliferation and survival of malignant T-cells and influences immune suppression [47–52]. PLCG1 promotes progression by supporting tumour growth and therapy resistance (53,54). TP53 mutations impair tumour suppression and contribute to therapy resistance and poor prognosis [53–59]. STAT3 promotes tumour cell survival, proliferation, immune evasion, and therapy resistance (62–64).

Furthermore, the immunogenicity of these SAAs (NCOR1, TRRAP, JAK3, PLCG1, TP53 and STAT3) was allele-dependent. It was generally highest for HLA-A*11:01 and HLA-A*02:01 and lowest for HLA-A*01:01 (see **Figure 7**). The immunogenicity of SAAs for all the alleles was comparable, with TRRAP showing the highest number of affinity peptides. These findings suggest that specific alleles may play a crucial role in shaping the immune response to SAAs in SS, with

implications for personalised immunotherapy approaches. Regarding the gene expression profile of the SAAs in normal human tissues, expression levels were generally low, except for STAT3 in certain tissues (see **Figure 8**). Therefore, targeting *STAT3* in SS immunotherapy may lead to off-target effects in these select tissues. Regarding the protein interaction partners, all SAAs exhibited a high number of protein interaction partners, emphasising their vital roles in SS (see **Figure 9**). With only JAK3 and STAT3 interacting with each other at the primary level, this interaction warrants further investigation to determine its role in SS.

Overall, despite the small patient sample size and limited demographics, this study's findings primarily contribute to our understanding of the peptidomic landscape in SS and highlight SAAs (NCOR1, TRRAP, JAK3, PLCG1, TP53) as potential targets for immunotherapeutic interventions. The immunotherapeutic potential is based on the SAAs high mutation rate in SS, HLA-I peptides presentation, high immunogenicity, low gene expression profiles in normal human tissue, and a high number of protein interaction partners. Including their role in cancer, as mentioned in the previous studies above. *STAT3* falls short in the SAAs list, as it is also highly expressed in some normal human tissues. However, its interaction with JAK3 at the primary level warrants future studies to determine the role of this interaction.

Disclosures

Author contributions
L.W.N. conceived the project, designed and performed the experiments, analysed and interpreted results, and wrote the paper. K.T-T contributed to the generation of LC-MS/MS data. P.W. conceived the project, contributed reagents/materials, and contributed to the interpretation of the results. All authors reviewed the results and approved the manuscript.

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
The study was supported by funds from the German Ministry for Research and Education (BMBF, 13N9197), the Berlin Cancer Society (WAFF200824), and the German Academic Exchange Service (DAAD). Many thanks to Dr. med. Christoph Gille, at the Institute of Biochemistry, Charité-Universitätsmedizin Berlin, for his support in Analysing Sub-Cellular locations and molecular functions of source proteins.

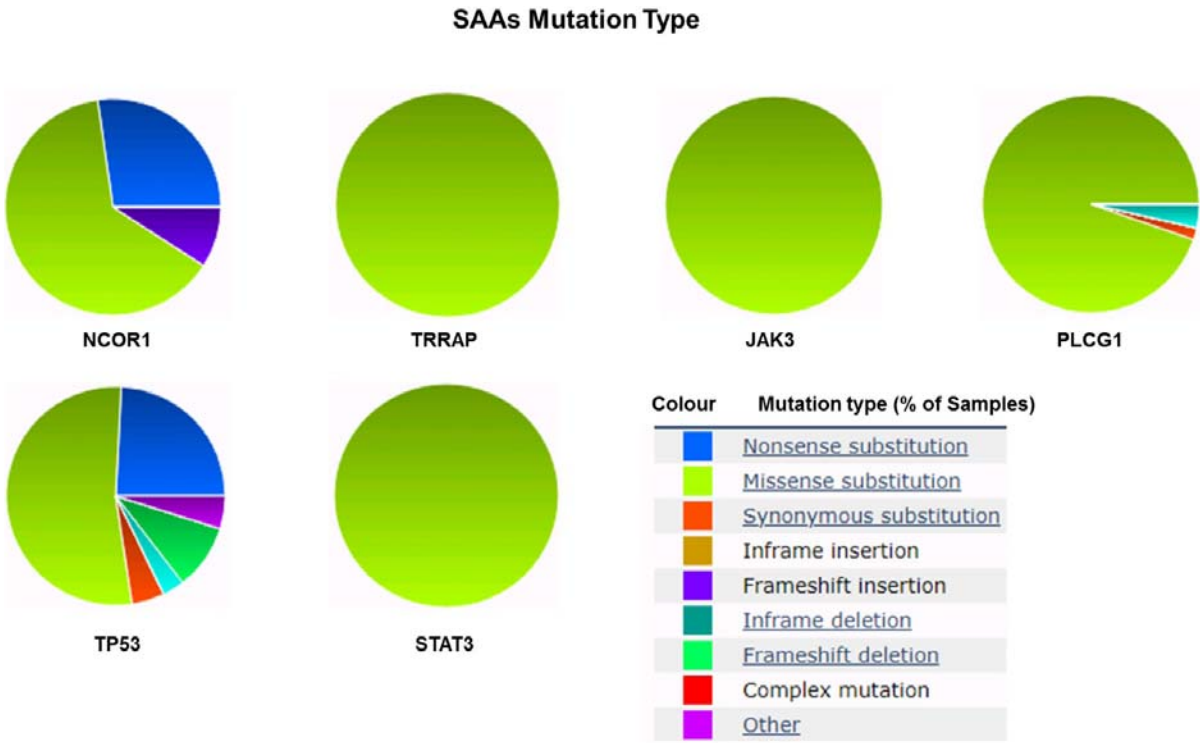
References

1. Alaggio R, Amador C. The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Lymphoid Neoplasms. Leukemia. 2022;36:1720–48. https://doi.org/10.1038/s41375-022-01620-2.
2. Willemze R, Cerroni L, Kempf W, Berti E, Facchetti F. The 2018 update of the WHO-EORTC classification for primary cutaneous lymphomas. Blood. 2019;133:1703–14. https://doi.org/10.1182/blood-2018-11-881268.
3. Agar NS, Wedgeworth E, Crichton S, Mitchell TJ, Cox M, Ferreira S, et al. Survival outcomes and prognostic factors in mycosis fungoides/Sézary syndrome: validation of the revised International Society for Cutaneous Lymphomas/European Organisation for Research and Treatment of Cancer staging proposal. J Clin Oncol. 2010;28:4730–9. https://doi.org/10.1200/jco.2009.27.7665.
4. Scarisbrick JJ, Prince HM, Vermeer MH, Quaglino P, Horwitz S, Porcu P, et al. Cutaneous Lymphoma International Consortium Study of Outcome in Advanced Stages of Mycosis Fungoides and Sézary Syndrome: Effect of Specific Prognostic Markers on Survival and Development of a Prognostic Model. J Clin Oncol. 2015;33:3766–73. https://doi.org/10.1200/jco.2015.61.7142.
5. Jawed SI, Myskowski PL, Horwitz S, Moskowitz A, Querfeld C. Primary cutaneous T-cell lymphoma (mycosis fungoides and Sézary syndrome): part II. Prognosis, management, and future directions. J Am Acad Dermatol. 2014;70:223.e1-17; quiz 240–2. https://doi.org/10.1016/j.jaad.2013.08.033.
6. Querfeld C, Zain J, Rosen ST. Primary Cutaneous T-Cell Lymphomas: Mycosis Fungoides and Sezary Syndrome. Cancer Treat Res. 2019;176:225–48. https://doi.org/10.1007/978-3-319-99716-2_11.
7. Stuver R, Geller S. Advances in the treatment of mycoses fungoides and Sézary syndrome: a narrative update in skin-directed therapies and immune-based treatments. Front Immunol. 2023;14:1284045. https://doi.org/10.3389/fimmu.2023.1284045.
8. Roccuzzo G, Giordano S, Fava P, Pileri A, Guglielmo A, Tonella L, et al. Immune Check Point Inhibitors in Primary Cutaneous T-Cell Lymphomas: Biologic Rationale, Clinical Results and Future Perspectives. Front Oncol. 2021;11:733770. https://doi.org/10.3389/fonc.2021.733770.
9. Larocca C, Kupper T. Mycosis Fungoides and Sézary Syndrome: An Update. Hematol Oncol Clin North Am. 2019;33:103–20. https://doi.org/10.1016/j.hoc.2018.09.001.
10. Buus TB, Willerslev-Olsen A. Single-cell heterogeneity in Sézary syndrome. Blood Advances. 2018;2:2115–26. https://doi.org/10.1182/bloodadvances.2018022608.
11. Litvinov IV, Tetzlaff MT, Thibault P, Gangar P, Moreau L, Watters AK, et al. Gene expression analysis in Cutaneous T-Cell Lymphomas (CTCL) highlights disease heterogeneity and potential diagnostic and prognostic indicators. Oncoimmunology. 2017;6:e1306618. https://doi.org/10.1080/2162402x.2017.1306618.

12. Vergnolle I, Douat-Beyries C, Boulinguez S, Rieu JB. CD158k and PD-1 expressions define heterogeneous subtypes of Sezary syndrome. Blood Advances. 2022;6:1813–25. https://doi.org/10.1182/bloodadvances.2021005147.
13. Bassani-Sternberg M, Bräunlein E, Klar R, Engleitner T, Sinitcyn P, Audehm S, et al. Direct identification of clinically relevant neoepitopes presented on native human melanoma tissue by mass spectrometry. Nat Commun. 2016;7:13404. https://doi.org/10.1038/ncomms13404.
14. Caron E, Kowalewski DJ, Chiek Koh C, Sturm T, Schuster H, Aebersold R. Analysis of Major Histocompatibility Complex (MHC) Immunopeptidomes Using Mass Spectrometry. Mol Cell Proteomics. 2015;14:3105–17. https://doi.org/10.1074/mcp.O115.052431.
15. Hellinger R, Sigurdsson A. Peptidomics. Nature Reviews Methods Primers. 2023;3.25 https://doi.org/10.1038/s43586-023-00205-2.
16. Kalaora S, Barnea E, Merhavi-Shoham E, Qutob N, Teer JK, Shimony N, et al. Use of HLA peptidomics and whole exome sequencing to identify human immunogenic neo-antigens. Oncotarget. 2016;7:5110–7. https://doi.org/10.18632/oncotarget.6960.
17. Shapiro IE, Bassani-Sternberg M. The impact of immunopeptidomics: From basic research to clinical implementation. Semin Immunol. 2023;66:101727. https://doi.org/10.1016/j.smim.2023.101727.
18. Vaughan K, Xu X, Caron E, Peters B, Sette A. Deciphering the MHC-associated peptidome: a review of naturally processed ligand data. Expert Rev Proteomics. 2017;14:729–36. https://doi.org/10.1080/14789450.2017.1361825.
19. Nyambura LW, Jarmalavicius S, Baleeiro RB. Diverse HLA-I Peptide Repertoires of the APC Lines MUTZ3-Derived Immature and Mature Dendritic Cells and THP1-Derived Macrophages. The Journal of Immunology. 2016;197:2102–9. https://doi.org/10.4049/jimmunol.1600762.
20. Demine R, Walden P. Sequit: software for de novo peptide sequencing by matrix-assisted laser desorption/ionization post-source decay mass spectrometry. Rapid Commun Mass Spectrom RCM. England; 2004;18:907–13. https://doi.org/10.1002/rcm.1420.
21. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Res. England; 2023;51:D523–31. https://doi.org/10.1093/nar/gkac1052.
22. Mishra GR, Suresh M, Kumaran K, Kannabiran N, Suresh S, Bala P, et al. Human protein reference database--2006 update. Nucleic Acids Res. 2006;34:D411-4. https://doi.org/10.1093/nar/gkj141.
23. Sondka Z, Dhir NB, Carvalho-Silva D. COSMIC: a curated database of somatic variants and clinical data for cancer. Nucleic Acids Research. 2024;52:D1210-d1217. https://doi.org/10.1093/nar/gkad986.
24. Vita R, Mahajan S, Overton JA, Dhanda SK, Martini S, Cantrell JR, et al. The Immune Epitope Database (IEDB): 2018 update. Nucleic Acids Res. England; 2019;47:D339–43. https://doi.org/10.1093/nar/gky1006.
25. Gonzalez-Galarza FF, McCabe A, Santos E, Jones J, Takeshita L, Ortega-Rivera ND, et al. Allele frequency net database (AFND) 2020 update: gold-standard data classification, open access genotype data and new query tools. Nucleic Acids Res. 2020;48:D783-d788. https://doi.org/10.1093/nar/gkz1029.
26. Stelzer G, Rosen N, Plaschkes I, Zimmerman S, Twik M, Fishilevich S, et al. The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. Curr Protoc Bioinforma. United States; 2016;54:1.30.1-1.30.33. https://doi.org/10.1002/cpbi.5.
27. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. England; 2019;47:D607–13. https://doi.org/10.1093/nar/gky1131.
28. Jarmalavicius S, Welte Y, Walden P. High immunogenicity of the human leukocyte antigen peptidomes of melanoma tumor cells. J Biol Chem. 2012;287:33401–11. https://doi.org/10.1074/jbc.M112.358903.
29. Pritchard AL, Hastie ML, Neller M, Gorman JJ, Schmidt CW, Hayward NK. Exploration of peptides bound to MHC class I molecules in melanoma. Pigment Cell Melanoma Res. 2015;28:281–94. https://doi.org/10.1111/pcmr.12357.
30. Schellens IM, Hoof I, Meiring HD, Spijkers SN, Poelen MC, van Gaans-van den Brink JA, et al. Comprehensive Analysis of the Naturally Processed Peptide Repertoire: Differences between HLA-A and B in the Immunopeptidome. PLoS One. 2015;10:e0136417. https://doi.org/10.1371/journal.pone.0136417.
31. Fissolo N, Haag S, de Graaf KL, Drews O, Stevanovic S, Rammensee HG, et al. Naturally presented peptides on major histocompatibility complex I and II molecules eluted from central nervous system of multiple sclerosis patients. Mol Cell Proteomics. 2009;8:2090–101. https://doi.org/10.1074/mcp.M900001-MCP200.
32. Brito Baleeiro R, Liu P, Chard Dunmall LS, Di Gioia C, Nagano A, Cutmore L. Personalized neoantigen viro-immunotherapy platform for triple-negative breast cancer. Journal for ImmunoTherapy of Cancer. 2023;11:e007336. https://doi.org/10.1136/jitc-2023-007336.
33. Bassani-Sternberg M, Pletscher-Frankild S, Jensen LJ, Mann M. Mass spectrometry of human leukocyte antigen class I peptidomes reveals strong effects of protein abundance and turnover on antigen presentation. Mol Cell Proteomics. 2015;14:658–73. https://doi.org/10.1074/mcp.M114.042812.
34. Rock KL, Farfán-Arribas DJ, Colbert JD, Goldberg AL. Re-examining class-I presentation and the DRiP hypothesis. Trends Immunol. 2014;35:144–52. https://doi.org/10.1016/j.it.2014.01.002.
35. Abedin SA, Thorne JL, Battaglia S, Maguire O, Hornung LB, Doherty AP, et al. Elevated NCOR1 disrupts a network of dietary-sensing nuclear receptors in

bladder cancer cells. Carcinogenesis. 2009;30:449–56. https://doi.org/10.1093/carcin/bgp005.
36. Fozzatti L, Park JW, Zhao L, Willingham MC, Cheng SY. Oncogenic Actions of the Nuclear Receptor Corepressor (NCOR1) in a Mouse Model of Thyroid Cancer. PLoS One. 2013;8:e67954. https://doi.org/10.1371/journal.pone.0067954.
37. Kim H, Park SH. TNFα Enhances Tamoxifen Sensitivity through Dissociation of ERα-p53-NCOR1 Complexes in ERα-Positive Breast Cancer. Cancers. 2021;13(11):2601. https://doi.org/10.3390/cancers13112601.
38. Zhang Z, Yamashita H, Toyama T, Sugiura H, Ando Y, Mita K, et al. NCOR1 mRNA is an independent prognostic factor for breast cancer. Cancer Lett. 2006;237:123–9. https://doi.org/ 10.1016/j.canlet.2005.05.046.
39. Shu S, Li Z, Liu L, Ying X, Zhang Y, Wang T, et al. HPV16 E6-Activated OCT4 Promotes Cervical Cancer Progression by Suppressing p53 Expression via Co-Repressor NCOR1. Front Oncol. 2022;12:900856. https://doi.org/10.3389/fonc.2022.900856.
40. St-Jean S, De Castro AC, Lecours M, Jones C, Rivard N, Rodier F. NCOR1 Sustains Colorectal Cancer Cell Growth and Protects against Cellular Senescence. Cancers. 2021;13(17):4414. https://doi.org/10.3390/cancers13174414.
41. Tan J, Zhang S, Li L, Mu J, Wang Z, Zhang L, et al. Abnormally localized DLK1 interacts with NCOR1 in non-small cell lung cancer cell nuclear. Biosci Rep. 2019;39(12):BSR20192362. https://doi.org/10.1042/bsr20192362.
42. Detilleux D, Raynaud P, Pradet-Balade B, Helmlinger D. The TRRAP transcription cofactor represses interferon-stimulated genes in colorectal cancer cells. Elife. 2022;11:e69705. https://doi.org/10.7554/eLife.69705.
43. Kang KT, Kwon YW, Kim DK, Lee SI, Kim KH, Suh DS, et al. TRRAP stimulates the tumorigenic potential of ovarian cancer stem cells. BMB Rep. 2018;51:514–9. https://doi.org/10.4048/jbc.2016.19.1.61.
44. Kang KT, Shin MJ, Moon HJ, Choi KU, Suh DS. TRRAP Enhances Cancer Stem Cell Characteristics by Regulating NANOG Protein Stability in Colon Cancer Cells. International Journal of Molecular Sciences. 2023;24(7):6260. https://doi.org/10.3390/ijms24076260.
45. Kwan SY, Sheel A, Song CQ, Zhang XO, Jiang T, Dang H, et al. Depletion of TRRAP Induces p53-Independent Senescence in Liver Cancer by Down-Regulating Mitotic Genes. Hepatology. 2020;71:275–90. https://doi.org/10.1002/hep.30807.
46. Wang J, Shan M, Liu T, Shi Q, Zhong Z, Wei W, et al. Analysis of TRRAP as a Potential Molecular Marker and Therapeutic Target for Breast Cancer. J Breast Cancer 2016;19:61–7. https://doi.org/10.4048/jbc.2016.19.1.61.
47. Jiang Y, Li T. Morphine in Combination with Ketamine Improves Cervical Cancer Pain and Suppresses Immune Function via the JAK3/STAT5 Pathway. Journal of Healthcare Engineering. 2022;2022:9364365. https://doi.org/10.1155/2022/9364365.
48. Jin J, Guo Q, Xie J, Jin D, Zhu Y. Combination of MEK Inhibitor and the JAK2-STAT3 Pathway Inhibition for the Therapy of Colon Cancer. Pathol Oncol Res. 2019;25:769–75. https://doi.org/10.1007/s12253-019-00592-6.
49. Li SD, Ma M, Li H, Waluszko A, Sidorenko T, Schadt EE, et al. Cancer gene profiling in non-small cell lung cancers reveals activating mutations in JAK2 and JAK3 with therapeutic implications. Genome Med. 2017;9:89. https://doi.org/ 10.1186/s13073-017-0478-1.
50. Van Allen EM, Golay HG, Liu Y, Koyama S, Wong K, Taylor-Weiner A, et al. Long-term Benefit of PD-L1 Blockade in Lung Cancer Associated with JAK3 Activation. Cancer Immunol Res. 2015;3:855–63. https://doi.org/10.1158/2326-6066.cir-15-0024.
51. Wu T, Yu J, Wang C, Jin Y, Zheng X, Chen L, et al. Novel Potent EGFR-JAK3 Dual-Target Inhibitor that Overcomes KRAS Mutation Resistance in Colorectal Cancer. Anticancer Agents Med Chem. 2023;23(4):440–9. https://doi.org/10.2174/1871520622666220609112816.
52. Zhou C, Chen L, Chen R, Xu F, Huang Z, Huang R, et al. miR-4486 enhances cisplatin sensitivity of gastric cancer cells by restraining the JAK3/STAT3 signalling pathway. J Chemother. 2022;34:35–44. https://doi.org/ 10.1080/1120009x.2021.1936957.
53. Canale M, Andrikou K, Priano I, Cravero P, Pasini L, Urbini M. The Role of TP53 Mutations in EGFR-Mutated Non-Small-Cell Lung Cancer: Clinical Significance and Implications for Therapy. Cancers (Basel) 2022;14(5):1143. https://doi.org/10.3390/cancers14051143.
54. Chen J, Chang X, Li X, Liu J, Wang N, Wu Y, et al. The heterogeneous impact of targeted therapy on the prognosis of stage III/IV colorectal cancer patients with different subtypes of TP53 mutations. Cancer Medicine. 2023;12:21920–32. https://doi.org/10.1002/cam4.6766.
55. Liu B, Yi Z, Guan Y, Ouyang Q, Li C, Guan X, et al. Molecular landscape of TP53 mutations in breast cancer and their utility for predicting the response to HER-targeted therapy in HER2 amplification-positive and HER2 mutation-positive amplification-negative patients. Cancer Medicine. 2022;11(14):2767–78. https://doi.org/ 10.1002/cam4.4652.
56. Liu J, Gao J. Efficacy of immunotherapy as second-line or later-line therapy and prognostic significance of KRAS or TP53 mutations in advanced non-small cell lung cancer patients. Eur J Cancer Prev. 2023;32:590–9. https://doi.org/10.1097/cej.0000000000000799.
57. Michikawa C, Torres-Saavedra PA, Silver NL, Harari PM, Kies MS, Rosenthal DI, et al. Evolutionary Action Score of TP53 Analysis in Pathologically High-Risk Human Papillomavirus-Negative Head and Neck Cancer From a Phase 2 Clinical Trial: NRG Oncology Radiation Therapy Oncology Group 0234. Advances in Radiation Oncology. 2022;7(6):100989. https://doi.org/ 10.1016/j.adro.2022.100989.
58. Roeper J, Christopoulos P, Falk M, Heukamp LC, Tiemann M, Stenzinger A, et al. TP53 co-mutations as

an independent prognostic factor in 2nd and further line therapy-EGFR mutated non-small cell lung cancer IV patients treated with osimertinib. Transl Lung Cancer Res. 2022;11:4–13. https://doi.org/10.21037/tlcr-21-754.
59. Sun H, Ren P, Chen Y, Lan L, Yan Z, Yang Y, et al. Optimal therapy for concomitant EGFR and TP53 mutated non-small cell lung cancer: a real-world study. BMC Cancer. 2023;23:198. https://doi.org/ 10.1186/s12885-023-10637-4.
60. Abdelhamid D, Abdel-Aziz M, Li X, Jiang W, Dong S, Li W, et al. STAT3 Inhibitors: A Novel Insight for Anticancer Therapy of Pancreatic Cancer. Biomolecules 2022;12(10):1450. https://doi.org/10.3390/biom12101450.
61. Allam A, Yakou M, Pang L, Ernst M, Huynh J. Exploiting the STAT3 Nexus in Cancer-Associated Fibroblasts to Improve Cancer Therapy. Front Immunol. 2021;12:767939. https://doi.org/10.3389/fimmu.2021.767939.
62. Dong J, Cheng XD, Zhang WD, Qin JJ. Recent Update on Development of Small-Molecule STAT3 Inhibitors for Cancer Therapy: From Phosphorylation Inhibition to Protein Degradation. Journal of Medicinal Chemistry. 2021;64(13):8884–8915. https://doi.org/10.1021/acs.jmedchem.1c00629.



Supplementary Figure 1.

ORIGINAL PAPER

JMS Journal of Medical Science

Clostridioides difficile infection after surgical myocardial revascularisation: unravelling risk factors and impact on postoperative outcomes

Maciej Łuczak
Department of Cardiac Surgery, Józef Struś Hospital, Poznań, Poland
https://orcid.org/0009-0000-3660-7275
Corresponding author: mluczak@szpital-strusia.poznan.pl

Krzysztof Greberski
Department of Cardiac Surgery, Józef Struś Municipal Hospital, Poznań, Poland
Faculty of Health Sciences, Poznan University of Medical Sciences, Poznań, Poland
https://orcid.org/0000-0003-1002-4738

Marian Burysz
Department of Cardiac Surgery, Regional Specialist Hospital, Grudziądz, Poland
Thoracic Research Centre, Collegium Medicum Nicolaus Copernicus University, Innovative Medical Forum, Bydgoszcz, Poland
https://orcid.org/0009-0003-1257-0510

Bartłomiej Perek
Department of Cardiac Surgery and Transplantology, Poznan University of Medical Sciences, Poznań, Poland
https://orcid.org/0000-0003-2398-9571

Radosław Jarząbek
Department of Cardiac Surgery, Józef Struś Municipal Hospital, Poznań, Poland
https://orcid.org/0000-0002-2842-3318

Paweł Bugajski
Department of Cardiac Surgery, Józef Struś Municipal Hospital, Poznań, Poland
Faculty of Health Sciences, Poznan University of Medical Sciences, Poznań, Poland
https://orcid.org/0000-0003-1207-2979

https://doi.org/10.20883/medical.e1384

Keywords: *Clostridioides difficile*, cardiac surgery, risk factors, postoperative impact, antibiotic usage, geographical analysis, hospitalization time

Received 2025-08-26
Accepted 2025-08-10
Published 2025-09-26

How to Cite: Łuczak M, Greberski K, Burysz M, Perek B, Jarząbek R, Bugajski P. *Clostridioides difficile* infection after surgical myocardial revascularisation: unravelling risk factors and impact on postoperative outcomes. Journal of Medical Science. 2025 September;94(3);e1384. https://doi.org/10.20883/medical.e1384

ABSTRACT

Aim. *Clostridioides difficile* infection (CDI) poses a significant threat to postoperative cardiac surgery patients. This study examines the impact of specific pre-, intra-, and post-operative factors, along with geographical considerations, on *Clostridioides difficile* (CD) incidence and its consequences. The study aims to identify factors contributing to increased CD prevalence in cardiac surgery units.

Material and methods. A single-centre cohort of 3502 patients undergoing surgical myocardial revascularisation between January 2013 and March 2018 was analysed, with 48 diagnosed with CDI. Preoperative risk factors include the use of broad-spectrum antibiotics, advanced age, comorbidities, and prolonged hospital stays. Intraoperatively, attention is given to catheter-related issues, mechanical ventilation, and the use of blood products. Postoperatively, the study assesses CDI's impact on recovery, complications, and outcomes. A geographical analysis explores regional variation in CDI incidence.

Results. Results indicate a CDI incidence of 1.37%, aligning with existing literature trends. Demographically matched controls show no significant differences in age, gender, or location. Higher *Body Mass Index* and lower left ventricular ejection fraction are identified as significant risk factors. Laboratory findings indicate elevated CRP levels and increased platelet count associated with CDI. Postoperative CDI significantly prolongs hospitalisation time. EuroSCORE II values are higher in the CDI group, though not statistically significant.
Conclusions. The study offers a comprehensive understanding of CDI dynamics in cardiac surgery, emphasising the need for tailored preventive measures. Specific risk factors and regional variations underscore the importance of vigilant monitoring and early intervention. Future research should include larger cohorts and explore gut microbiota for refined strategies.

Introduction

Clostridioides difficile (CD) is the leading cause of antibiotic-associated diarrhoea, healthcare-associated diarrhoea, and colitis, representing a significant risk for postoperative patients [1–3]. This gram-positive, strictly anaerobic, spore-forming bacillus is widely present in the environment, with asymptomatic colonisation observed in approximately 2% of healthy adults and up to 14% of the elderly population [1]. Despite ongoing efforts to curb nosocomial transmission, both the incidence and severity of *Clostridioides difficile* infection (CDI) have increased globally in recent years [4]. The use of broad-spectrum antibiotics remains the principal risk factor, compounded by advanced age, comorbidities, extended hospital stays, and the use of proton pump inhibitors or histamine-2 receptor antagonists [2,5]. Certain geographic areas, notably the Northeastern United States, have been identified as regions with particularly high CDI prevalence [5,6].

Surgical patients, particularly those undergoing cardiac procedures, are exposed to additional infection risk factors, including catheter-related complications, prolonged mechanical ventilation, substantial blood product transfusions, indwelling catheter drainage, and open surgical fields [7]. The clinical presentation of CDI ranges from asymptomatic colonisation and mild diarrhoea to severe, life-threatening conditions such as fulminant colitis with sepsis, pseudomembranous colitis, toxic megacolon, transmural pancolitis requiring colectomy, and multi-organ failure [1–4]. The reported incidence of CD-related diarrhoea following surgical interventions varies widely, ranging from 0.3% to 8.4% [3].

The rising number of CDI cases observed in the cardiac surgery unit has prompted a comprehensive investigation aimed at evaluating the influence of specific preoperative, intraoperative, and postoperative factors – as well as patients' geographic origin – on the risk of CDI. The study further seeks to assess the downstream impact of CDI on postoperative recovery and outcomes.

Factors under scrutiny

To comprehensively evaluate the multifactorial nature of CDI following cardiac surgery, the present study examines preoperative conditions, intraoperative factors, and postoperative outcomes. Key preoperative risk factors under investigation include the use of broad-spectrum antibiotics, advanced age, coexisting medical conditions, and prolonged hospitalisation. Intraoperatively, the analysis focuses on catheter-associated complications, extended mechanical ventilation, high-volume transfusion of blood products, persistent drainage via indwelling catheters, and the presence of open surgical cavities. In the postoperative phase, the study explores how CDI influences recovery, with particular attention to complication rates and the potential need for additional interventions.

Geographical considerations

In addition to patient-related factors, this study considers geographic location as a potential contributor to CDI incidence. Building on prior findings that identify certain regions as CDI hotspots, we explore whether similar regional disparities exist within cardiac surgery settings. This analysis moves beyond simple geographic correlation, aiming instead to identify contextual or systemic factors that may underlie higher local prevalence rates.

Impact on postoperative course

The final dimension of this study focuses on the postoperative impact of CDI. By analysing how

CDI influences recovery patterns, the incidence of complications, and overall clinical outcomes, we aim to offer clinicians actionable insights that support risk reduction and optimise postoperative care pathways.

In summary, this investigation provides a comprehensive assessment of CDI following cardiac surgery, with particular attention to its multifactorial risk profile and postoperative consequences. Our findings aim to deepen the understanding of CDI in this high-risk population and inform the development of targeted preventive and management strategies in cardiac surgical practice.

Material and methods

Patient selection and diagnostic criteria

This retrospective study was conducted at the Department of Cardiac Surgery, J. Strus Municipal Hospital in Poznan, and covered the period from January 2013 to March 2018. During this time, 3502 patients underwent surgical myocardial revascularisation. Among them, 48 cases of CDI were identified. For comparative analysis, a control group of 52 patients without CDI was selected, matched for key demographic parameters.

The diagnosis of CDI was based on the presence of clinical symptoms and laboratory confirmation of CD toxin in stool samples [8]. For patients presenting with diarrhoea during hospitalisation, defined according to World Health Organisation criteria as three or more loose or liquid stools per day or an increase relative to the patient's regular pattern [9], nucleic acid amplification testing was performed. Stool specimens were analysed using the C. diff Quik Chek Complete assay (TECHLAB, USA).

Retrospective statistical analysis

The retrospective analysis encompassed a broad range of variables, including demographic and laboratory data, preoperative clinical status, surgical characteristics, procedure type and timing, intraoperative durations, and the quantity of transfused blood products. Outcomes assessed included in-hospital mortality, defined as death occurring during the same hospitalisation as the cardiac surgery, and total length of stay.

The study was conducted in accordance with the Declaration of Helsinki and received approval from the Local Bioethics Committee of the Poznan University of Medical Sciences (approval date: July 3, 2024; ID: KB-533/24). The committee classified the research as a non-interventional, retrospective study based on analysis of existing medical records. Data were extracted from the electronic records of the Department of Cardiac Surgery at J. Strus Multispecialty Municipal Hospital in Poznan between July and August 2024. All personally identifiable information was anonymised at the point of extraction, and patients were assigned sequential numerical codes.

Perioperative antibiotic therapy

All patients received standard perioperative antibiotic prophylaxis, consisting of cefazolin administered at a weight-adjusted dose. The initial dose was given one hour before the start of surgery, and antibiotic coverage was continued for 48 hours following the procedure.

Severity stages of CDI and treatment protocol

The severity of CDI was classified into three categories: non-severe (white blood cell count ≤15,000 cells/mL and serum creatinine <1.5 mg/dL), severe (white blood cell count ≥15,000 cells/mL or serum creatinine >1.5 mg/dL), and fulminant, defined by the presence of hypotension or shock, ileus, or megacolon [10]. Treatment followed the current guidelines of the European Society of Clinical Microbiology and Infectious Diseases [11]. Patients with confirmed CDI based on stool toxin testing were isolated from asymptomatic individuals. Therapeutic regimens included metronidazole and vancomycin, in accordance with established recommendations. Notably, none of the patients in the study group progressed to fulminant CDI.

This structured approach to patient selection, diagnostic confirmation, and treatment standardisation provides the methodological basis for evaluating the clinical impact of CDI in the context of cardiac surgery.

Statistical analysis

Statistical analyses were adapted to the scale and distribution of each variable. Quantitative data were presented as means accompanied by minimum and maximum values, while qualitative variables were expressed as absolute numbers and percentages.

The normality of continuous variables was evaluated using the Shapiro-Wilk test. Variables with normal distribution and homogeneity of variance were compared using the Student's t-test for independent samples. In contrast, non-normally distributed variables were assessed using the Mann-Whitney U test.

For categorical variables, statistical significance was determined using Pearson's chi-square test for comparisons involving two groups, and the Fisher-Freeman-Halton test for variables with more than two subgroups.

A p-value of ≤ 0.05 was considered statistically significant. All analyses were performed using Statistica 13 software (StatSoft, USA).

Results

Incidence of CDI

Out of the 3502 patients subjected to analysis, CDI was identified in 48 individuals, representing an incidence of 1.37%.

Demographic characteristics

The control group, meticulously selected to be demographically comparable, exhibited no statistically significant differences in terms of age p-value = 0.4724 and gender p-value = 0.2949 when compared to the CDI group (see **Table 1**). Similarly, an exploration of the county's location concerning Poznan p-value = 0.2171 and the ZIP code p-value = 0.4069 (see **Table 2**) revealed no discernible relationship between the region of residence and the occurrence of CDI.

Comorbidities and their correlation with CDI

No significant differences were observed between the two study groups concerning the impact of diabetes, hypertension, pulmonary hypertension, hyperlipidemia, kidney failure, asthma, chronic lung disease, cerebrovascular diseases, peripheral vascular diseases, past heart attack, and nicotine use on the risk of CDI (see **Table 3**).

It is noteworthy that both study groups exhibited a high prevalence of hypertension (87.5% and 94.2%) and hyperlipidemia (91.7% and 92.3%).

Table 1. Demographic characteristics of patients in the study, comparing those with CDI to those without CDI.

Coefficient	Patients with CDI (*N* = 48)	Patients without CDI (*N* = 52)	*p*-value
Age [years]	67 [51–87]	68 [52–83]	0.4724 [1]
Gender (female/male)	8 [16.7%]/40 [83.3%]	5 [9.6%]/47 [90.4%]	0.2949 [2]

[1] Student's t-test; [2] Pearson's chi-square test; CDI – *Clostridioides difficile* infection

Table 2. Distribution of CDI and non-CDI patients according to geographic location and ZIP code.

Coefficient		Patients with CDI (*N* = 48)	Patients without CDI (*N* = 52)	*p*-value
Geographical location	Poznań county	19 [39.6%]	22 [42.3%]	0.2171 [1]
	On the east side	11 [22.9%]	12 [23.1%]	
	On the south side	4 [8.3%]	11 [21.2%]	
	On the west side	10 [20.8%]	5 [9.6%]	
	On the north side	2 [4.2%]	2 [3.8%]	
	Other location	2 [4.2%]	0 [0.0%]	
ZIP code	56-xxx	1 [2.1%]	0 [0.0%]	0.4069 [1]
	60-xxx	5 [10.4%]	7 [13.5%]	
	61-xxx	4 [8.3%]	3 [5.8%]	
	62-xxx	23 [47.9%]	25 [48.1%]	
	63-xxx	1 [2.1%]	6 [11.5%]	
	64-xxx	13 [27.1%]	11 [21.2%]	
	66-xxx	1 [2.1%]	0 [0.0%]	

[1] Pearson's chi-square test; CDI – *Clostridioides difficile* infection; ZIP – Zone Improvement Plan

These findings highlight the prevalent comorbidities within the studied population (see **Table 3**).

Platelet count and C-reactive protein levels

Statistical significance was attained for a higher number of platelets and an elevated level of C-reactive protein in preoperative laboratory parameters (see **Table 4**), as depicted in **Figure 1.**

European heart surgical risk assessment system (EuroSCORE)

Values derived from the EuroSCORE were not statistically significant p-value = 0.8930 but were higher in patients with CDI (Table 5). Conversely, statistical analysis revealed that patients with a higher Body Mass Index (BMI) (*p*-value = 0.0028) and lower left ventricular ejection frac-

Table 3. Prevalence of comorbidities and health indicators in patients with CDI and patients without CDI.

Coefficient	Patients with CDI (*N* = 48)	Patients without CDI (*N* = 52)	*p*-value
Diabetes	16 [38.1%]	15 [28.9%]	0.3430 [1]
Hypertension	42 [87.5%]	49 [94.2%]	0.2400 [1]
Pulmonary hypertension	1 [2.1%]	1 [1.9%]	0.9544 [1]
Hyperlipidemia	44 [91.7%]	48 [92.3%]	0.9060 [1]
Kidney failure	2 [4.17%]	1 [1.9%]	0.5111 [1]
Asthma	4 [8.3%]	3 [5.8%]	0.6156 [1]
Chronic lung disease	0 [0.0%]	1 [1.9%]	0.3342 [1]
Cerebrovascular diseases	12 [25.0%]	16 [30.8%]	0.5209 [1]
Peripheral vascular diseases	10 [20.8%]	13 [25.0%]	0,6208 [1]
Number of heart attacks experienced	0.48 [0–2]	0.44 [0–2]	0.7409 [2]
Current nicotinism	10 [20.8%]	11 [21.2%]	0.9686 [1]

[1] Pearson's chi-square test; [2] Mann-Whitney test; CDI – *Clostridioides difficile* infection

Table 4. Laboratory parameters and clinical characteristics in patients with CDI and patients without CDI.

Coefficient		Patients with CDI (*N* = 48)	Patients without CDI (*N* = 52)	*p*-value
RBC [10^{12}/L]		4.46 [2.58–5.50]	4.57 [2.84–5.51]	0.3865 [3]
WBC [10^{9}/L]		8.53 [4.10–22.30]	8.09 [3.00–16.10]	0.7431 [3]
PLT [10^{9}/L]		257.36 [104.00–759.00]	212.33 [62.00–390.00]	**0.0203** [1]
Hemoglobin [mmol/L]		7.56 [4.05–9.07]	7.95 [0.03–9.07]	0.4169 [3]
Na [mmol/L]		139.45 [129.00–147.00]	140.45 [134.00–145.00]	0.1269 [3]
K [mmol/L]		4.36 [3.60–5.45]	4.45 [3.32–6.28]	0.0991 [3]
Creatinine [µmol/L]		103.94 [54.00–200.00]	99.94 [56.00–229.00]	0.8685 [3]
HbA1c		6.51 [4.90–11.20]	6.21 [4.90–9.40]	0.3379 [3]
CRP [mg/L]		24.77 [0.50–282.10]	4.17 [0.50–74.40]	**0.0142** [3]
BMI [kg/m^2]		29.92 [21.43–37.77]	27.70 [22.39–36.57]	**0.0028** [1]
CCS	Class I	2 [4.1%]	1 [1.9%]	0.5106 [4]
	Class II	33 [68.75%]	33 [63.5%]	
	Class III	12 [25.0%]	18 [34.6%]	
	Class IV	1 [2.1%]	0 [0.0%]	
NYHA	Class I	0 [0.0%]	7 [13.5%]	0.0118 [4]
	Class II	0 [0.0%]	1 [1.9%]	
	Class III	38 [79.2%]	39 [75.0%]	
	Class IV	10 [20.8%]	5 [9.6%]	
LVEF [%]		47 [25–60]	50 [29–60]	**0.0051** [3]
Decreased creatinine clearance		19 [45.2%]	28 [53.9%]	0.4066 [2]

[1] Student's t-test; [2] Pearson's chi-square test; [3] Mann-Whitney test; [4] Fisher-Freeman-Halton test; BMI – Body Mass Index; CCS – Canadian Cardiovascular Society Angina Grading Scale; CDI – *Clostridioides difficile* infection; CRP – C-reactive protein; HbA1c – glycated hemoglobin; K – potassium; LVEF – left ventricular ejection fraction; Na – sodium, NYHA – New York Heart Association classification; PLT – platelet; RBC – red blood cells; WBC – white blood cells

tion (LVEF) (p-value = 0.0051) in the preoperative period were more prone to developing CDI during hospitalisation, as illustrated in **Figure 2** (see **Table 4**).

New York Heart Association (NYHA) scale of heart failure

The NYHA degree of heart failure was significantly higher in patients with CDI, p-value = 0.0118 (see **Table 4**). Conversely, parameters related to surgical characteristics, type and timing of surgery, intraoperative times, the number of transfused blood products, and in-hospital mortality did not exhibit statistical significance. However, postoperative CDI significantly extended the total hospitalisation time (p-value = 0.0001), as depicted in **Figure 3** and **Table 5**.

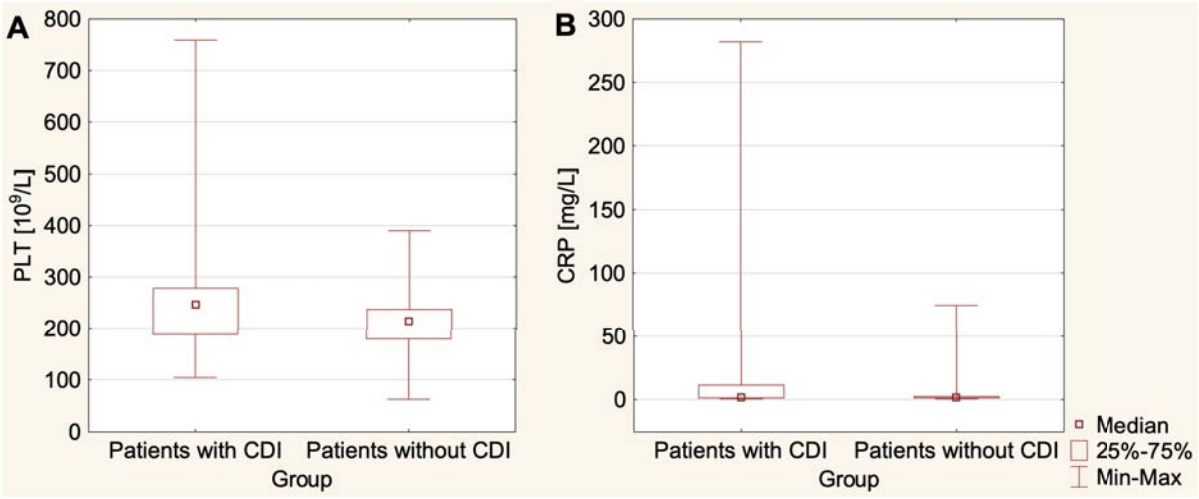


Figure 1. A: Platelet Counts (PLT [10^9/L]) in patients with CDI and patients without CDI. The boxplots depict the distribution of platelet counts, with median values and interquartile ranges indicated. The platelet count is significantly different between the two groups (p-value = 0.0203). **B:** C-reactive protein levels (CRP [mg/L]) in patients with CDI and patients without CDI. The boxplots illustrate the distribution of CRP levels, showing median values and interquartile ranges. A statistically significant difference is observed between the two groups (p-value = 0.0142).

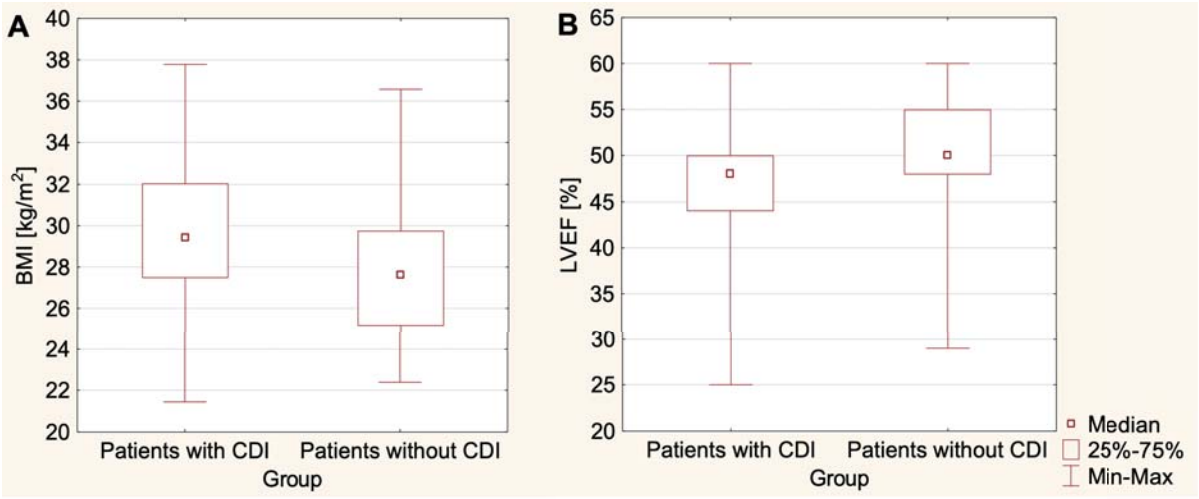


Figure 2. A: BMI Comparison between patients with CDI (N = 48) and without CDI (N = 52). The figure presents the distribution of Body Mass Index (BMI) in patients with CDI and those without CDI, with medians and interquartile ranges. **B:** Left Ventricular Ejection Fraction (LVEF) comparison between patients with CDI (N = 48) and without CDI (N = 52). The comparison of Left Ventricular Ejection Fraction (LVEF) between patients with CDI and those without CDI.

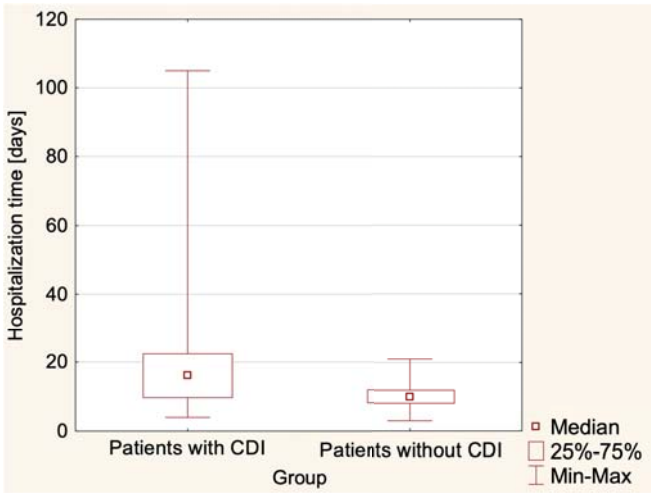


Figure 3. Hospitalisation time in patients with CDI (N = 48) and those without CDI (N = 52) was compared to examine the difference in hospitalisation time between patients with CDI and those without CDI. The boxplots present the distribution of hospitalisation time, including median values and interquartile ranges. Significant divergence is observed between the two groups, with a *p*-value of 0.0001.

Table 5. Surgical and procedural characteristics of patients with CDI and patients without CDI.

Coefficient		Patients with CDI (*N* = 48)	Patients without CDI (*N* = 52)	*p*-value
EuroSCORE [points]		3.6 [0,5–29.3]	2.3 [0.6–13.1]	0.8930 [3]
Urgency of operation	Elective	41 [85.4%]	47 [90.4%]	0.3389 [4]
	Urgent	4 [8.3%]	1 [1.9%]	
	Emergency	3 [6.3%]	4 [7.7%]	
	Salvage	0 [0.0%]	0 [0.0%]	
Preoperative ACS		11 [22.9%]	8 [15.4%]	0.3375 [2]
Number of arterial bypasses	0	3 [6.3%]	1 [2.0%]	0.5479 [4]
	1	42 [87.5%]	48 [96.0%]	
	2	3 [6.3%]	1 [2.0%]	
Number of venous bypasses	0	7 [14.6%]	2 [3.9%]	0.0661 [2]
	1	8 [16.7%]	4 [7.7%]	
	2	26 [54.2%]	30 [57.7%]	
	3	7 [14.6%]	16 [30.8%]	
Extracorrporeal circulation time [min]		77 [41–143]	75 [52–132]	0.9015 [3]
Aortic cross-clamp time [min]		44 [24–99]	41 [26–76]	0.7980 [3]
Transfusion	Red blood cells [units]	4 [0–20]	3 [0–10]	0.3715 [3]
	Plasma [units]	1 [0–12]	1 [0–8]	0.7826 [3]
Hospitalization time [days]		22 [5–106]	12 [4–22]	**0.0001** [3]

[1] Student's t-test; [2] Pearson's chi-square test, [3] Mann-Whitney test, [4] Fisher-Freeman-Halton test; ACS – acute coronary syndrome

Discussion

The incidence of CDI observed in this study, at 1.3%, is consistent with previous reports in cardiac surgery cohorts, indicating CDI as a significant postoperative complication. Our analysis showed no notable demographic differences between the CDI and control groups in terms of age, gender, or geographic distribution, supporting a stable comparison basis. In a similar study, Sanaiha et al. [12] reported a CDI incidence of 0.5% among patients undergoing elective cardiac surgeries between 2005 and 2016. They identified advanced age, female sex, and heart failure as significant risk factors for CDI development, while also noting that academic centres had the

highest incidence but the lowest mortality due to superior access to diagnostic and therapeutic resources.

Furthermore, Keshavamurthy et al. [13] reported a CDI incidence of 0.63% among cardiac surgery patients between 2005 and 2011, based on a large multicenter cohort. Their analysis emphasised that patients who developed CDI exhibited a significantly higher burden of comorbidities, including chronic kidney disease, heart failure, and diabetes. These patients also required more frequent surgical reinterventions and had a markedly increased in-hospital mortality compared to non-CDI counterparts.

Our findings are consistent with these observations. In our cohort, we observed that patients with higher BMI and LVEF were more likely to develop CDI, echoing previous studies that have linked obesity and impaired cardiac function to an increased risk of infectious complications. These associations may be explained by the proinflammatory state and immune dysregulation often seen in patients with metabolic syndrome and advanced heart failure, both of which can exacerbate the intestinal barrier dysfunction and dysbiosis that predispose to CDI.

In contrast to the findings by Chatterjee et al. [14], who reported no significant association between BMI and the severity of *Clostridioides difficile* infection, instead identifying female Hsex and hypoalbuminemia as stronger predictors of adverse outcomes, our results align with those of Mulki et al. [15], who demonstrated that a BMI greater than 35 kg/m² was independently associated with a 1.7-fold increase in the likelihood of developing severe CDI. This discrepancy may reflect differences in study populations, comorbidity burden, or definitions of disease severity.

Furthermore, the elevated NYHA classification observed among CDI patients in our cohort underscores the potential role of advanced heart failure in predisposing individuals to worse infectious outcomes. Méndez-Bailón et al. [16] similarly reported an increase in in-hospital mortality and CDI incidence in heart failure patients with complex comorbid profiles. Likewise, Mamic et al. [17] highlighted the adverse impact of CDI on heart failure–related hospitalisations, emphasising the importance of early identification and preventive strategies tailored to high-risk cardiac surgery populations.

Pathophysiological mechanisms

The increased susceptibility to *Clostridioides difficile* infection in cardiac surgery patients can be explained by several converging pathophysiological mechanisms. A key factor is the perioperative administration of broad-spectrum antibiotics, which disrupts gut microbiota composition and impairs colonisation resistance. Foley et al. [18] demonstrated that post-antibiotic disruption of microbial communities in a murine model modulates susceptibility to CDI in a strain-specific manner, showing that Lactobacillus acidophilus increases CDI risk. In contrast, Lactobacillus gasseri enhances colonisation resistance via bacteriocin-mediated effects and the enrichment of protective Muribaculaceae species.

Moreover, CD itself contributes to intestinal damage through its toxins, particularly toxin A, which disrupts epithelial integrity and induces apoptosis in intestinal cells. Gigli et al. [19] showed that exposure of Caco-2 cells to toxin A significantly reduced transepithelial resistance and tight junction protein expression, impairing mucosal barrier function. This confirms that toxin-mediated epithelial injury is a key driver of CDI pathogenesis, especially in postoperative patients with compromised gut function.

Additional contributing factors include perioperative immunosuppression, use of opioids and proton pump inhibitors, and hemodynamic changes during cardiopulmonary bypass, which may exacerbate dysbiosis and compromise epithelial defence. A better understanding of these mechanisms is essential for developing targeted prophylactic strategies, including microbiota-preserving antibiotic regimens, judicious use of gut-disruptive medications, and adjunctive therapies supporting barrier function.

Biomarkers and CDI risk

Analysis of laboratory biomarkers in our cohort revealed a strong association between elevated C-reactive protein (CRP) and platelet counts and the incidence of CDI, reinforcing the known link between systemic inflammation and infectious risk. These findings are consistent with a study by Nseir et al. [20], which demonstrated that elevated CRP levels, the neutrophil-to-lymphocyte ratio, and mean platelet volume were significantly associated with both recurrence and mortality in CDI patients. The authors proposed that these

inflammatory markers may reflect host immune response dysregulation and mucosal injury severity.

In our patient group, the presence of elevated CRP and thrombocytosis in CDI cases suggests a hyperinflammatory state that may predispose individuals to worse clinical outcomes. These parameters are readily available and cost-effective, making them attractive candidates for early risk stratification in surgical populations. Incorporating such biomarkers into perioperative surveillance protocols may facilitate earlier diagnosis, targeted interventions, and potentially improved prognosis.

Further prospective studies are warranted to evaluate the predictive value of these and other inflammatory or immune-derived markers—such as procalcitonin, interleukin-6, or faecal calprotectin—in the context of CDI following cardiac surgery. Understanding the interplay between systemic and mucosal inflammation may offer novel insights into disease progression and recovery dynamics.

Preventive strategies, healthcare implications, and broader postoperative considerations

Given the substantial impact of CDI on postoperative outcomes and healthcare resource utilisation, the implementation of effective preventive strategies in cardiac surgery patients is essential. Antibiotic stewardship plays a pivotal role in minimising broad-spectrum antimicrobial exposure, thereby preserving gut microbiota diversity. Additionally, interventions aimed at supporting intestinal microbial balance—such as selective digestive decontamination or the use of carefully selected probiotic strains—may hold promise. However, their efficacy in the cardiac surgery population requires further investigation.

Early identification of high-risk individuals is equally important. Enhanced screening protocols and standardised postoperative monitoring—especially in elderly patients or those with immunosuppression—may facilitate timely recognition of CDI and mitigate complications. In our cohort, CDI was associated with significantly prolonged hospitalisation, consistent with previous reports. Kiersnowska et al. [21] described the substantial economic burden of CDI in Polish hospitals, emphasising increased treatment costs and extended inpatient care. Similarly, Crabtree et al. [22] identified CDI as a contributing factor to prolonged mechanical ventilation, extended ICU stays, and overall longer hospitalisations in cardiac surgery patients. These data reinforce the urgent need for robust infection prevention and early intervention frameworks tailored to surgical populations.

The complexity of postoperative care in cardiac surgery extends beyond infectious complications. Rare but serious non-infectious events continue to challenge clinicians. We previously reported a case of Abiotrophia defectiva endocarditis requiring urgent mitral valve surgery due to diagnostic delays, highlighting the importance of early pathogen identification and targeted microbiological evaluation in atypical presentations [23]. Likewise, stress-induced Takotsubo syndrome can clinically mimic acute heart failure after valvular or coronary interventions, necessitating careful differential diagnosis in early postoperative decompensation [24]. Late complications may also arise, such as ascending aortic injury secondary to a dislocated sternal wire nearly two decades post-Ravitch procedure, manifesting with tamponade and cardiogenic shock [25].

While these cases are unrelated to CDI, they illustrate the critical need for comprehensive postoperative vigilance. Given the frequently nonspecific and delayed presentation of CDI, clinicians should maintain broad diagnostic awareness and integrate infection risk into the wider context of post-cardiac surgery surveillance.

Future research in this field should prioritise large, multicenter prospective trials integrating microbiota profiling better to understand the dynamics of gut dysbiosis in postoperative CDI. Investigating the role of specific probiotic or prebiotic therapies, alongside the development of predictive models that incorporate clinical, demographic, and microbiome-derived variables, may enable personalised risk stratification and ultimately improve surgical outcomes.

Perspectives

Despite the comprehensive nature of our study, certain limitations should be acknowledged. The single-centre design and relatively small sample

size may limit the generalizability of our findings. Additionally, a more in-depth exploration of the gut microbiota and its role in CDI development could provide further insights into preventive strategies.

Conclusion

In conclusion, our study highlights the multifactorial nature of *Clostridioides difficile* infection in patients undergoing cardiac surgery, underscoring the interplay between demographic, comorbid, and perioperative risk factors. In particular, elevated *body mass index* and reduced left ventricular ejection fraction emerged as relevant correlates of CDI susceptibility in this population. These findings support more individualised risk stratification and targeted preventive approaches in the perioperative setting.

Moreover, the observed association between CDI and prolonged hospitalisation reinforces the clinical and economic burden of this complication and emphasises the importance of early identification and intervention. Future research involving larger, multicenter cohorts and incorporating comprehensive clinical, microbiological, and microbiome data is warranted to refine risk prediction models further and inform evidence-based prevention strategies tailored to the needs of cardiac surgery patients.

Disclosures

Authors' contributions
Łuczak Maciej: conceptualisation, methodology, formal analysis, visualisation, writing – original draft. Greberski Krzysztof: conceptualisation, data curation, methodology, writing – original draft. Burysz Marian: writing – review & editing. Perek Bartłomiej: writing – review & editing. Jarząbek Radosław: conceptualization, data curation. Bugajski Paweł: conceptualisation, supervision, validation. All authors: read and approved the final version of the manuscript.

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

References

1. Murphy C, Vernon M, Cullen M. Intravenous immunoglobulin for resistant Clostridium difficile infection. Age Ageing. 2006 Jan;35(1):85-6. https://doi.org/10.1093/ageing/afi212. Epub 2005 Nov 22. PMID: 16303776.
2. De Roo AC, Regenbogen SE. Clostridium difficile Infection: An Epidemiology Update. Clin Colon Rectal Surg. 2020 Mar;33(2):49-57. https://doi.org/10.1055/s-0040-1701229. Epub 2020 Feb 25. PMID: 32104156; PMCID: PMC7042002.
3. Vondran M, Schack S, Garbade J, Binner C, Mende M, Rastan AJ, Borger MA, Schroeter T. Evaluation of risk factors for a fulminant Clostridium difficile infection after cardiac surgery: a single-center, retrospective cohort study. BMC Anesthesiol. 2018 Sep 27;18(1):133. https://doi.org/10.1186/s12871-018-0597-2. PMID: 30257648; PMCID: PMC6158878.
4. Sartelli M, Di Bella S, McFarland LV, Khanna S, Furuya-Kanamori L, Abuzeid N, et al. 2019 update of the WSES guidelines for management of Clostridioides (Clostridium) difficile infection in surgical patients. World J Emerg Surg. 2019 Feb 28;14:8.
5. Eze P, Balsells E, Kyaw MH, Nair H. Risk factors for Clostridium difficile infections - an overview of the evidence base and challenges in data synthesis. J Glob Health. 2017 Jun;7(1):010417. https://doi.org/10.7189/jogh.07.010417. PMID: 28607673; PMCID: PMC5460399.
6. Zilberberg MD, Shorr AF, Wang L, Baser O, Yu H. Development and Validation of a Risk Score for Clostridium difficile Infection in Medicare Beneficiaries: A Population-Based Cohort Study. J Am Geriatr Soc. 2016 Aug;64(8):1690-5. https://doi.org/10.1111/jgs.14236. Epub 2016 Jun 13. PMID: 27295521.
7. Gelijns AC, Moskowitz AJ, Acker MA, Argenziano M, Geller NL, Puskas JD, Perrault LP, Smith PK, Kron IL, Michler RE, Miller MA, Gardner TJ, Ascheim DD, Ailawadi G, Lackner P, Goldsmith LA, Robichaud S, Miller RA, Rose EA, Ferguson TB Jr, Horvath KA, Moquete EG, Parides MK, Bagiella E, O'Gara PT, Blackstone EH; Cardiothoracic Surgical Trials Network (CTSN). Management practices and major infections after cardiac surgery. J Am Coll Cardiol. 2014 Jul 29;64(4):372-81. https://doi.org/10.1016/j.jacc.2014.04.052. PMID: 25060372; PMCID: PMC4222509.
8. Burke KE, Lamont JT. Clostridium difficile infection: a worldwide disease. Gut Liver. 2014 Jan;8(1):1-6. https://doi.org/10.5009/gnl.2014.8.1.1. Epub 2014 Jan 13. PMID: 24516694; PMCID: PMC3916678.
9. World Health Organization. Diarrhoeal disease. 2017 May 2. Retrieved from: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease
10. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis. 2018 Mar 19;66(7):e1-e48. https://doi.org/10.1093/cid/cix1085. PMID: 29462280; PMCID: PMC6018983.
11. Debast SB, Bauer MP, Kuijper EJ; European Society of Clinical Microbiology and Infectious Diseases. European Society of Clinical Microbiology and Infectious

Diseases: update of the treatment guidance document for Clostridium difficile infection. Clin Microbiol Infect. 2014 Mar;20 Suppl 2:1-26. https://doi.org/10.1111/1469-0691.12418. PMID: 24118601.
12. Sanaiha Y, Sareh S, Lyons R, Rudasill SE, Mardock A, Shemin RJ, Benharash P. Incidence, Predictors, and Impact of Clostridium difficile Infection on Cardiac Surgery Outcomes. Ann Thorac Surg. 2020 Nov;110(5):1580-1588. https://doi.org/10.1016/j.athoracsur.2020.03.037. Epub 2020 Apr 15. PMID: 32304688.
13. Keshavamurthy S, Koch CG, Fraser TG, Gordon SM, Houghtaling PL, Soltesz EG, Blackstone EH, Pettersson GB. Clostridium difficile infection after cardiac surgery: prevalence, morbidity, mortality, and resource utilization. J Thorac Cardiovasc Surg. 2014 Dec;148(6):3157-65.e1-5. https://doi.org/10.1016/j.jtcvs.2014.08.017. Epub 2014 Aug 14. PMID: 25242055.
14. Chatterjee T, Bansal S, Abuzar A, Hussain H, Gupta L. Is Increased BMI a Risk Factor for Developing Severe Clostridioides Difficile Infection? A Retrospective Study. J Community Hosp Intern Med Perspect. 2022 Nov 7;12(6):43-50. https://doi.org/10.55729/2000-9666.1123. PMID: 36816160; PMCID: PMC9924641.
15. Mulki R, Baumann AJ, Alnabelsi T, Sandhu N, Alhamshari Y, Wheeler DS, Perloff S, Katz PO. Body mass index greater than 35 is associated with severe Clostridium difficile infection. Aliment Pharmacol Ther. 2017 Jan;45(1):75-81. https://doi.org/10.1111/apt.13832. Epub 2016 Oct 28. PMID: 27790736.
16. Méndez-Bailón M, Jiménez-García R, Hernández-Barrera V, Miguel-Díez J, Miguel-Yanes JM, Muñoz-Rivas N, Lorenzo-Villalba N, Carabantes-Alarcon D, Zamorano-León JJ, Astasio-Arbiza P, Ortega-Molina P, López-de-Andrés A. Heart Failure Is a Risk Factor for Suffering and Dying of Clostridium difficile Infection. Results of a 15-Year Nationwide Study in Spain. J Clin Med. 2020 Feb 25;9(3):614. https://doi.org/10.3390/jcm9030614. PMID: 32106444; PMCID: PMC7141109.
17. Mamic P, Heidenreich PA, Hedlin H, Tennakoon L, Staudenmayer KL. Hospitalized Patients with Heart Failure and Common Bacterial Infections: A Nationwide Analysis of Concomitant Clostridium Difficile Infection Rates and In-Hospital Mortality. J Card Fail. 2016 Nov;22(11):891-900. https://doi.org/10.1016/j.cardfail.2016.06.005. Epub 2016 Jun 16. PMID: 27317844.
18. Foley MH, McMillan AS, O'Flaherty S, Thanissery R, Vanhoy ME, Fuller MG, Barrangou R, Theriot CM. Differential modulation of post-antibiotic colonization resistance to Clostridioides difficile by two probiotic Lactobacillus strains. mBio. 2025 Aug 13;16(8):e0146825. https://doi.org/10.1128/mbio.01468-25. Epub 2025 Jul 21. PMID: 40689613; PMCID: PMC12345269.
19. Gigli S, Seguella L, Pesce M, Bruzzese E, D'Alessandro A, Cuomo R, Steardo L, Sarnelli G, Esposito G. Cannabidiol restores intestinal barrier dysfunction and inhibits the apoptotic process induced by Clostridium difficile toxin A in Caco-2 cells. United European Gastroenterol J. 2017 Dec;5(8):1108-1115. https://doi.org/10.1177/2050640617698622. Epub 2017 Mar 13. PMID: 29238589; PMCID: PMC5721977.
20. Nseir W, Khamisy-Farah R, Amara A, Farah R. The Prognostic Value of Inflammatory Markers in Clostridium difficile-associated Diarrhea. Isr Med Assoc J. 2019 Oct;21(10):658-661. PMID: 31599506.
21. Kiersnowska Z, Lemiech-Mirowska E, Ginter-Kramarczyk D, Kruszelnicka I, Michałkiewicz M, Marczak M. Problems of Clostridium difficile infection (CDI) in Polish healthcare units. Ann Agric Environ Med. 2021 Jun 14;28(2):224-230. https://doi.org/10.26444/aaem/119321. Epub 2020 Apr 2. PMID: 34184502.
22. Crabtree T, Aitchison D, Meyers BF, Tymkew H, Smith JR, Guthrie TJ, Munfakh N, Moon MR, Pasque MK, Lawton J, Moazami N, Damiano RJ Jr. Clostridium difficile in cardiac surgery: risk factors and impact on postoperative outcome. Ann Thorac Surg. 2007 Apr;83(4):1396-402. https://doi.org/10.1016/j.athoracsur.2006.10.067. PMID: 17383346.
23. Wilawer M, Elikowski W, Greberski K, Ratajska PA, Welc NA, Lisiecka ME. Abiotrophia defectiva endocarditis - Diagnostic and therapeutic challenge: Case report. IDCases. 2023 Oct 8;34:e01906. https://doi.org/10.1016/j.idcr.2023.e01906. PMID: 37867569; PMCID: PMC10585279.
24. Elikowski W, Małek-Elikowska M, Greberski K, Rzymski S, Kołowrotkiewicz A, Furmaniuk J, Bugajski P. Takotsubo syndrome following mitral valve replacement and left anterior descending coronary artery bypass grafting. Pol Merkur Lekarski. 2019 Jan 28;46(271):36-41. PMID: 30810114.
25. Greberski K, Jarząbek R, Perek B, Łuczak M, Bugajski P. Exceptional life-threatening complication 19 years after Ravitch correction of pectus excavatum. J Card Surg. 2021 Oct;36(10):3971-3972. https://doi.org/10.1111/jocs.15889. Epub 2021 Aug 2. PMID: 34339529.

ORIGINAL PAPER

JMS Journal of Medical Science

Market Access Professionals in the Pharmaceutical Industry in the United Kingdom: Essential Roles, Knowledge, Skills, and Attributes

Clara T. Fatoye
Department of Health, Wellbeing and Social Care, Global Banking School, Manchester, United Kingdom
https://orcid.org/0000-0003-3509-5065
Corresponding author: Cfatoye@globalbanking.ac.uk

Gillian Yeowell
Department of Health Professions, Manchester Metropolitan University, Manchester, United Kingdom
https://orcid.org/0000-0003-3872-9799

Eula Miller
School of Nursing and Public Health, Manchester Metropolitan University, Manchester, United Kingdom
https://orcid.org/0000-0002-0609-2634

Isaac Odeyemi
Department of Health Professions, Manchester Metropolitan University, Manchester, United Kingdom
https://orcid.org/0000-0002-9037-9290

Chidozie Mbada
Department of Health Professions, Manchester Metropolitan University, Manchester, United Kingdom
https://orcid.org/0000-0003-3666-7432

https://doi.org/10.20883/medical.e1341

Keywords: market access, pharmaceutical industry, roles, skills, United Kingdom

Received 2025-07-08
Accepted 2025-08-13
Published 2025-09-25

How to Cite: Fatoye CT, Yeowell G, Miller E, Odeyemi I, Mbada C. Market Access Professionals in the Pharmaceutical Industry in the United Kingdom: Essential Roles, Knowledge, Skills, and Attributes. Journal of Medical Science. 2025 September;94(3);e1341. doi:10.20883/medical.e1341

ABSTRACT

Introduction. Clearly defined roles and educational standards are crucial for enhancing professional status and facilitating Market Access (MA) in the pharmaceutical industry (pharma). However, literature on this topic remains limited. This study explored MA professionals' roles within pharma in the United Kingdom (UK), including the knowledge, skills, competence, and other attributes required.

Materials and methods. A document analysis of UK MA job advertisements from pharmaceutical companies, National Health Service websites, and Google search engine was undertaken between February and August 2019, and updated between 1st and 31st August 2023. Thematic content analysis was used to analyse the data.

Results. Eighty advertisements met the inclusion criteria, classified by seniority: entry-level (44%), senior level (15%), pricing and reimbursement (11%), and head/director positions (30%). Entry-level roles displayed the most variability in titles. A degree in a relevant field was required for 86% of these positions, dropping to 38% for senior MA roles. While skills and activities varied, a strong understanding of healthcare systems and excellent communication skills were essential across all positions. The core attributes of MA professionals in pharma were defined by three MA components: 'right roles' (job responsibilities), 'right people' (reputation and skills), and 'right reward' (wages and remuneration).

Conclusions. This study is the first document analysis of MA job roles in pharma, focusing on their conceptualisation and definition. MA is an emerging profession characterised by diverse roles, varying entry requirements, and the need for collaboration across healthcare systems. The findings indicate that MA in pharma is still in the early stages of professionalisation and needs further development. To advance this evolution, developing competency frameworks, standardising job roles, and guiding curriculum design are recommended.

Introduction

Market access (MA) in healthcare is a complex and diverse concept, particularly in the pharmaceutical industry (pharma) [1–4]. Unlike typical goods influenced by supply and demand dynamics, the healthcare market poses unique challenges to the conventional economic model [1–3,5]. According to Gold [6], current frameworks for assessing access are person-based and fail to capture the complexity of the healthcare system and the varied structures of managed care organisations that integrate delivery and financing. Therefore, MA in pharma is crucial for enabling manufacturers to convey the value of their products to stakeholders before patient access. Its implications vary depending on whether it applies to private, public, or mixed healthcare systems, and the type of healthcare market organisation (centralised, decentralised, or fragmented) [1]. Effective MA demands collaboration among various stakeholders—healthcare providers, payers, regulatory bodies, and patient advocacy groups—each offering unique expertise and insights. Moreover, with the shift toward patient-centric care, companies must prioritise patient needs and outcomes in the MA process [7]. MA involves various processes to engage diverse stakeholders [8], with variations in healthcare systems across countries influencing its conceptualisation. For example, in the United Kingdom (UK), key gatekeepers include the Medicines and Healthcare Products Regulatory Agency, National Institute for Health and Care Excellence, and the National Health Service (NHS). In contrast, the US insurance system significantly influences the roles of regulatory agencies like the Food and Drug Administration and the Institute for Clinical and Economic Review [2].

Pharma, like any commercial industry, depends on profits for survival, with research and development, sales, and marketing traditionally driving success [8]. Increasing financial constraints on healthcare provision and the need for evidence to support healthcare decisions have made MA a key component of pharma. MA provides evidence related to patient needs, safety, efficacy, effectiveness, budget impact, and cost-effectiveness of technologies compared with existing treatments [9]. This involves the application of tools and methodologies such as cost-effectiveness modelling to assess value, budget impact analysis to evaluate affordability within healthcare systems, stakeholder mapping to identify and engage key decision-makers, and healthcare system analysis to align access strategies with NHS structures and policy priorities. Consequently, pharma has started employing MA professionals, and to navigate the dynamic regulatory environment, some have established MA functions as integral parts of their organisations, though few have dedicated MA teams with well-defined roles [8]. MA is associated with delivering value for money through pharmaceutical products [10]. From the manufacturer's perspective, MA aims to maximise patient access to their products [1,10]. However, MA can be oversimplified and confused with activities like obtaining licensing, market authorisation, medical representatives gaining access to healthcare professionals, ensuring product availability in pharmacies, and selecting promotion channels. This underscores the need for clearly defined MA roles and activities within pharma [1,8–10].

Establishing a professional identity by defining roles and developing educational standards is crucial for enhancing professional status. Evaluating the roles of emerging professionals like MA in pharma, along with the practical skills and knowledge of current practitioners, can inform the necessary educational standards, ultimately enhancing the preparedness and effectiveness of MA professionals. Due to the limited primary literature on the skills and roles of MA professionals in pharma, a document analysis (DA) of grey literature was undertaken to fill this gap. This grey literature offers valuable insights into the development of MA and the identity of its practitioners. According to Bowen [11], DA is a systematic process that can complement other data sources. This study explored the roles of MA professionals within pharma in the UK, including the knowledge (theoretical and contextual understanding), skills (practical and interpersonal abilities), competences acquired and expected (the integrated application of knowledge and skills in real-world settings), and other attributes required for an MA job.

Materials and methods

Methods

A DA of published UK MA job advertisements from pharma, NHS websites, and Google search

engine was conducted. These advertisements were public records, and did not require ethics approval [11,12], and were retrieved between February and August 2019 and updated between 1st and 31st August 2023. A job analysis template was used for this DA. Duties, tasks, and responsibilities associated with MA roles, as well as the knowledge, abilities, and skills required, were gleaned from the advertisements.

Search strategy

The search terms used were "market access jobs" and "MA jobs." The selected websites (NHS, pharmaceutical companies, Google) were deemed the most common sites for advertising MA jobs during the data collection period. The selected pharma websites included Pharmaphorum, CarrotPharma, and Pharmiweb.jobs, as they were top-ranked for advertising MA jobs during the data collection period. MA jobs advertised outside the UK were excluded.

Data extraction

An adapted template from O'Leary [12] was used for data extraction, following an eight-step framework:

1. Create a list of text to explore relevant keywords: The keywords used were 'market access jobs' and 'MA jobs.' All MA jobs advertised in the UK (in English only), regardless of hierarchical level, from entry-level to top managerial roles, were considered. Job roles in health economics and similar roles were excluded.
2. Consider how texts would be accessed: Online MA job advertisements were gleaned using a job analysis data extraction template.
3. Acknowledge and address biases (inclusion and exclusion): Eligibility criteria were set based on a preliminary cursory search. CF retrieved documents based on the eligibility criteria, and GY and CM checked the search strategy and criteria. MA jobs lacking person specification details and required skills, or outside the scope of MA, were excluded.
4. Develop appropriate skills for research: CF, a PhD candidate with work experience in MA, and the other authors (GY, EM, CM, and IO), experienced academics/researchers in research methodology, conducted the study.
5. Consider strategies for ensuring credibility: Data were obtained from reputable pharmaceutical companies' websites, NHS websites, and Google search. Duplicate job advertisements were removed from search results.
6. Ensure data specificity to the study's objective: Sensitive keywords 'MA jobs' or 'market access jobs' were used for data identification. Data were sourced from pre-determined relevant sources based on a cursory literature review.
7. Consider ethical issues: An exempt approval was granted by Faculty Ethics, Manchester Metropolitan University, UK, as the study was considered low-risk being an analysis of publicly available documents.
8. Explore content: A job analysis template was used in this study. Data were gleaned on the job role advertised, the website where the job was published, the date the job data was retrieved, the remuneration for the job, the job location, the required job skills, the job hierarchy, and key features or elements of the role.

Data analysis

DA is typically regarded as a qualitative analysis method [11]. Thematic Content Analysis (TCA) was employed to analyse the data. TCA was used to identify key themes across the dataset by grouping conceptually similar codes into themes. The data were quantified in terms of frequency to examine the presence, meanings, and relationships of text, codes, and relevant themes.

Results

Eighty MA job advertisements were compiled from February to August 2019 (n = 60) and updated between 1st and 31st August 2023 (n = 20) (see **Figure 1**). Three main MA components were identified: Role Advertised, Skills and Activities, and Rewards.

Role advertised

The job roles were categorised as: entry-level (44%), senior-level (15%), pricing and reimbursement (P&R) (11%), and head and director roles (30%). Entry-level roles exhibited the most variability in job titles. The entry-level role with the highest frequency was the MA consultant (29%). Senior MA roles included senior MA manager (25%) and senior analyst MA (17%). P&R roles

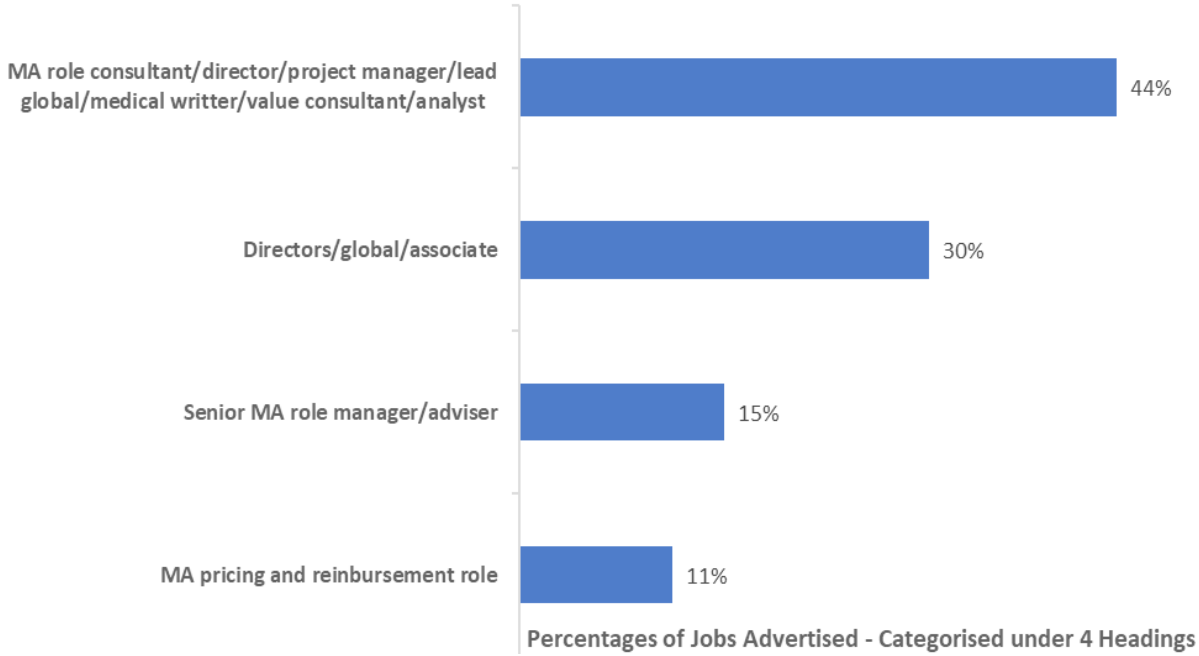


Figure 1. Total number of MA Jobs Advertised.

included pricing and MA consultant (33%) and MA and pricing specialist (33%). Head and director roles included global category manager (17%) and MA director (13%) (see **Table 1**).

Skills and activities

For entry-level roles, key skills include understanding healthcare systems and managing stakeholder collaborations (98%), and generating new business through client partnerships in MA, health economics, and pharmaceuticals (98%). Others include managing day-to-day deliverables of projects (95%), demonstrating healthcare value (95%), and communicating P&R (93%).

For senior MA roles, essential skills include ownership in resolving methodological issues like P&R (92%), effective communication to build trust and identify opportunities (92%), a strong understanding of healthcare systems (92%), and project management skills (92%).

In P&R roles, key required skills include understanding healthcare systems and managing stakeholder collaborations (100%). Generating new business through client collaboration in MA and health economics is also vital (100%). Other important skills are communicating P&R (89%) and managing project deliverables (89%).

For head and director roles, the key skills required included managing the transition from pre-launch to post-launch and developing effective launch strategies (100%). Other important skills were team management (92%) and strong organisational and technical abilities (83%) (see **Table 2**).

Rewards

For entry-level roles, the top reward was accrue holiday (53%), followed by performance bonuses (45%) and personal development opportunities (40%). For senior roles, the most common reward was a competitive salary and bonus (£104,600.00–£209,200.00) (54%), along with opportunities to work with international professionals (46%) and career development (46%). In P&R roles, the standout reward was a care flexible spending account (99%), with a friendly work environment (88%) also noted. For head and director roles, key rewards included a challenging environment with high-performance rewards (25%) and competitive salary and benefits (25%) (see **Table 3**).

Discussion

This study investigated the roles of MA professionals within the pharmaceutical industry through a DA of MA job advertisements. Three

Table 1. Frequency distribution of all advertised roles.

Roles advertised	Frequency (%)
MA Entry-Level Role (n = 35)	
Market access consultant	10(29)
Market access manager	5(14)
Medical writer market access	4(11)
Analyst health market access	1(3)
Business development manager – health economics	1(3)
Consultant market access	1(3)
Health economics and MA project manager	1(3)
Market access analyst	1(3)
Market access editor	1(3)
National market access associate	1(3)
National MA lead	1(3)
Associate value consultant MA	1(3)
Health economics and MA graduate	1(3)
Market access and pharmaceutical sales	1(3)
Market access and project manager	1(3)
Market access and business development manager	1(3)
Payer value and patients access manager	1(3)
Business manager – HEOR (Health Economic and Outcomes Research) and MA	1(3)
Rare disease MA manager	1(3)
Senior MA Role Advertised (n = 12)	
Senior MA manager	3(25)
Senior analyst MA	2(17)
Senior MA adviser	2(17)
Market access senior analyst	1(8)
Senior global manager of MA	1(8)
Senior MA manager	1(8)
Senior manager UK MA	1(8)
Senior UK MA manager	1(8)
Pricing and Reimbursement role Advertised (n = 9)	
Pricing and MA consultant	3(33)
MA and pricing specialist	3(33)
Pricing and MA Associate	1(11)
Pricing and MA	1(11)
MA and pricing managing consultant	1(11)
MA Head & Directors Role Advertised (n = 24)	
Global category manager	4(17)
Market access director	3(13)
Principal Consultant MA	3(13)
Head of MA	2(8)
Director/Associate director of HEOR & MA	2(8)
Director international MA	2(8)
Business development head MA	1(4)
Market access VP life science	1(4)
Head MA	1(4)
Associate director, HEOR & MA	1(4)
Associate director pricing and MA	1(4)
Director MA	1(4)
Global MA director	1(4)
UK MA director	1(4)

Keys: HEOR – Health Economics and Outcomes Research; MA – Market Access; UK – United Kingdom; VP – Vice President

Table 2. Entry Level Role: Skills and Activities.

Skills and Activities	Frequency (%)
MA Entry Level Skills and Activities (n = 35)	
Understanding of different healthcare system and managing collaborations with stakeholders	34(98)
Generate new business through collaboration with clients for MA, HE, pharma, and healthcare policy	34(98)
Managing day to day deliverables of projects including time and budget	33(95)
Expertise in healthcare value demonstration including HTA, Pricing and reimbursement submissions & KPIs	33(95)
Communicating price and reimbursement and making submissions to HTA agencies	32(93)
Leadership capabilities with passion to develop others within the team	32(93)
Strategic insight, creative insight, creative mind-set, and active listening & paying attention to detail	31(91)
Record of accomplishment of strong analytical skills and been able to work with analyst on the team	31(91)
Degree and a post graduate degree in relevant fields such as MA, HE, life science and public health and PhD. Meaning degree can be BSc, MSc and PhD	30(86)
Qualitative and quantitative research including systematic reviews	29(84)
Active business and clients, stakeholder's relationship to ensure high quality deliverables	28(80)
Creating and delivery solutions for clients to inform development of value proposition through MA and medical communication	28(80)
Communication, interpersonal and management skills	26(75)
Team player, mentoring other team members with strong work ethics	25(73)
Computer and statistical analysis skills with experience of using STATA/SAS, Excel, and spreadsheet	24(70)
Global value dossier, market access tools and client deliverables	23(66)
Interpretations of clinical trials and observational studies	20(57)
Experience of working with the NHS and it goals, NICE & MA insight	8(23)
Minimum of 3 years' experience	6(16)
Fluent in English	4(11)
Willingness and ability to travel and current full driving licence	2(5)
Another European language	1(2)
Senior MA Skills and Activities (n = 12)	
Experience of taking ownership & resolving methodological problems including pricing & reimbursement	11(92)
Proven track records of effective communication to generate trust and tactics for MA opportunities	11(92)
Strong understanding of healthcare systems liaising with relevant business and external affairs & global strategy	11(92)
Project management and High understanding of HTA submissions & working independently at senior level	11(92)
Proven capabilities to represent at NICE, NHS on cross system initiatives & developing KPIs	10(85)
Excellent negotiation skills and Project management including day-to-day delivery of projects & prepare dossiers	10(85)
Ensuring that barriers to product uptake & early adoption including the use of Excel & spreadsheet	9(77)
Experience in MA role in pharma sector and in multi-disciplinary, matrix and global context	9(77)
Providing line management, mentoring and technical leadership on portfolio of projects	8(69)
Analysing data, rolling out tools, materials, and projects to drive access and drawing conclusions	7(62)
Having a post graduate qualification in scientific discipline & experience with qualitative and quantitative research	5(38)
Extremely strong written and spoken English	4(31)
MA Pricing & Reimbursement Skills and Activities (n = 9)	
Understanding of different healthcare system and managing collaborations with stakeholders	9(100)
Generate new business through collaboration with clients for MA, HE, pharma, and healthcare policy	9(100)
Communicating price and reimbursement and making submissions to HTA agencies	8(89)
Managing day to day deliverables of projects including time and budget	8(89)
Qualitative and quantitative research including systematic reviews	7(78)
Expertise in healthcare value demonstration including HTA, Pricing and reimbursement submissions	7(78)
Active business and clients, stakeholder's relationship to ensure high quality deliverables	6(67)
Creating and delivery solutions for clients to inform development of value proposition through MA and medical communication	6(67)
Team player, mentoring other team members with strong work ethics & and ability to attend medical and commercial conferences	5(56)
Leadership capabilities with passion to develop others within the team	5(56)
Degree and a post graduate degree in relevant fields such as MA, HE, life science and public health	3(33)
Record of accomplishment of strong analytical skills and been able to work with analyst on the team	3(33)
Global value dossier, market access tools and client deliverables	3(33)
Communication, interpersonal and management skills & to build professional relationship with team members	2(22)

Table 2. (Continued).

Skills and Activities	Frequency (%)
Computer and statistical analysis skills with experience of using STATA/SAS, Excel, and spreadsheet	2(22)
Interpretations of clinical trials and observational studies & performing qualitative and quantitative analysis	2(22)
Strategic insight and creative mind-set and able to create clients ready report	1(11)
MA Head and Director Skills and Activities (n = 24)	
Bringing assets from prelaunch to post launch develop through implement effective launch strategy including responses to clients' queries	24(100)
Managing and leading team of well-trained analysis and consultants on MA pathways for a variety of products	22(92)
Excellent organisational skills and technical skills including Working with MA to implement, sustain and optimise pricing, access, and reimbursements	20(83)
Develop and implement effective MA strategies ensure project management such as pharma, biotech, medical devices projects	18(75)
Responsible for strategic insight and development of HTA as set out by NICE and demonstrate initiative	18(75)
Conducting research activities in various therapy areas and initiative on clinical trials and observational studies	16(67)
Degree in life science, medical degree, health economics, mathematics, epidemiology, and Biostatistics	14(58)
Collaborating with stakeholders and ensure that the local market activities comply with regulatory agencies	14(58)
Effective building relationship with internal team and stakeholders	13(54)
Excellent presentation, communication skills including clients influencing skills, value dossier, payer value propositions	13(54)
Responsible for managing multiple projects Working with senior leaders across healthcare business unit to ensure integration	10(42)
Experience of 2 to 8 years	6(25)

Keys: HE – BSc – Bachelor of Sciences; Health Economics; HTA – Health Technology Assessment; KPI – Key performance indicator; MA – Market Access; MSc – Master of Science; NHS – National Health Science; NICE – National Institute for Health and Care Excellence; PhD – Doctor of Philosophy; SAS – Statistical Analysis System; STATA – Statistics and Data

main MA components emerged: roles, skills and activities, and rewards. These were re-labelled as the 'right roles' (job responsibilities), 'right people' (skills and activities), and 'right reward' (wages and remuneration) to align with a related scoping review on the conceptualisation and role of MA in pharma [13].

Right roles

The advertised roles included entry-level, senior-level, P&R, and head and director roles. Entry-level roles were the most common, offering opportunities to learn about market dynamics, pricing strategies, and stakeholder management [14–16]. These roles often assist with research, data analysis, and strategy development. However, the lack of consistency in job titles suggests that the profession is in the early stages of professionalisation. Compared to other professions, MA in pharma is relatively new and may be described as a 'semi-profession' [17]. This is because it currently shares characteristics of a semi-profession, such as no specific training requirement, shorter training periods, and less autonomy from supervision [17].

Many healthcare occupations have transitioned to professional status through the development of educational standards and professional certificates. For example, nursing and physiotherapy have evolved from occupations to professions [18,19]. Nursing and physiotherapy have registered titles for entry roles, such as 'newly qualified nurse' and 'junior physiotherapist,' with fixed starting pay bands, unlike MA in pharma where the starting pay point is negotiated [18,19]. Similarly, the transition to professional status for the UK pharmacy profession between 1880 and 1905 involved lower-status professionals engaging in mnemonic work to maintain professional purity and collectively mobilising to influence professional bodies dominated by higher status peers [20]. Considering that MA is a relatively new field in the pharma, Koch [21] suggested integrating MA into health economics in research and development.

The 'P&R' role was the least advertised MA role. This role requires skills and competencies in pricing and market consultation. P&R in pharma refer to establishing a price and obtaining a positive reimbursement decision for a new product

Table 3. Rewards offered for the different MA roles.

MA Entry Level Reward (n = 35)	Frequency (%)
Holidays accrued	19(53)
Performance related bonus/Bonus £21900/ Salary 40k to 73k and pension	16(45)
Personal development	14(40)
Competitive salary/statutory payment	12(33)
Working alongside talented professionals	10(28)
Other incentives	9(26)
Healthcare	9(26)
Flexible working including flexible working abroad – hybrid	7(21)
MA Senior Role Reward (n = 12)	
Excellent bonus/competitive salary 104,600.00 – 209,200.00	7(54)
Opportunity to work alongside international & group of talented professionals and personal development	6(46)
Career development opportunities and enhancement	6(46)
International experience of working with other companies such as San Francisco & Singapore and top 100 companies	2(16)
Excellent health &wellness programmes such as Yoga, meditation & summer day out	1(8)
MA pricing & Reimbursement Rewards (n = 9)	
Care and flexible spending account	9(99)
Incentives & friendly work environment	8(88)
Competitive compensation package/Bonus incentive	6(67)
Fast paced career progression	3(33)
Opportunity to develop internal engagement including line management role	2(22)
Ability to manage multiple work streams	2(22)
Retirement/Salary range $104,600.00 – $209,200.00	2(22)
Medical, dental insurance	1(11)
MA Head & Vice President and Director & Associate Reward (n = 24)	
A challenging and fun environment with High performance reward system	6(25)
Competitive salary and benefits	6(25)
BUPA health Insurance including partners and children cover	5(21)
Company shares option scheme including Health insurance	5(21)
Employer matched pension scheme 5%	5(21)
Free gym membership	5(21)
Clear and solid foundation for future growth including excellent career prospect	4(17)
Cycle to work scheme	1(4)
Excellent long-term position	1(4)

[8,9,21,22]. P&R are crucial in MA because they determine whether pharmaceutical products can reach patients and be financially viable [23]. Considering that P&R measures affect the capacity of pharma to sell their products, the right person with competence and experience is needed for these roles. Berndt and Newhouse [23] highlight how federal legislation in the United States, insurance systems, and distribution logistics significantly impact drug prices and utilisation, thus requiring effective P&R strategies to ensure that pharmaceutical products are accessible to patients while maintaining the economic sustainability of pharmaceutical companies.

The MA head and director roles were the last in the category. The 'global category manager' was the most frequently advertised job role in this category. Holders of this role need deep industry knowledge, understanding of market trends, research and data-driven insights, and robust payer relationships [14]. Overall, there were different 'right roles' with diverse tasks, yet all aimed towards ensuring patient access to pharmaceutical products. MA roles are not uniform and often vary significantly depending on therapeutic area, product lifecycle stage, and organisational context [24–26]. For example, MA professionals in oncology may need to engage more deeply with accelerated access schemes, orphan drug policies, and complex evidence packages compared to their counterparts in primary care, where the focus is often on cost-effectiveness and the

overall impact on the population [25,27 28]. Additionally, responsibilities change throughout the product lifecycle: pre-launch roles generally prioritise early value strategy, evidence generation, and stakeholder mapping, while post-launch roles focus on maintaining access, managing real-world evidence (RWE), and adapting to shifting payer requirements [29–31].

The roles and competencies identified in stakeholder engagement, Health Technology Assessment (HTA) navigation, and evidence communication are particularly relevant to real-world challenges. These include overcoming local formulary restrictions by creating tailored value propositions, utilising RWE to support reimbursement in areas with limited data and negotiating managed entry agreements that align clinical uncertainty with payer risk-sharing mechanisms [32–35]. The type of employer also shapes role expectations; large pharmaceutical companies may offer more specialised MA functions with access to internal health economics and outcomes research (HEOR) and policy teams, whereas consultancies often require broader, cross-functional expertise to support multiple clients across diverse markets. These contextual differences underscore the need for flexible, role-specific competencies within any framework aimed at professionalising MA [36–38].

Right people

The theme 'right people' refers to MA professionals. Employing or outsourcing MA roles to the right people is crucial for achieving patient access to products. A candidate's reputation in skills and experience is highly desirable to pharmaceutical employers. MA professionals need experience in different therapy areas and exposure to health economics.

MA professionals handle various responsibilities, including crafting the product's value proposition, conducting health economics and outcomes research, and managing price negotiations. They also engage with stakeholders like healthcare professionals and patient advocacy groups [8,9,21]. The findings show that understanding different healthcare systems and managing collaborations with stakeholders were the most commonly required skills. These requirements resonate with the field realities of an MA professional in pharma.

Other essential attributes required include external stakeholder engagement, such as mapping and prioritising decision-makers across NHS structures, tailoring value messages to payer needs, and facilitating advisory boards or consultations with patient groups. Additionally, strong data analytics capabilities are essential. This includes interpreting RWE, conducting budget impact analyses, and modelling cost-effectiveness to guide pricing and access strategies [39–42]. In addition to traditional competencies, modern MA roles increasingly require proficiency in digital tools and data-driven approaches [43–45]. Thus, the integration of RWE, patient-reported outcomes, and AI-enabled analytics is transforming how value is demonstrated and communicated [43–45]. For instance, RWE supports the validation of value post-launch [46,47], while patient-reported outcomes capture patient-centric outcomes that are critical for decision-making [48,49]. Thus, MA professionals are crucial to product launch success because they enable priced and well-timed product fulfilment for appropriate patients.

Additionally, the use of AI-driven tools enhances forecasting, segmentation, and stakeholder targeting in MA [50,51]. These capabilities are becoming essential for MA professionals for navigating complex access environments and aligning with the evolving expectations of payers. Also, the changing regulatory landscape driven by digital health innovations, the growing use of RWE, adaptive licensing pathways, and the transformation of HTA processes require MA professionals to develop new skills. These skills include the ability to interpret and apply RWE in regulatory and reimbursement submissions, navigate digital health policy frameworks, and engage with adaptive HTA models that emphasise early access and iterative evidence generation. Consequently, regulatory literacy, data governance, and cross-functional collaboration are becoming essential components of the MA skillset [52,53].

Furthermore, the MA strategy entails providing clinical and economic evidence and bargaining with healthcare access stakeholders. A combination of regulatory frameworks, market dynamics, and innovation pressures shapes the pharma's financial and policy landscape. Companies must navigate complex pricing and reimbursement

systems, which vary significantly across countries and impact drug accessibility and affordability [54].

The job adverts indicated that MA professionals must interpret and understand the healthcare system and collaborate with other professionals to achieve organisational goals. Entry-level MA professionals are typically field workers who ensure better patient access to pharmaceutical products. Usually, MA professionals in pharma play a key role in marketing and managing companies by enhancing interactions with various business and non-business actors to ensure MA for ethical drugs. These relational interactions are critical in navigating the regulatory environment, acquiring knowledge, and establishing legitimacy, viewed from an industrial marketing perspective [55]. Other roles requiring core skills and competencies include negotiating price/reimbursement and obtaining authorisation for new products [56]. Guercini et al. [57] stressed that conceptualising MA in pharma should involve engaging the company in a network of relationships with other actors to sustain a national healthcare system.

No specific academic requirement was necessary for an MA entry role, other than holding a degree in a broadly relevant field. Instead of needing skills specific to MA jobs, candidates were expected to possess a transversal skillset that applies across various backgrounds and contexts [58,59]. This suggests that persons with varying academic backgrounds, especially in life sciences, can work as MA professionals. This finding aligns with a related scoping review [15] and another report [56]. Currently, MA roles increasingly reflect the 'T-shaped' model, where professionals combine deep expertise in MA (vertical) with broad, cross-disciplinary skills (horizontal) essential for navigating complex healthcare challenges [60]. The multidisciplinary nature of the knowledge and skills possessed by these professionals enables them to have unique insights into their tasks [61].

The lack of a restricted academic background to qualify as an MA professional in pharma is a testament to its semi-professional status. Due to the diverse skill requirements and varying activities for MA entry roles, there is a need to establish MA functions, especially in emerging markets, where the complex and dynamic healthcare landscape confounds product approval and uptake [3,8,58]. Adopting a conceptualised framework could help establish MA as a profession [8]. Batt, Tavares, and Williams [62] emphasise that competency frameworks are crucial for defining workforce attributes, facilitating professional mobility, and assessing expertise. The authors emphasise the need for tailored development processes for competency frameworks in healthcare professions to ensure valid outcomes.

Also, Fallis et al. [63] advocate for the adoption of more structured and contextually appropriate approaches to the development and reporting of competency frameworks, in response to the methodological inconsistencies identified across the existing literature. Thus, as is familiar with other emerging fields, professionalisation of MA requires the establishment of formal structures, including accreditation bodies, certification pathways, and standardised curricula [64,65]. For instance, the European Market Access University Diploma in France, and organisations like the Market Access Society, International Market Access Society, and the International Society for Pharmacoeconomics and Outcomes Research, a leading global professional society for HEOR, which plays a central role in MA, provide recognised training and certifications. Incorporating these trainings into career pathways, along with university-affiliated programmes and industry-led training, could help establish a formally recognised and regulated MA profession.

Right reward

The MA component of "right reward" encompasses remuneration, salary, wages, and other entitlements. The study found that remuneration varied across different MA roles. Only a few job adverts specified exact salaries, with most emphasising "competitive benefits" or a "competitive compensation package." The most frequently mentioned reward was the ability to accrue holiday, suggesting a flexible working pattern. Senior MA roles offered a competitive salary range between £104,600.00 and £209,200.00. P&R roles were associated with a "care flexible spending account," allowing employees to set aside pre-tax money to cover healthcare and dependent care expenses. Head and director roles were linked to high-performance reward systems and competitive salary and benefits, indicating that employers value and recognise exceptional performance.

Rewards are typically tangible or transactional, often fixed and lacking personalisation, with a focus on outcomes rather than employee behaviour. The literature distinguishes between financial rewards, such as raises, bonuses, and benefits, and nonfinancial rewards, like autonomy, flexibility, and recognition [56]. Offering encouraging rewards and recognition can help retain employees in the long term, enabling them to advance in their careers. Studies among other health professionals have shown that remuneration is a strong predictor of job retention and satisfaction [18,19,64]. Specifically, organisational commitment, job satisfaction, and employee retention in the pharma industry are significantly associated with compensation and remuneration [65,66].

This study represents the first DA of MA job roles aimed at understanding how the MA in pharma is conceptualised. The findings highlight the necessity for defining professional roles, skills, and knowledge to meet the diverse and evolving demands of the MA in pharma. These insights can inform the development of educational standards for a field that currently lacks them, better preparing individuals for industry challenges and enhancing healthcare quality.

However, this DA is limited by its exclusive reliance on publicly available job advertisements from selected UK-based websites, which may introduce sample bias and restrict the generalisability of findings beyond the UK context or across different recruitment platforms. Moreover, by excluding international roles, the analysis does not capture regional variations in MA roles, competencies, regulatory expectations, or healthcare system structures that shape the profession globally. Therefore, future research should incorporate broader geographic and platform diversity to enhance the applicability and relevance of findings across different contexts. A further limitation of this study is the temporal distribution of data, with 60 of 80 job postings from 2019 and only 20 from 2023. This imbalance may limit the study's ability to capture recent shifts in MA roles following COVID-19 and amid ongoing regulatory changes. This study focuses on advertised MA roles in the UK, and does not incorporate the perspectives of hiring managers, MA team leads, or HR professionals who define these roles in practice. Future research could incorporate these perspectives to enhance document-based analyses.

Conclusions

A Master's in Pharmacy is an emerging profession with heterogeneous job roles and titles, variable entry requirements, and requires a demonstrable ability to understand and collaborate in different healthcare systems. The findings suggest that the MA in pharma is in the early stages of professionalisation and needs further development. To advance this evolution, developing industry-wide competency frameworks, establishing standardised job taxonomies, and creating educational guidelines to inform MA curricula and training pathways are recommended.

Disclosures

Authors' contributions
Conceptualisation and methodology – C.F., G.Y., E.M. and C.M.: Data curation – C.F., G.Y.: Formal analysis. C.F., G.Y. and C.M.: Writing – Original Draft. C.F., G.Y., E.M., I.O. and C.M.: Supervision – G.Y., E.M., I.O. and C.M.: All authors read and approved the final version of the manuscript.

Ethical approval, registration
An exempt ethical approval was granted for this study by the Faculty Ethics at the Manchester Metropolitan University (Registration number -1474, approved on 22/10/2024).

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

References

1. Jarosławski S, Toumi M. Market access agreements for pharmaceuticals in Europe: diversity of approaches and underlying concepts. BMC Health Serv Res 2011;11:259. https://doi.org/10.1186/1472-6963-11-259.
2. Zheng C, Ha J, Hydery T, Westrich K. HTA51 Impact of ICER Assessments on Payer Decision Making in the United States. Value in Health 2023;26:S268. https://doi.org/10.1016/j.jval.2023.03.1480.
3. Pereira CSSM. Examining the Role of Market Access in the Pharmaceutical Industry: A Scoping Review of European Practices (Master's thesis, Universidade de Lisboa (Portugal)). 2023. Retrieved April 09 2025. https://repositorio.ulisboa.pt/bitstream/10451/64350/1/MICF_Carolina_Pereira.pdf.
4. Chaudhury H. Market Access focus: What does modern market access really mean? 2018. Retrieved February 20 2024, from https://pf-media.co.uk/in-depth/market-access-focus-what-does-modern-market-access-really-mean.

5. Chowhan SS. Pharma's future in developing market: A look at market access, elements, and trends. Rabindra Bharati University Journal of Economics. 2024;8:122-130.
6. Gold M. Beyond coverage and supply: measuring access to healthcare in today's market. Health Serv Res. 1998 Aug;33(3 Pt 2):625-52; discussion 681-4. PMID: 9685110; PMCID: PMC1975648.
7. Kockaya G. Collaborative Approaches to Market Access: Working with Stakeholders. Health Policy. 2024. https://marketaccesstoday.com/collaborative-approaches-to-market-access-working-with-stakeholders.
8. Kumar A, Juluru K, Thimmaraju PK, Reddy J, Patil A. Pharmaceutical market access in emerging markets: concepts, components, and future. Journal of Market Access & Health Policy 2014;2:25302. https://doi.org/10.3402/jmahp.v2.25302.
9. Van Nooten F, Holmstrom S, Green J, Wiklund I, Odeyemi IAO, Wilcox TK. Health economics and outcomes research within drug development. Drug Discovery Today 2012;17:615–22. https://doi.org/10.1016/j.drudis.2012.01.021.
10. Scherer FM. Pricing, Profits, and Technological Progress in the Pharmaceutical Industry. Journal of Economic Perspectives 1993;7:97–115. https://doi.org/10.1257/jep.7.3.97.
11. Bowen GA. Document Analysis as a Qualitative Research Method. Qualitative Research Journal 2009;9:27–40. https://doi.org/10.3316/QRJ0902027.
12. O'Leary Z. The essential guide to doing your research project (2nd ed.). Thousand Oaks, CA: SAGE Publications, Inc. 2014.
13. Fatoye C, Yeowell G, Miller E, Odeyemi I, Mbada C. Conceptualisation and Role of Market Access in Pharmaceutical Industry: A Scoping Review. JMAHP 2024;12:81–99. https://doi.org/10.3390/jmahp12020007.
14. Communication. How to get started in Market Access. 2024. https://www.rxcomms.com/learning/guide-to-getting-started-in-market-access#:~:text=The%20Market%20Access%20Specialist%20is,ensuring%20compliance%20with%20regulatory%20standards. Retrieved February 20 2024
15. Acquis. The Intersection of Pricing, Reimbursement, and Market Access Strategies. 2023. Retrieved November 11, 2023 from https://www.acquisconsulting.com/our-thinking/pricing-reimbursement-market-access-strategy.
16. Bruni DS, Verona G. Dynamic Marketing Capabilities in Science-based Firms: an Exploratory Investigation of the Pharmaceutical Industry. British J of Management 2009;20. https://doi.org/10.1111/j.1467-8551.2008.00615.x.
17. Turner A. The professional status of community pharmacists: an exploration of the perceptions of community pharmacists and the general public (Doctoral dissertation, Aston University). 2017.
18. Adams D, Miller BK. Professionalism in nursing behaviors of nurse practitioners. Journal of Professional Nursing 2001;17:203–10. https://doi.org/10.1053/jpnu.2001.25913.
19. Swisher LLD, and Page CG. Professionalism in physical therapy: History, practice, and development. Philadelphia: Elsevier, 2005, pp 23–44.
20. Currie G, Wild A, Lockett A. Contestation over a Profession's Memory: The UK Pharmacy Profession, 1880–1905. Journal of Management Studies. 2025; p 1-33.
21. Koch MA. Pharmaceutical Market Access: current state of affairs and key challenges – results of the Market Access Launch Excellence Inventory (MALEI). Journal of Market Access & Health Policy 2015;3:29679. https://doi.org/10.3402/jmahp.v3.29679.
22. Garrison L, Towse A. Value-Based Pricing and Reimbursement in Personalised Healthcare: Introduction to the Basic Health Economics. JPM 2017;7:10. https://doi.org/10.3390/jpm7030010.
23. Berndt ER, and Newhouse JP. Pricing and reimbursement in US pharmaceutical markets (No. w16297). National Bureau of Economic Research. 2010. https://www.nber.org/system/files/working_papers/w16297/w16297.pdf.
24. IJzerman MJ, Steuten LMG. Early Assessment of Medical Technologies to Inform Product Development and Market Access: A Review of Methods and Applications. Applied Health Economics and Health Policy 2011;9:331–47. https://doi.org/10.2165/11593380-000000000-00000.
25. Pauwels K, Huys I, Casteels M, Simoens S. Industry Perspectives on Market Access of Innovative Drugs: The Relevance for Oncology Drugs. Front Pharmacol 2016;7. https://doi.org/10.3389/fphar.2016.00144.
26. Panteli D, Eckhardt H, Nolting A, Busse R, Kulig M. From market access to patient access: overview of evidence-based approaches for the reimbursement and pricing of pharmaceuticals in 36 European countries. Health Res Policy Syst. 2015 Sep 25;13:39. https://doi.org/10.1186/s12961-015-0028-5.
27. Baird LG, Banken R, Eichler HG, Kristensen FB, Lee DK, Lim JC, Lim R, Longson C, Pezalla E, Salmonson T, Samaha D, Tunis S, Woodcock J, Hirsch G. Accelerated access to innovative medicines for patients in need. Clin Pharmacol Ther. 2014 Nov;96(5):559-71. https://doi.org/10.1038/clpt.2014.145. Epub 2014 Jul 9.
28. Vallano A, Pontes C, Agustí A. The challenges of access to innovative medicines with limited evidence in the European Union. Front Pharmacol 2023;14:1215431. https://doi.org/10.3389/fphar.2023.1215431.
29. Fatoye C, Betts A, Odeyemi A, Fatoye F, Odeyemi I. PNS260 Problems Experienced By Involving Patients at Post-Launch STAGE of Pharmaceutical Product Development. Value in Health 2020;23:S684–5. https://doi.org/10.1016/j.jval.2020.08.1704.
30. Rabea M. Understanding the Factors that Impact the Pre-Launch Phase and New Product Launch Excellence in the Pharmaceutical Industry. AJIBM 2022;12:88–122. https://doi.org/10.4236/ajibm.2022.121007.
31. Mota RDO, Godinho Filho M, Ganga GMD, Da Silva JMN, Mendes GHDS. Assessing the drivers and capabilities for faster product launch: a scale for time to-

market reduction in start-ups. R&D Management 2025;55:127–64. https://doi.org/10.1111/radm.12688.
32. Facey KM, Rannanheimo P, Batchelor L, Borchardt M, De Cock J. Real-world evidence to support Payer/HTA decisions about highly innovative technologies in the EU—actions for stakeholders. Int J Technol Assess Health Care 2020;36:459–68. https://doi.org/10.1017/S026646232000063X.
33. Ogayemi C, Filani OM, Osho GO. A Market Access Optimization Model for New Drug Indications in Emerging Pharmaceutical Markets. IJMRGE 2022;3:699–710. https://doi.org/10.54660/.IJMRGE.2022.3.3.699-710.
34. Dravida S, Dabral A, Sharma J. Chapter 4: Challenge in the use of real world evidence. In: Real-World Evidence in the Pharmaceutical Landscape. 2021. Gatekeeper Press.
35. Claire R, Elvidge J, Hanif S, Goovaerts H, Rijnbeek PR, Jónsson P, Facey K, Dawoud D. Advancing the use of real world evidence in health technology assessment: insights from a multi-stakeholder workshop. Front Pharmacol 2024;14:1289365. https://doi.org/10.3389/fphar.2023.1289365.
36. Auwal FI, Clark EJ, Copeland CS, McClelland GR. From insight to impact: a qualitative study on pharmaceutical industry professionals' perceptions of the value of patient engagement. Current Medical Research and Opinion 2025;41:1261–75. https://doi.org/10.1080/03007995.2025.2537888.
37. Pizzi LT, Onukwugha E, Corey R, Albarmawi H, Murray J. Competencies for Professionals in Health Economics and Outcomes Research: The ISPOR Health Economics and Outcomes Research Competencies Framework. Value in Health 2020;23:1120–7. https://doi.org/10.1016/j.jval.2020.04.1834.
38. Palatino V. How to get and develop a career in a market access role in the medical devices' industry. In Career options in the pharmaceutical and biomedical industry: An insider's guide, 2023; pp. 119-136.
39. Thomas JR. Job and Career Opportunities in the Pharmaceutical Industry: An Overview. In: Thomas JR, Saso L, Van Schravendijk C, editors. Career Options in the Pharmaceutical and Biomedical Industry, Cham: Springer International Publishing; 2023, p. 33–84. https://doi.org/10.1007/978-3-031-14911-5_3.
40. Hubbart JA. The Importance of Stakeholder Engagement Across Market Domains. J Glob Entrep Manage 2024;2:1–7. https://doi.org/10.59462/JGEM.2.1.106.
41. Umrania O, Jagtap NS. Optimising Pharmaceutical Sales and Marketing with Data Analytics. 2024 IEEE 3rd World Conference on Applied Intelligence and Computing (AIC), Gwalior, India: IEEE; 2024, p. 412–7. https://doi.org/10.1109/AIC61668.2024.10730850.
42. Rath B, and Kar SK. Real-World Data Analytics in Global Pharmaceutical Marketing. IUP Journal of Knowledge Management. 2016;14(2):48–59
43. Uzhakova (Née Sabirzyanova) N, Fischer S. Data-Driven Enterprise Architecture for Pharmaceutical R&D. Digital 2024;4:333–71. https://doi.org/10.3390/digital4020017.
44. Holmér GW, and Gamage IH. Development of a data-driven marketing strategy for an online pharmacy. 2022; Master Thesis. https://www.diva-portal.org/smash/get/diva2:1682303/FULLTEXT01.pdf
45. Kumar SA, Ananda Kumar TD, Beeraka NM, Pujar GV, Singh M, Narayana Akshatha HS, Bhagyalalitha M. Machine Learning and Deep Learning in Data-Driven Decision Making of Drug Discovery and Challenges in High-Quality Data Acquisition in the Pharmaceutical Industry. Future Med Chem 2022;14:245–70. https://doi.org/10.4155/fmc-2021-0243.
46. Akhmetov I, Ramaswamy R, Akhmetov I, Thimmaraju P. Market Access Advancements and Challenges in "Drug-Companion Diagnostic Test" Co-Development in Europe. JPM 2015;5:213–28. https://doi.org/10.3390/jpm5020213.
47. Olson DW. Market testing and postlaunch evaluation for consumer goods. The PDMA Handbook of New Product Development, 2007; pp.479-496.
48. Du Plessis D, Sake J-K, Halling K, Morgan J, Georgieva A, Bertelsen N. Patient Centricity and Pharmaceutical Companies: Is It Feasible? Ther Innov Regul Sci 2017;51:460–7. https://doi.org/10.1177/2168479017696268.
49. Zou KH, Salem LA, Ray A. Real-World Evidence in a Patient-Centric Digital Era. 1st ed. New York: Chapman and Hall/CRC; 2022. https://doi.org/10.1201/9781003017523.
50. Saini JPS, Thakur A, Yadav D. AI-driven innovations in pharmaceuticals: optimizing drug discovery and industry operations. RSC Pharm 2025;2:437–54. https://doi.org/10.1039/D4PM00323C.
51. Shraddha Dingre, Vishal Girbane, Sujit Garudkar, Priyanka Todakari, Dhanashri Borawake, Meera Deshmukh. Integrating artificial intelligence in pharmaceutical marketing: Enhancing engagement and efficiency. World J Adv Eng Technol Sci 2025;15:526–39. https://doi.org/10.30574/wjaets.2025.15.3.0948.
52. Jen M-H, Mt-Isa S, Chu H, Bridges I, Cai Z, Dunger-Baldauf C, Guan J, Ma J, Ren M, Wang Z, He W. The Current Landscape of HTA Framework and Key Challenges. Statistics in Biopharmaceutical Research 2025:1–15. https://doi.org/10.1080/19466315.2025.2495307.
53. Ströhmer N. The Role of Real-World Evidence in Health Technology Assessment Decision-Making: Methodological Challenges and Recommendations From International Health Systems (Master's thesis, Universidade NOVA de Lisboa (Portugal)). 2024.
54. Schweitzer SO, Lu ZJ. Pharmaceutical Economics and Policy. vol. 1. Oxford University Press; 2018. https://doi.org/10.1093/oso/9780190623784.001.0001.
55. Runfola A, Guercini S, Milanesi M. Network interactions for pharmaceutical market access: findings from an explorative research. JBIM 2021;36:174–86. https://doi.org/10.1108/JBIM-07-2020-0371.
56. Sendyona S, Odeyemi I, Maman K. Perceptions and factors affecting pharmaceutical market access: results from a literature review and survey of stakeholders in different settings. Journal of Market Access & Health Policy 2016;4:31660. https://doi.org/10.3402/jmahp.v4.31660.
57. Guercini S, Milanesi M, Runfola A. Bridges to sustainable health systems: public-private interaction

for market access. JBIM 2020;35:1929–39. https://doi.org/10.1108/JBIM-11-2019-0475.
58. De Cosmo LM. The Strategic Role of Sales Management for Market Access in the Pharmaceutical Sector. ESJ 2018;14. https://doi.org/10.19044/esj.2018.v14n34p146.
59. Malviya N, Malviya S, Dhere M. Transformation of Pharma Curriculum as Per the Anticipation of Pharma Industries-Need to Empower Fresh Breeds with Globally Accepted Pharma Syllabus, Soft Skills, AI and Hands-on Training. Ind J Pharm Edu Res 2023;57:320–8. https://doi.org/10.5530/ijper.57.2.41.
60. Conley SN, Foley RW, Gorman ME, Denham J, Coleman K. Acquisition of T-shaped expertise: an exploratory study. Social Epistemology 2017;31:165–83. https://doi.org/10.1080/02691728.2016.1249435.
61. Hansen, M. T. T-shaped stars: The backbone of IDEO's collaborative culture. Interview with IDEO CEO Tim Brown. Chief Executive. https://chiefexecutive.net/ideo-ceo-tim-brown-t-shaped-stars-the-backbone-of-ideoaes-collaborative-culture__trashed. 2010, January 21.
62. Batt AM, Tavares W, Williams B. The development of competency frameworks in healthcare professions: a scoping review. Adv in Health Sci Educ 2020;25:913–87. https://doi.org/10.1007/s10459-019-09946-w.
63. Fallis D, Irwin S, Cervero R, Durning S. Frameworks to Guide Faculty Development for Health Professions Education: A Scoping Review. J Contin Educ Health Prof 2022;42:180–9. https://doi.org/10.1097/CEH.0000000000000376.
64. Randolph DS. Predicting the effect of extrinsic and intrinsic job satisfaction factors on recruitment and retention of rehabilitation professionals. J Healthc Manag. 2005 Jan-Feb;50(1):49-60; discussion 60. PMID: 15729907.
65. Atif A, Kashif Ur Rehman, Ijaz Ur Rehman, Muhammad AK, Asad AH. Impact of organizational commitment on job satisfaction and employee retention in pharmaceutical industry. Afr J Bus Manage 2011;5:7316–24. https://doi.org/10.5897/AJBM10.1296.
66. Alam SMdT. Factors Affecting Job Satisfaction, Motivation and Turnover Rate of Medical Promotion Officer (MPO) in Pharmaceutical Industry: A Study Based in Khulna City. Asian Bus Rev 2012;1:126–31. https://doi.org/10.18034/abr.v1i2.131.

REVIEW PAPER

JMS Journal of Medical Science

Heme Oxygenase-1-Targeted Cancer Therapy: At the Crossroads of Cytoprotection and Tumour Progression

Marzieh Raeispour
Doctoral School, Department of Toxicology, Poznan University of Medical Sciences, Poznań, Poland
https://orcid.org/0009-0004-6744-2855

Marek Murias
Department of Toxicology, Poznan University of Medical Sciences, Poznań, Poland
https://orcid.org/0000-0002-2903-4912

Corresponding author: marek.murias@ump.edu.pl

https://doi.org/10.20883/medical.e1358

Keywords: heme oxygenase-1, cancer progression, oxidative stress, ferroptosis, tumour angiogenesis, chemoresistance

Received 2025-07-25
Accepted 2025-09-01
Published 2025-09-25

How to Cite: Raeispour M, Murias M. Heme Oxygenase-1-Targeted Cancer Therapy: At the Crossroads of Cytoprotection and Tumour Progression. Journal of Medical Science. 2025 September;94(3);e1358. doi:10.20883/medical.e1358

ABSTRACT

Introduction. HO-1 is a stress-responsive enzyme involved in cellular protection against oxidative damage, inflammation, and tissue injury. However, in cancer, its cytoprotective functions may paradoxically support cancer progression, immune evasion, and therapy resistance.
Material and methods. This review explores current findings on HO-1's dual role in cancer biology. We analysed studies addressing its function in redox regulation, angiogenesis, immune modulation, iron metabolism, and its impact on treatment response. Particular focus was placed on HO-1's downstream metabolites (CO, biliverdin/bilirubin, and iron) and their influence on tumour development.
Results. HO-1 contributes to cellular defence by limiting reactive oxygen species and supporting DNA repair. However, its overexpression in tumours promotes survival signalling, angiogenesis (via VEGF and HIF-1α), metabolic reprogramming, and resistance to apoptosis and chemotherapy. Additionally, HO-1 regulates ferroptosis by modulating intracellular iron and lipid peroxidation. The Nrf2/HO-1 axis is frequently upregulated in tumours, enhancing antioxidant capacity and undermining therapeutic efficacy. Preclinical studies show that HO-1 inhibition—via gene silencing, small molecules, or combination with chemotherapy and photodynamic therapy—can restore treatment sensitivity and suppress tumour growth.
Conclusions. HO-1 plays a context-dependent, dual role in cancer as both a protector and promoter. Therapeutic targeting of HO-1 holds promise but requires precision to avoid disrupting its protective roles in normal tissues. Further research should aim to develop selective, tumour-specific HO-1 inhibitors and integrate them into combination treatment strategies.

Biological Role of HO-1

Cytoprotective Role of HO-1

HO-1 is a key component of the endogenous cellular defence system against oxidative stress and tissue injury, playing a multifaceted cytoprotective role in various physiological and pathological contexts. HO-1 is an inducible enzyme that catalyses the first and rate-limiting step in heme degradation, producing biliverdin, free iron, and

CO. Each of these products contributes to cellular homeostasis and protection. Biliverdin is rapidly converted into bilirubin, one of the most potent endogenous antioxidants, which neutralises ROS and prevents lipid peroxidation [1]. CO, despite its known toxicity at high levels, functions as a signalling molecule at physiological concentrations, inhibiting excessive inflammation and apoptosis via modulation of the mitogen-activated protein kinase (MAPK) and NF-κB pathways [1,2]. The free iron released during heme breakdown induces ferritin expression, thereby limiting Fenton reaction-mediated oxidative damage [3]. HO-1 expression is upregulated in response to diverse stressors, including hypoxia, pro-inflammatory cytokines, heavy metals, and UV radiation. Experimental models have demonstrated that elevated HO-1 levels protect tissues, including the liver, heart, kidneys, lungs, and central nervous system, from ischaemia-reperfusion injury, toxins, and inflammation [2]. *In vivo*, HO-1 overexpression reduces necrosis and apoptosis, limits leukocyte infiltration, and promotes tissue repair. HO-1 also modulates immune responses by influencing the function of T lymphocytes and macrophages, thereby fostering a tolerogenic immune profile and supporting regeneration mechanisms relevant to inflammatory diseases and transplant tolerance. Therefore, this effect is highly context-dependent; in specific cancer types, persistent HO-1 overexpression may paradoxically promote tumour cell survival and therapy resistance by enhancing antioxidant defences and inhibiting apoptosis [3]. Importantly, maintaining a balance in HO-1 activity is crucial, as chronic, uncontrolled overexpression of this enzyme has been observed in some cancers, where it enhances cancer cell survival and treatment resistance, posing a challenge for the development of targeted therapies [4].

In summary, HO-1 functions as a central cytoprotective mechanism by orchestrating antioxidant defence, immune modulation, and iron homeostasis. Its diverse biological effects make it a promising therapeutic target in conditions such as ischaemia-reperfusion injury, autoimmune disease, neurodegeneration, and cardiovascular pathology, while also demanding caution due to its potential pro-tumorigenic role, unlike earlier reviews published so far [5,6]. This work highlights novel insights into HO-1 regulation by exosomes, its dual role in ferroptosis, and its interplay with photodynamic therapy and immunotherapy, providing an updated perspective on the therapeutic potential of HO-1.

Anti-inflammatory effects

HO-1 and its metabolic byproducts exert antioxidant and anti-inflammatory effects by scavenging reactive oxygen species and limiting lipid peroxidation. Among its byproducts, carbon monoxide, generated during heme catabolism, acts as a signalling molecule that inhibits key inflammatory pathways such as NF-κB. This leads to reduced expression of pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β. A notable example of HO-1's anti-inflammatory relevance is andrographolide, a plant-derived compound that ameliorates ulcerative colitis by activating the Nrf2 (nuclear factor erythroid 2-related factor 2)/HO-1 pathway. Activation of Nrf2 leads to upregulation of HO-1, reduction of oxidative stress, and suppression of inflammatory cytokines, including IL-23, IL-17, and TNF-α. Andrographolide also restores levels of major antioxidant defence players like glutathione and superoxide dismutase. These effects are likely mediated through Nrf2 activation, rather than direct upregulation of antioxidant genes. These effects are reversed by the Nrf2 inhibitor ML385, confirming the central role of the Nrf2/HO-1 axis in mediating its protective action [7].

CO itself significantly contributes to immune modulation. Both endogenous CO production and pharmacological HO-1 induction suppress inflammatory cytokines (e.g., TNF-α, IL-17), while simultaneously increasing anti-inflammatory mediators, among which are IL-10 and IL-22 in colonic tissue. Inhibition of HO-1 attenuates these effects, highlighting CO's immunoregulatory potential [8]. Overall, HO-1 and its byproducts, such as CO, demonstrate potent anti-inflammatory and antioxidant actions by modulating key signalling pathways and cytokine production. This underscores its therapeutic relevance in inflammatory diseases and tissue protection.

Vascular regulation

HO-1 plays a critical role in regulating vascular dynamics within the tumour microenvironment, particularly in angiogenesis and vascular remodelling. As part of the cellular antioxidant defence system, HO-1 expression is strongly upregulated

under oxidative and inflammatory conditions, frequently observed in tumours. One of the key mechanisms involves the upregulation of vascular endothelial growth factor (VEGF) through interaction with the HIF-1α pathway under hypoxia. This promotes neovascularisation, facilitating tumour growth and nutrient supply. However, under specific contexts, excessive HO-1 expression can paradoxically suppress VEGF signalling, reflecting its context-dependent role in regulating the vascular system. HO-1-derived carbon monoxide promotes endothelial cell survival by inhibiting apoptosis through the NF-κB and MAPK pathways, while also downregulating adhesion molecules, including intercellular adhesion molecule 1 and vascular cell adhesion molecule 1. This dampens immune cell infiltration, creating an immunosuppressive microenvironment conducive to cancer progression [4].

Exosome-mediated signalling has emerged as another layer of HO-1. Tumour-derived exosomes can carry regulatory molecules such as microRNAs, long non-coding RNAs, and proteins that influence HO-1 expression and downstream signalling in recipient stromal and endothelial cell regulation. In prostate cancer, tumour-derived exosomes enriched with regulatory RNAs activate HO-1 pathways in neighbouring stromal or endothelial cells, promoting angiogenesis and resistance to anti-androgen therapy [9]. These findings highlight the importance of intercellular communication in reinforcing HO-1-mediated tumour adaptations. In line with these findings, HO-1 has been shown to modulate endothelial cell proliferation, migration, and tube formation, primarily through VEGF and activation of transcription factors such as NF-κB and Nrf2. While inhibition of HO-1 can effectively reduce angiogenesis and tumour growth in some settings, it may also impair endothelial integrity or increase oxidative stress in others.

Nevertheless, systemic HO-1 inhibition may impair endothelial function in normal tissues, increase oxidative stress, and compromise vascular perfusion, necessitating careful therapeutic targeting. Therefore, any therapeutic strategy targeting HO-1 must carefully consider the tumour type, vascular context, and potential systemic side effects [4].

Interestingly, cannabinoids, for example, cannabidiol (CBD), modulate vascular smooth muscle cell behaviour through HO-1 induction via ROS-dependent mechanisms. While HO-1 contributes to cytoprotection, CBD's antiproliferative and antimigratory effects appear to be both receptor-dependent and -independent. Notably, inhibition of HO-1 enhances the antiproliferative action of CBD, suggesting a complex interplay between ROS signalling and vascular regulation [10].

Finally, HO-1 metabolites—CO and bilirubin—contribute directly to vascular homeostasis. CO stimulates VEGF expression and endothelial proliferation. At the same time, bilirubin, with potent antioxidant activity, protects vascular structures and limits leukocyte adhesion and migration [11]. In summary, HO-1 plays a pivotal, highly context-dependent role in shaping tumour vasculature. It supports angiogenesis, preserves endothelial cell integrity, and contributes to immune evasion. This dual functionality makes HO-1 both a potential therapeutic ally and a risk factor, underscoring the need for precise, tumour-specific modulation in anticancer strategies.

Iron homeostasis

HO-1 is a key regulator of iron homeostasis, particularly within the tumour microenvironment, where it catalyses the degradation of heme into biliverdin, carbon monoxide, and free iron. This process not only supports cellular antioxidant defence but also influences several metabolic pathways linked to cancer progression and therapy response. By breaking down heme, HO-1 facilitates iron recycling within tissues. Although essential for local iron turnover, HO-1 is not the primary contributor to systemic iron needed for haematopoiesis. In HO-1 knockout mouse models, iron abnormally accumulates in the liver and kidneys while circulating levels decrease, leading to impaired systemic iron regulation [12]. Under normal conditions, intracellular iron is either exported via ferroportin or safely stored in ferritin. However, excessive HO-1 activity increases intracellular free iron, which can overwhelm detoxification systems and lead to reactive oxygen species production via Fenton reactions, ultimately driving lipid peroxidation and DNA damage. While HO-1 generally protects tumour cells from oxidative injury, under certain stress conditions, for instance, impaired ferritin buffering or increased ROS, excess iron can trigger ferroptosis, a form of regulated, iron-dependent cell death. Ferrop-

tosis is triggered by iron accumulation but also depends on the presence of polyunsaturated fatty acids (PUFAs) in membranes and the depletion of protective systems, such as glutathione or the glutathione peroxidase 4 enzyme [5]. This paradox highlights HO-1's dual role: promoting survival in early tumorigenesis by supporting redox homeostasis, yet potentially inducing cell death when iron accumulation exceeds protective thresholds. Notably, HO-1 is part of a broader antioxidant network regulated by transcription factors, particularly Nrf2, which is often activated in cancer. This activation frequently results from mutations in genes such as Kelch-like ECH-associated protein 1 (KEAP1) or nuclear factor, erythroid 2–like 2, leading to constitutive Nrf2 signalling that enhances antioxidant capacity and promotes the survival of cancer cells. Through the Nrf2/HO-1 axis, cancer cells can adapt to oxidative stress, enhance glutathione production, and resist chemotherapy or radiotherapy. However, this same system can be exploited therapeutically. Under certain conditions, HO-1-driven increases in intracellular iron may be redirected to promote ferroptosis, offering a strategic vulnerability in tumours resistant to conventional therapies [5].

In summary, HO-1 maintains a delicate balance between iron recycling and oxidative stress. While it protects against iron-mediated damage in normal physiology, its dysregulation in tumours may contribute to both survival and susceptibility. Understanding this balance is critical for leveraging HO-1 as a therapeutic target—either by enhancing its protective role or tipping it toward ferroptotic cell death in cancer therapy.

A dual role of HO-1 in cancer

HO-1 plays a complex and paradoxical role in cancer, acting as both a tumour suppressor and a promoter of cancer progression depending on the stage of tumorigenesis and the cellular context. This duality highlights the intricate interplay between oxidative stress, cellular metabolism, and survival mechanisms in cancer biology. This duality is also influenced by HO-1's subcellular localisation (cytosolic vs mitochondrial vs nuclear), stage-specific expression, and interactions with microenvironmental stressors such as hypoxia, inflammation, and immune cell activity [13].

HO-1 as a tumour suppressor

While HO-1 is often associated with cancer progression due to its antioxidant and cytoprotective functions, emerging evidence suggests that it can also exert tumour-suppressive effects, particularly in early stages of carcinogenesis. A key regulatory mechanism involves TRC8, an endoplasmic reticulum-associated E3 ubiquitin ligase with tumour-suppressive function. TRC8 targets HO-1 for ubiquitination and proteasomal degradation, limiting its accumulation and oncogenic potential. Loss of TRC8 leads to elevated HO-1 levels, which in turn enhance tumour cell proliferation, migration, and invasion. Conversely, reduced HO-1 expression is associated with increased oxidative stress, G2/M cell cycle arrest, and activation of DNA damage checkpoints—highlighting its role in redox homeostasis and genome surveillance [14]. These tumour-suppressive properties of HO-1 appear particularly relevant in the early phases of tumorigenesis, when genomic instability and oxidative damage are major drivers of malignant transformation. In addition, HO-1 and its metabolic product carbon monoxide contribute to genomic stability by mitigating ROS-induced damage and supporting DNA repair mechanisms. HO-1 has been shown to facilitate the activation of key DNA repair kinases such as ATM and ATR, which are essential for homologous recombination and non-homologous end joining. CO further enhances this protective effect by promoting efficient DNA repair, thereby reducing the accumulation of mutations that could drive malignant transformation or premature cellular senescence. Significantly, HO-1 and CO may modulate apoptotic responses to DNA damage, allowing time for repair before irreversible cell death is initiated [15]. Collectively, these findings suggest that HO-1 can function as a tumour suppressor by maintaining oxidative balance, promoting DNA integrity, and regulating cell cycle progression. Its interaction with tumour-suppressive regulators such as TRC8 underscores the relevance of post-translational control in limiting early tumour development.

HO-1 as a tumour promoter

Despite its well-established cytoprotective and anti-inflammatory functions, HO-1 is frequently overexpressed in various malignancies and is now widely recognised as a key pro-tumorigen-

ic factor. Emerging evidence demonstrates that HO-1 promotes cancer progression by shaping a microenvironment that favours immune evasion, metabolic adaptation, angiogenesis, metastasis, and resistance to therapy [5]. HO-1 facilitates cancer cell survival and proliferation primarily by modulating redox homeostasis and preserving mitochondrial integrity under stress. Its enzymatic degradation of heme produces biliverdin, bilirubin, CO, and ferrous iron—each of which contributes to tumorigenesis. Biliverdin and its reduced form, bilirubin, act as potent antioxidants that scavenge ROS, shielding tumour cells from oxidative damage and apoptosis [5]. CO, in turn, activates pro-survival pathways, including the MAPK cascade and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) signalling, while promoting angiogenesis via soluble guanylate cyclase/cyclic guanosine monophosphate and VEGF pathways and supporting anti-apoptotic gene expression [4]. Beyond redox control, HO-1 reprograms iron metabolism in tumour cells. The enzymatic release of free iron from heme degradation increases intracellular Fe^{2+} levels, which can initially be sequestered by ferritin, serving an antioxidant buffering function. However, when iron accumulation exceeds cellular capacity, redox-active iron promotes the Fenton reaction, leading to ROS generation, DNA damage, genomic instability, and potentially oncogenic transformation [5]. HO-1 further contributes to immune evasion by regulating macrophage polarisation, suppressing dendritic cell maturation, and enhancing the activity of regulatory T cells. Its metabolic byproducts, CO and bilirubin, inhibit the expression of pro-inflammatory cytokines (e.g., IL-12, TNF-α) and promote the production of anti-inflammatory mediators such as IL-10 and TGF-β, thereby dampening effective immune surveillance [5]. Moreover, HO-1 has been shown to facilitate epithelial-to-mesenchymal transition, a critical mechanism underlying tumour invasion and metastasis, particularly in breast and prostate cancer models [13]. HO-1 also plays a pivotal role in the metabolic reprogramming of cancer cells. It induces glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of the PPP, enhancing NADPH production and supporting glutathione regeneration. This shift not only strengthens antioxidant defences but also facilitates anabolic biosynthesis and tumour survival under metabolic stress.

Additionally, activation of the CO/cystathionine β-synthase pathway by HO-1 decreases 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3 methylation, further diverting glucose flux into the PPP to sustain redox balance and proliferation, even under hypoxia [16]. The enzyme's contribution to therapy resistance is well documented. Overexpression of HO-1 in tumour cells confers protection against chemotherapy and radiotherapy by mitigating treatment-induced oxidative stress. This protection is mediated through Nrf2-dependent antioxidant responses, the upregulation of cyclin-dependent kinase inhibitor 1, and the suppression of caspase-3 activation [17,18]. Conversely, pharmacological or genetic inhibition of HO-1 has been shown to sensitise tumour cells to chemotherapeutic agents such as doxorubicin, cisplatin, and paclitaxel [13]. An especially intriguing aspect of HO-1 biology is its subcellular localisation. In some tumour types, notably prostate cancer, HO-1 is aberrantly translocated to the nucleus, where it loses its enzymatic activity but gains non-canonical regulatory functions. Nuclear HO-1 is associated with increased proliferation, resistance to apoptosis, enhanced oxidative metabolism, and recurrence, particularly under hypoxic conditions [13]. While CO may paradoxically sensitise tumour cells to genotoxic agents by increasing oxidative stress, HO-1 simultaneously protects surrounding healthy tissues from damage, highlighting the enzyme's context-dependent duality [19]. Altogether, these findings highlight the complexity of HO-1 as a potential tumour promoter. Its ability to modulate redox balance, immune function, metabolism, and therapeutic response makes it a compelling but challenging target in cancer therapy. Depending on the tumour type, cellular context, and disease stage, HO-1 may shift from a protective enzyme to an oncogenic driver, underscoring the importance of tumour-specific context when evaluating its role. Indeed, while some research groups emphasise the protective functions of HO-1 in preventing oxidative damage, maintaining genomic stability, and limiting early tumour initiation, others consistently report that its persistent overexpression promotes angiogenesis, immune evasion, and therapy resistance. This divergence reflects the highly context-dependent nature of HO-1 biology. In our view, the preponderance of recent evidence

indicates that in advanced malignancies, HO-1 functions predominantly as a tumour promoter. In contrast, its protective role appears more relevant in normal tissues and at the earliest stages of carcinogenesis.

Role of HO-1 in angiogenesis and Nrf2 pathway activation

HO-1 expression is strongly induced under hypoxic conditions, where it stabilises hypoxia-inducible factors (HIF-1α and HIF-2α), leading to upregulation of angiogenic mediators such as VEGF and COX-2. This promotes neovascularisation, fuelling tumour growth and metastasis. In contrast, under normoxia, HO-1 overexpression has a limited impact on angiogenesis, highlighting the importance of oxygen tension in its pro-angiogenic effects. Pharmacological inhibition of HO-1—for example, with ZnPP—has been shown to reduce VEGF levels and microvessel density both *in vitro* and *in vivo*, confirming its functional relevance [20,21]. The Nrf2/HO-1 axis, a key regulator of oxidative stress responses, also plays a central role in tumour angiogenesis. In hypoxia, Nrf2 activation leads to increased HO-1 expression and enhanced VEGF production. Compounds such as dextran sulphate and brusatol, which inhibit Nrf2, effectively suppress the expression of HO-1 and VEGF in gastric cancer models, thereby reducing their angiogenic potential. Conversely, tert-butylhydroquinone, a known Nrf2 activator, promotes angiogenesis—an effect that can be reversed by dextran sulphate [22]. These findings suggest that modulating Nrf2 signalling can significantly impact tumour vascularisation. Huang and coworkers [23] further demonstrated that hypoxia-induced Nrf2 activation increases HO-1 and VEGF levels, with CO serving as an intermediary pro-angiogenic signal. Analysis of tumour tissues revealed that Nrf2, HO-1, and VEGF expression positively correlate with microvessel density and tumour grade. The Cancer Genome Atlas datasets support these observations, showing co-expression of these genes in more aggressive cancers. Although Nrf2 is essential for maintaining redox homeostasis under physiological conditions, its persistent activation in tumours provides cancer cells with a survival advantage, enhancing resistance to chemotherapy and facilitating metastasis [24]. HO-1, as a downstream effector of Nrf2, contributes to this adaptation by inhibiting apoptosis and inducing cytoprotective autophagy. Pharmacological HO-1 inhibitors, such as ZnPP and SnPP, as well as gene-silencing strategies, have been shown to restore treatment sensitivity; however, their use is limited by potential off-target effects and the loss of HO-1's protective role in normal tissues [25]. In conclusion, the Nrf2/HO-1 signalling axis is a key driver of tumour angiogenesis under hypoxic stress, promoting vascular remodelling, immune evasion, and therapy resistance. Although targeting this pathway holds significant therapeutic promise, successful intervention will require context-specific approaches that minimise harm to normal cells while disrupting tumour-supportive angiogenesis.

Therapeutic implications of targeting HO-1

Given its dual role in cancer biology, HO-1 represents both a therapeutic target and a clinical challenge. Overexpression of HO-1 has been observed in various tumour types, where it contributes to proliferation, invasion, and metastasis via key oncogenic pathways, including MAPK/ERK and p38 signalling. Pharmacological inhibition of HO-1 reduces oxidative stress, lowers protein carbonylation, and suppresses tumour growth *in vitro* and *in vivo*. For example, the small-molecule inhibitor 2-[2-(4-bromophenyl)ethyl]-2-[(1H-imidazol-1-yl) methyl]-1,3-dioxolane hydrochloride has demonstrated significant anticancer activity and synergised with chemotherapy agents such as Taxol, enhancing tumour regression and reducing metastatic spread [26]. However, targeting HO-1 is complicated by its essential cytoprotective functions in healthy tissues. Systemic inhibition may disrupt antioxidant defence, provoke toxicity, and interfere with physiological immune regulation. Moreover, tumours may activate compensatory survival pathways when HO-1 is suppressed, limiting therapeutic effectiveness. An additional layer of complexity arises in cancers influenced by hormonal signalling, such as prostate cancer, where androgen deprivation has been associated with increased HO-1 expression, potentially undermining therapeutic response [26,27]. Gene-silencing strategies, such as small interfering RNA or short hairpin RNA, offer more specific approaches but often suffer from incomplete inhibition, off-target effects, and delivery challenges—especially when targeting tumour tissue while sparing vital organs like the

liver and kidneys. Despite these limitations, HO-1 inhibition remains a promising approach, particularly when combined with standard therapies, such as chemotherapy, radiotherapy, or immunotherapy. Preclinical studies have consistently shown that disrupting HO-1 signalling can restore chemosensitivity, enhance oxidative damage in tumour cells, and improve treatment outcomes [18,28,29].

In summary, effective targeting of HO-1 requires a context-specific and selective approach—one that suppresses its tumour-promoting activity while preserving protective functions in normal tissues. Future therapeutic strategies should aim to develop tumour-specific delivery systems, identify predictive biomarkers of HO-1 dependence, and explore rational combination therapies. Optimising HO-1 modulation could significantly improve therapeutic efficacy and expand the arsenal of targeted cancer treatments.

HO-1 inhibitors: mechanisms and therapeutic potential

Direct enzymatic inhibition

HO-1 inhibitors mainly block the catalytic activity of the enzyme, which involves the breakdown of heme to CO, biliverdin, and free iron. These inhibitors often act through competitive mechanisms, binding to the enzyme's active site and preventing heme interaction. Metalloporphyrins, for example, mimic the heme structure and bind to the catalytic domain of HO-1. By competing with heme, these inhibitors block enzymatic activity, leading to an accumulation of free heme, which acts as a pro-oxidant, inducing oxidative stress and apoptosis, especially in cancer cells. A critical structural feature for HO-1 inhibition is the presence of an azole nucleus (such as imidazole) that coordinates with the iron atom in the enzyme's active site. The therapeutic potential of these inhibitors lies in their ability to reverse HO-1's anti-apoptotic and pro-angiogenic effects, thereby reducing tumour proliferation, enhancing chemotherapy efficacy, and increasing oxidative stress within tumour cells [30]. It must, therefore, always be considered that direct enzymatic inhibitors competitively block HO-1's catalytic function, elevate intracellular heme, and promote pro-oxidant conditions that sensitise cancer cells to apoptosis and therapy.

Impact on downstream products of HO-1

Pharmacological inhibition of HO-1, such as with ZnPPIX or PEG-ZnPPIX, elevates oxidative stress and reduces angiogenesis by decreasing its downstream products. Bilirubin protects cells from oxidative damage, while CO, a key signalling molecule, contributes to tumour growth and treatment resistance by activating anti-apoptotic pathways and promoting angiogenesis. Additionally, free iron from heme breakdown can cause cytotoxicity via ROS formation if it accumulates excessively; however, cancer cells often upregulate iron regulation mechanisms to counteract this. HO-1 inhibition disrupts this balance, leading to increased oxidative stress and iron-mediated damage in cancer cells [6]. Blocking HO-1 -derived metabolites removes cytoprotective effects, weakens tumour antioxidant defences, and enhances oxidative stress and cytotoxicity.

Iron dysregulation and oxidative stress

HO-1 inhibition also targets tumour survival by disrupting iron homeostasis. Hyperactive HO-1 increases Fe^{2+} release from excessive heme degradation, producing hydroxyl radicals through the Fenton reaction and elevating iron-dependent oxidative stress. Tumours counteract this by increasing ferritin expression and activating survival pathways such as the Nrf2-Keap1 axis, protecting against ferroptosis. HO-1 inhibitors like ZnPPIX and SnPPIX can suppress the Nrf2-HO-1-FTH1 (FTH1 – ferritin heavy chain 1) pathway, increasing free iron and triggering lipid peroxidation and ferroptosis. Inhibition of HO-1 also upregulates transferrin receptor 1 (TFR1) and increases iron uptake, exacerbating iron dyshomeostasis and promoting cell death. Combining HO-1 suppression with ferroptosis-inducing drugs like Ras-selective lethal 3 MQC and Iristin further enhances therapeutic effects [31–34]. Targeting HO-1 disrupts iron regulation, triggers ferroptosis, and amplifies oxidative stress to overcome tumour resistance.

Future therapeutic directions and clinical perspectives

Inhibition of tumour growth and angiogenesis

HO-1 promotes tumour cell survival by supporting angiogenesis and counteracting oxidative

stress. Carbon monoxide, a product of heme degradation by HO-1, activates pro-survival pathways including the MAPK cascade and soluble guanylyl cyclase, facilitating proliferation and vascularisation. Concurrently, biliverdin and bilirubin function as potent antioxidants, scavenging reactive oxygen species that would otherwise impair tumour cell viability. Pharmacological or genetic inhibition of HO-1 disrupts these cytoprotective mechanisms, leading to reduced tumour cell proliferation, increased apoptosis, and suppression of angiogenic signalling. Studies have shown that HO-1 inhibition decreases cell viability in breast, melanoma, and pancreatic cancer models and can potentiate the efficacy of conventional chemotherapeutics such as paclitaxel or cisplatin [30,35]. Thus, HO-1 represents a promising target for impairing tumour vascular support and enhancing therapeutic responses.

Overcoming chemoresistance

HO-1 overexpression is frequently associated with resistance to chemotherapy, as tumour cells exploit its antioxidant and cytoprotective functions to withstand treatment-induced oxidative stress. Inhibiting HO-1 depletes intracellular antioxidant reserves, thereby resensitising tumour cells to chemotherapeutic agents. Combination therapies involving HO-1 inhibitors and cytotoxic drugs such as cisplatin or doxorubicin have been shown to enhance apoptosis and reduce tumour viability [6]. In breast carcinoma cell lines, HO-1 upregulation is linked to increased autophagy, which contributes to drug resistance. Silencing HO-1 expression or targeting upstream signalling pathways, such as PI3K/Akt, can inhibit autophagy and restore chemosensitivity [36]. Similar effects are reported in brain tumours, neuroblastoma, pancreatic ductal adenocarcinoma, and gastric cancer, where HO-1 inhibition enhances apoptosis, reduces immunosuppression, and improves prognosis [37–40]. Suppressing HO-1 disrupts tumour cell defence mechanisms, reverses chemoresistance, and enhances the cytotoxicity of anticancer drugs.

Inducing apoptosis in cancer cells

Inhibition of HO-1 promotes apoptosis through multiple mechanisms, including the accumulation of free heme, induction of oxidative stress, caspase activation, and mitochondrial dysfunction. HO-1 typically supports mitochondrial quality control and redox balance; however, its suppression compromises mitochondrial integrity, making tumour cells more susceptible to stress-induced apoptosis [25]. While HO-1 also influences mitochondrial quality control by regulating oxidative stress, its suppression can increase mitochondrial damage in tumour cells, compromising their survival under stress [41]. Interestingly, HO-1's dual role means that its inhibition can either prevent antioxidant protection or induce ferroptosis by increasing lipid peroxidation.

Indeed, HO-1 has emerged as a critical regulator of ferroptosis—a distinct, iron-dependent form of programmed cell death marked by lipid peroxidation. The enzymatic activity of HO-1 breaks down heme into free ferrous iron (Fe^{2+}), biliverdin, and carbon monoxide. Elevated intracellular iron levels can catalyse the Fenton reaction, enhancing ROS production and triggering lipid peroxidation, which are hallmarks of ferroptosis. Activation of HO-1 (e.g., via haemin or carbon monoxide-releasing molecules) sensitises cancer cells to ferroptosis inducers such as erastin. In contrast, pharmacological inhibition of HO-1 (e.g., with ZnPP) reduces ferroptotic cell death, suggesting a dose- and context-dependent role of HO-1 in this pathway. While HO-1-derived biliverdin and bilirubin serve as antioxidants that may mitigate ferroptotic damage, excessive HO-1 activity can overwhelm redox buffering systems and promote cell death. Moreover, the transcriptional regulation of HO-1 by Nrf2 links oxidative stress to ferroptosis: Nrf2 activation induces HO-1 expression in response to redox imbalance, which paradoxically can increase cellular susceptibility to iron-driven lipid peroxidation in cancer cells. The subcellular localisation of HO-1 further modulates its function—mitochondrial HO-1 contributes to redox regulation and cellular adaptation to stress, whereas nuclear HO-1 is associated with aggressive tumour behaviour and poor prognosis [13]. Thus, modulation of HO-1 activity—whether to promote apoptosis via mitochondrial damage or to induce ferroptosis through iron overload—represents a potent therapeutic avenue. Collectively, these mechanisms demonstrate how HO-1 inhibition selectively impairs tumour cell survival pathways, providing an effective strategy for inducing programmed cell death in cancer cells.

Combination with immunotherapy and PDT

Photodynamic therapy (PDT) relies on the generation of ROS to eliminate tumour cells. However, HO-1 mitigates PDT-induced oxidative damage by neutralising ROS and promoting survival signalling, thus reducing treatment efficacy. Inhibitors such as ZnPPIX have been shown to sensitise tumours to PDT by lowering the levels of cytoprotective HO-1 metabolites [6,42]. Recent advances in nanomedicine and combination therapies aim to overcome this limitation. For example, ZnPP@FQOS (a tumour microenvironment-responsive organosilica hybrid nanoformulation) enhances ROS production, downregulates HO-1, and stimulates dendritic cell maturation and cytotoxic T lymphocyte activation, thereby augmenting both PDT and immunotherapy effects [43]. Combining HO-1 inhibition with PDT and immunotherapy sensitises tumours to oxidative damage and enhances immune-mediated tumour clearance.

In summary, therapeutic strategies targeting HO-1 can inhibit tumour growth, overcome chemoresistance, induce apoptosis, and improve the efficacy of adjunct therapies, including immunotherapy and PDT. However, the dual nature of HO-1 necessitates a context-specific approach that maximises antitumor effects while minimising potential harm to normal tissues. Ongoing research into selective inhibitors and tumour-targeted delivery systems will be critical for translating these insights into effective clinical interventions (see **Table 1**).

Divergent therapeutic perspectives on HO-1

Different groups have reached contrasting conclusions regarding the therapeutic targeting of HO-1. While some suggest that HO-1 induction may confer beneficial anti-inflammatory and cytoprotective effects, others argue that inhibition is necessary to overcome therapy resistance and sensitise tumours to chemotherapy, radiotherapy, or photodynamic therapy. Based on the integration of recent preclinical evidence, we consider selective HO-1 inhibition, particularly in combination with other therapeutic modalities, to be the more promising strategy in the oncological setting.

Table 1. Dual role of HO-1: protective in normal cells vs. tumour-promoting in cancer cells.

Mechanism	Role of HO-1 in Normal Cells	Role of HO-1 in Cancer Cells
Redox homeostasis and ROS scavenging	Maintains redox homeostasis and eliminates ROS, preventing oxidative DNA damage and mutations that could lead to cancer.	Neutralises ROS generated during chemotherapy/radiotherapy, reducing DNA damage and enabling resistance.
DNA repair	Activated in response to ROS to promote DNA repair and genome stability via pathways such as ATM/ATR and BRCA1.	Enhances DNA repair after therapy-induced damage, contributing to chemoresistance and radioresistance.
Cytoprotective and anti-inflammatory functions	Reduces oxidative stress and inflammation, supports mitochondrial integrity, and promotes cell survival.	Promotes tumour cell survival under stress, including drug-induced stress.
Antioxidant heme metabolites	Biliverdin and bilirubin scavenge ROS; CO mediates anti-inflammatory signalling and cytoprotection.	CO signalling promotes cell survival, inhibits apoptosis, and facilitates treatment resistance.
Role of HO-1 in Cancer Cells only		
Therapy resistance	Overexpression correlates with poor prognosis and therapy resistance; it increases after exposure to therapy-induced ROS.	
Autophagy regulation	Regulates autophagy via Beclin-1, p62, LC3B-I/II, aiding in survival and resistance.	
Anti-apoptotic effects	Increases anti-apoptotic proteins (e.g., Bcl-xL), suppresses apoptotic pathways (e.g., caspase-3), promotes survival in response to targeted drugs (e.g., rapamycin, sorafenib).	
Proliferation, angiogenesis, metastasis	Supports mitochondrial biogenesis and metabolic adaptation; promotes angiogenesis via VEGF and enhances metastasis.	
Subcellular localization	Nuclear HO-1 is associated with malignancy and poor prognosis; mitochondrial HO-1 supports metabolic flexibility and redox control.	

ATM, ataxia telangiectasia mutated; ATR, ATM- and Rad3-related; BRCA1, breast cancer 1, early onset; Beclin-1, Bcl-2–interacting protein 1; p62, sequestosome 1 (SQSTM1); LC3B-I/II, microtubule-associated protein 1 light chain 3 beta-I/II; Bcl-xL, B-cell lymphoma-extra large; caspase-3, cysteine-aspartic acid protease-3

Conclusion

HO-1 is a paradoxical regulator in cancer biology. It simultaneously exerts cytoprotective, antioxidant, anti-apoptotic, and immunomodulatory functions that can either safeguard tissues or promote cancer progression. The long-standing debate over whether HO-1 acts as a "friend or foe" in tumours has been discussed in previous reviews [5,6]. Our synthesis builds upon this discussion by incorporating recent advances that substantially refine this duality. In particular, we highlight novel aspects such as the contribution of HO-1 to ferroptosis, the impact of its subcellular localisation (nuclear versus mitochondrial HO-1) on tumour aggressiveness and therapy response, and the regulation of HO-1 signalling through tumour-derived exosomes. These mechanisms provide additional layers of complexity not fully addressed in earlier literature and point to emerging opportunities for therapeutic exploitation.

Significantly, the interpretation of HO-1's role depends heavily on tumour type, disease stage, and microenvironmental context. While some groups emphasise the protective effects of limiting oxidative damage and maintaining genomic stability, others demonstrate that persistent HO-1 overexpression promotes angiogenesis, immune evasion, and therapy resistance. Based on the weight of current evidence, we consider sustained HO-1 activity in advanced malignancies to be predominantly tumour-promoting. In contrast, its physiological role in normal tissues and early carcinogenesis remains protective. This context-specific duality must therefore inform future therapeutic strategies.

Looking ahead, several directions appear particularly promising. First, selective inhibition of HO-1 should be investigated as a means to overcome drug resistance, enhance ferroptosis, and improve the efficacy of chemotherapies and radiotherapies. Second, combining HO-1 modulation with photodynamic therapy and immunotherapy represents a timely avenue, supported by recent nanomedicine-based approaches. Third, understanding the non-canonical functions of nuclear HO-1 and its role in metabolic reprogramming may open new therapeutic frontiers. Finally, the identification of biomarkers predicting tumour dependence on HO-1 could enable patient stratification and personalised interventions. In summary, while the dual role of HO-1 has been recognised for more than a decade, our review distinguishes itself by highlighting cutting-edge insights into ferroptosis, exosomal signalling, subcellular localisation, and combined treatment approaches. We conclude that selective and context-specific inhibition of HO-1 in advanced cancers holds the most significant translational potential, whereas its activation may remain advantageous in non-malignant pathologies. Fully exploiting HO-1 as a therapeutic target will require reconciling divergent observations. Still, we believe that its integration into rational combination strategies offers one of the most promising directions for the future of cancer therapy.

Glossary

HO-1, heme oxygenase-1; CO, carbon monoxide; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; HIF-1a, hypoxia-inducible factor 1-alpha; VEGF, vascular endothelial growth factor; FTH1, ferritin heavy chain 1; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; PPP, pentose phosphate pathway; CLs, cytotoxic T lymphocytes; DCs, dendritic cells; PD-L1, programmed death-ligand 1; OB-24, 2-[2-(4-bromophenyl)ethyl]-2-[(1H-imidazol-1-yl)methyl]-1,3-dioxolane hydrochloride; ZnPP, zinc protoporphyrin IX; SnPP, tin protoporphyrin IX; PDT, photodynamic therapy; TNF-α, tumour necrosis factor alpha; IL-10, interleukin-10; IL-6, interleukin-6.

Disclosures

The author's contribution
Both authors (MR and MM) contributed equally to the conceptualisation, literature search, analysis and interpretation of the data, as well as to writing, reviewing and editing the manuscript. Both authors have read and approved the final version of the manuscript.

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
This work was supported by the National Science Centre, Poland, grant number UMO-2021/43/B/NZ7/02476 (granted to M.M.).

References

1. Ryter SW, Alam J, Choi AMK. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev. 2006 Apr;86(2):583–650. https://doi.org/10.1152/physrev.00011.2005.
2. Consonni FM, Incerti M, Bertolotti M, Ballerini G, Garlatti V, Sica A. Heme catabolism and heme oxygenase-1-expressing myeloid cells in pathophysiolo-

gy. Front Immunol. 2024 Oct;15:1433113. https://doi.org/10.3389/fimmu.2024.1433113.
3. Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. Annu Rev Pharmacol Toxicol. 2010 Feb;50(1):323–54. https://doi.org/10.1146/annurev.pharmtox.010909.105600.
4. Luu Hoang KN, Anstee JE, Arnold JN. The diverse roles of heme oxygenase-1 in tumor progression. Front Immunol. 2021 Mar;12:658315. https://doi.org/10.3389/fimmu.2021.658315.
5. Chiang SK, Chen SE, Chang LC. A Dual role of heme oxygenase-1 in cancer cells. Int J Mol Sci. 2018 Dec;20(1):39. https://doi.org/10.3390/ijms20010039.
6. Jozkowicz A, Was H, Dulak J. Heme oxygenase-1 in tumors: is it a false friend? Antioxid Redox Signal.. 2007 Dec;9(12):2099–117. https://doi.org/10.1089/ars.2007.1659.
7. Shu L, Fu H, Pi A, Feng Y, Dong H, Si C, Li S, Zhu F, Zheng P, Zhu Q. Protective effect of andrographolide against ulcerative colitis by activating Nrf2/HO-1 mediated antioxidant response. Front Pharmacol. 2024 Jul;15:1424219. https://doi.org/10.3389/fphar.2024.1424219.
8. Sheikh SZ, Hegazi RA, Kobayashi T, Onyiah JC, Russo SM, Matsuoka K, Sepulveda AR, Li F, Otterbein LE, Plevy SE. An anti-inflammatory role for carbon monoxide and heme oxygenase-1 in chronic Th2-mediated murine colitis. J Immunol. 2011 May;186(9):5506–13. https://doi.org/10.4049/jimmunol.1002433.
9. Zhang Y, Chen B, Xu N, Xu P, Lin W, Liu C, Huang P. Exosomes Promote the transition of androgen-dependent prostate cancer cells into androgen-independent manner through up-regulating the heme oxygenase-1. Int J Nanomedicine. 2021 Jan; 16:315–27. https://doi.org/10.2147/IJN.S281710.
10. Schwartz M, Böckmann S, Hinz B. Up-regulation of heme oxygenase-1 expression and inhibition of disease-associated features by cannabidiol in vascular smooth muscle cells. Oncotarget. 2018 Oct;9(77):34595–616. https://doi.org/10.18632/oncotarget.26191.
11. Nitti M, Ivaldo C, Traverso N, Furfaro AL. Clinical significance of heme oxygenase 1 in tumor progression. Antioxidants. 2021 May;10(5):789. https://doi.org/10.3390/antiox10050789.
12. Poss KD, Tonegawa S. Heme oxygenase 1 is required for mammalian iron reutilization. Proc Natl Acad Sci USA. 1997 Sep;94(20):10919–24. https://doi.org/10.1073/pnas.94.20.10919.
13. Chiang SK, Chen SE, Chang LC. The role of HO-1 and its crosstalk with oxidative stress in cancer cell survival. Cells. 2021 Sep;10(9):2401. https://doi.org/10.3390/cells10092401.
14. Lin PH, Lan WM, Chau LY. TRC8 suppresses tumorigenesis through targeting heme oxygenase-1 for ubiquitination and degradation. Oncogene. 2013 May;32(18):2325–34. https://doi.org/10.1038/onc.2012.244.
15. Otterbein LE, Hedblom A, Harris C, Csizmadia E, Gallo D, Wegiel B. Heme oxygenase-1 and carbon monoxide modulate DNA repair through ataxia-telangiectasia mutated (ATM) protein. Proc Natl Acad Sci USA. 2011 Aug;108(35):14491–6. https://doi.org/10.1073/pnas.1102295108.
16. Yamamoto T, Takano N, Ishiwata K, Ohmura M, Nagahata Y, Matsuura T, Kamata A, Sakamoto K, Nakanishi T, Kubo A, Hishiki T, Suematsu M. Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. Nat Commun. 2014 Mar;5(1):3480. https://doi.org/10.1038/ncomms4480.
17. Kongpetch S, Kukongviriyapan V, Prawan A, Senggunprai L, Kukongviriyapan U, Buranrat B. Crucial role of heme oxygenase-1 on the sensitivity of cholangiocarcinoma cells to chemotherapeutic agents. PLoS One. 2012 Apr;7(4):e34994. https://doi.org/10.1371/journal.pone.0034994.
18. Liu YS, Li HS, Qi DF, Zhang J, Jiang XC, Shi K, Zhang XJ, Zhang XH. Zinc protoporphyrin IX enhances chemotherapeutic response of hepatoma cells to cisplatin. World J Gastroenterol. 2014;20(26):8572–82. https://doi.org/10.3748/wjg.v20.i26.8572.
19. Wegiel B, Gallo D, Csizmadia E, Harris C, Belcher J, Vercellotti GM, Penacho N, Seth P, Sukhatme V, Ahmed A, Pandolfi PP, Helczynski L, Bjartell A, Persson JL, Otterbein LE. Carbon monoxide expedites metabolic exhaustion to inhibit tumor growth. Cancer Research. 2013 Dec 1;73(23):7009–21. https://doi.org/10.1158/0008-5472.CAN-13-1075.
20. Birrane G, Li H, Yang S, Tachado SD, Seng S. Cigarette smoke induces nuclear translocation of heme oxygenase 1 (HO-1) in prostate cancer cells: nuclear HO-1 promotes vascular endothelial growth factor secretion. Int J Oncol.. 2013 Jun;42(6):1919–28. https://doi.org/10.3892/ijo.2013.1910.
21. Miyake M, Fujimoto K, Anai S, Ohnishi S, Kuwada M, Nakai Y, Inoue T, Matsumura Y, Tomioka A, Ikeda T, Tanaka N, Hirao Y. Heme oxygenase-1 promotes angiogenesis in urothelial carcinoma of the urinary bladder. Oncol Rep. 2011 Mar;25(3):653-60. https://doi.org/10.3892/or.2010.1125.
22. Xu Y, Yang Y, Huang Y, Ma Q, Shang J, Guo J, Cao X, Wang X, Li M. Inhibition of Nrf2/HO-1 signaling pathway by Dextran Sulfate suppresses angiogenesis of Gastric Cancer. J Cancer. 2021 Jan;12(4): 1042-1060. https://doi.org/10.7150/jca.50605.
23. Huang Y, Yang Y, Xu Y, Ma Q, Guo F, Zhao Y, Tao Y, Li M, Guo J. Nrf2/HO-1 axis regulates the angiogenesis of gastric cancer via targeting VEGF. Cancer Manag Res.. 2021 Apr; 13:3155–69. https://doi.org/10.2147/CMAR.S292461.
24. Furfaro AL, Traverso N, Domenicotti C, Piras S, Moretta L, Marinari UM, Pronzato MA, Nitti M. The Nrf2/HO-1 axis in cancer cell growth and chemoresistance. Oxid Med Cell Longev. 2016 Jan;2016:1958174. https://doi.org/10.1155/2016/1958174.
25. Wang H, Cheng Q, Bao L, Li M, Chang K, Yi X. Cytoprotective role of heme oxygenase-1 in cancer chemoresistance: focus on antioxidant, antiapoptotic, and pro-autophagy properties. Antioxidants. 2023 Jun;12(6):1217. https://doi.org/10.3390/antiox12061217.
26. Alaoui-Jamali MA, Bismar TA, Gupta A, Szarek WA, Su J, Song W, Xu Y, Xu B, Liu G, Vlahakis JZ, Roman G,

Jiao J, Schipper HM. A novel experimental heme oxygenase-1–targeted therapy for hormone-refractory prostate cancer. Cancer Res. 2009 Oct ;69(20):8017–24. https://doi.org/10.1158/0008-5472.CAN-09-0419.
27. Mayerhofer M, Gleixner KV, Mayerhofer J, Hoermann G, Jaeger E, Aichberger KJ, Ott RG, Greish K, Nakamura H, Derdak S, Samorapoompichit P, Pickl WF, Sexl V, Esterbauer H, Schwarzinger I, Sillaber C, Maeda H, Valent P. Targeting of heat shock protein 32 (Hsp32)/heme oxygenase-1 (HO-1) in leukemic cells in chronic myeloid leukemia: a novel approach to overcome resistance against imatinib. Blood. 2008 Feb;111(4):2200–10. https://doi.org/10.1182/blood-2006-11-055723.
28. Berberat PO, Dambrauskas Z, Gulbinas A, Giese T, Giese N, Künzli B, Autschbach F, Meuer S, Büchler MW, Friess H. Inhibition of heme oxygenase-1 increases responsiveness of pancreatic cancer cells to anticancer treatment. Clin Cancer Res. 2005 May;11(10):3790–8. https://doi.org/10.1158/1078-0432.CCR-04-2159.
29. Sass G, Leukel P, Schmitz V, Raskopf E, Ocker M, Neureiter D, Meissnitzer M, Tasika E, Tannapfel A, Tiegs G. Inhibition of heme oxygenase 1 expression by small interfering RNA decreases orthotopic tumor growth in livers of mice. Int J Cancer. 2008 Sep;123(6):1269–77. https://doi.org/10.1002/ijc.23695 .
30. Salerno L, Floresta G, Ciaffaglione V, Gentile D, Margani F, Turnaturi R, Rescifina A, Pittalà V. Progress in the development of selective heme oxygenase-1 inhibitors and their potential therapeutic application. Eur J Med Chem. 2019 Apr;167:439–53. https://doi.org/10.1016/j.ejmech.2019.02.027.
31. Consoli V, Sorrenti V, Grosso S, Vanella L. Heme oxygenase-1 signaling and redox homeostasis in physiopathological conditions. Biomolecules. 2021 Apr;11(4):589. https://doi.org/10.3390/biom11040589.
32. Kwon MY, Park E, Lee SJ, Chung SW. Heme oxygenase-1 accelerates erastin-induced ferroptotic cell death. Oncotarget. 2015 Sep;6(27):24393–403. https://doi.org/10.18632/oncotarget.5162.
33. McDonagh AF. The biliverdin–bilirubin antioxidant cycle of cellular protection: Missing a wheel? Free Radic Biol Med. 2010 Sep ;49(5):814–20. https://doi.org/10.1016/j.freeradbiomed.2010.06.001.
34. Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, Igarashi K. Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. Proc Natl Acad Sci USA. 2004 Feb;101(6):1461–6. https://doi.org/10.1073/pnas.0308083100.
35. Morita T, Kourembanas S. Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. J Clin Invest. 1995 Dec;96(6):2676–82. https://doi.org/10.1172/JCI118334.
36. Pei L, Kong Y, Shao C, Yue X, Wang Z, Zhang N. Heme oxygenase-1 induction mediates chemoresistance of breast cancer cells to pharmorubicin by promoting autophagy via PI 3K/Akt pathway. J Cell Mol Med. 2018 Nov;22(11):5311–21. https://doi.org/10.1111/jcmm.13800.
37. Ahmad IM, Dafferner AJ, O'Connell KA, Mehla K, Britigan BE, Hollingsworth MA, Abdalla MY. Heme oxygenase-1 inhibition potentiates the effects of nab-paclitaxel-gemcitabine and modulates the tumor microenvironment in pancreatic ductal adenocarcinoma. Cancers. 2021 May;13(9):2264. https://doi.org/10.3390/cancers13092264.
38. Barbagallo I, Giallongo C, Volti GL, Distefano A, Camiolo G, Raffaele M, Salerno L, Pittalà V, Sorrenti V, Avola R, Di Rosa M, Vanella L, Di Raimondo F, Tibullo D. Heme oxygenase inhibition sensitizes neuroblastoma cells to carfilzomib. Mol Neurobiol. 2019 Feb;56(2):1451–60. https://doi.org/10.1007/s12035-018-1133-6.
39. Ren QG, Yang SL, Li PD, Xiong JR, Fang X, Hu JL, Wang QS, Chen RW, Chen YS, Wen L, Peng M. Low heme oxygenase-1 expression promotes gastric cancer cell apoptosis, inhibits proliferation and invasion, and correlates with increased overall survival in gastric cancer patients. Oncol Rep. 2017 Nov;38(5):2852–8. https://doi.org/10.3892/or.2017.5967.
40. Sferrazzo G, Di Rosa M, Barone E, Li Volti G, Musso N, Tibullo D, Barbagallo I. Heme oxygenase-1 in central nervous system malignancies. J Clin Med. 2020 May;9(5):1562. https://doi.org/10.3390/jcm9051562.
41. Shi J, Yu J, Zhang Y, Wu L, Dong S, Wu L, Wu L, Du S, Zhang Y, Ma D. PI3K/Akt pathway-mediated HO-1 induction regulates mitochondrial quality control and attenuates endotoxin-induced acute lung injury. Lab Invest. 2019 Dec;99(12):1795–809. https://doi.org/10.1038/s41374-019-0286-x.
42. Nowis D, Legat M, Grzela T, Niderla J, Wilczek E, Wilczynski GM, Głodkowska E, Mrówka P, Issat T, Dulak J, Józkowicz A, Waś H, Adamek M, Wrzosek A, Nazarewski S, Makowski M, Stokłosa T, Jakóbisiak M, Gołab J. Heme oxygenase-1 protects tumor cells against photodynamic therapy-mediated cytotoxicity. Oncogene. 2006 Jun;25(24):3365–74. https://doi.org/10.1038/sj.onc.1209378.
43. Zou J, Jiang C, Hu Q, Jia X, Wang S, Wan S, Mao Y, Zhang D, Zhang P, Dai B, Li Y. Tumor microenvironment-responsive engineered hybrid nanomedicine for photodynamic-immunotherapy via multi-pronged amplification of reactive oxygen species. Nat Commun. 2025 Jan;16(1):424. https://doi.org/10.1038/s41467-024-55658-0.

REVIEW PAPER

JMS Journal of Medical Science

Therapeutic *Targeting of Low-density lipoprotein receptor-related protein-1* (*LRP1*) gene in Ovarian Cancer: A Comprehensive Review

R.B. Devi Krishna
Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India
https://orcid.org/0009-0000-3466-9800

Nandini Krishnamurthy
Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India
https://orcid.org/0009-0000-2710-3577

Sanjana Murali
Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India
https://orcid.org/0009-0006-3549-0080

Preet Agarwal
Department of Gynaecology, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India
https://orcid.org/0000-0001-9907-5747

Elizabeth Rani Chellappan
Department of Biotechnology, Hindustan College of Arts & Science, Chennai, Tamil Nadu, India
https://orcid.org/0009-0000-6664-363X

Leena Dennis Joseph
Department of Pathology, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India
https://orcid.org/0000-0002-9395-2961

Banu Keerthana
Department of Pathology, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India
https://orcid.org/0000-0001-7858-8666

Andrea Mary Francis
Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India
https://orcid.org/0000-0003-2260-7199

Corresponding author: andreamary@sriramachandra.edu.in

https://doi.org/10.20883/medical.e1221

Keywords: ovarian cancer, *LRP1*, proliferation, tumour, therapeutic target, clinical implication

Received 2025-02-04
Accepted 2025-08-28
Published 2025-09-22

How to Cite: Krishna RBD, Krishnamurthy N, Murali S, Agarwal P, Chellappan ER, Joseph LD, Keerthana B, Francis AM. Therapeutic Targeting of *Low-density lipoprotein receptor-related protein-1* (*LRP1*) gene in Ovarian Cancer: A Comprehensive Review. Journal of Medical Science. 2025 September;94(3);e1221. doi:10.20883/medical.e1221

ABSTRACT

Low-density lipoprotein receptor-related protein 1 (*LRP1*, also known as *CD91*) is a multifunctional endocytic and cell-signalling receptor widely expressed in various cell types, including neurons, fibroblasts, hepatocytes, muscle cells, astrocytes, and tumour cells. It maintains cellular homeostasis by mediating interactions with extracellular matrix proteins, growth factors, and proteases. These interactions enable *LRP1* to act as a co-receptor that modulates and influences signalling pathways associated with cell migration, survival, and proliferation. In ovarian cancer, LRP1 is frequently activated and contributes to tumour progression. Functional studies have demonstrated that LRP1 promotes these malignant traits through activation of PI3K/Akt and MAPK/ERK pathways, while its inhibition suppresses proliferation and invasion. This review aims to comprehensively examine the functional role of LRP1 in ovarian cancer, with particular emphasis on its capacity to regulate tumour cell migration and invasion through key molecular pathways. Understanding these mechanisms may provide insights into novel therapeutic strategies for ovarian cancer treatment.

Introduction

Ovarian cancer (OC) is one of the most lethal gynaecologic malignancies, primarily due to late-stage diagnosis [1]. It originates from ovarian tissue or, more commonly, from adjacent structures such as the fallopian tubes or the peritoneal lining, which are connected to the ovaries. The ovary consists of three distinct cell types: stromal, germ, and epithelial. When epithelial cells undergo abnormal growth, they can proliferate uncontrollably, leading to tumour formation [2,3]. According to the Global Cancer Observatory 2022, OC accounts for approximately 6.7 cases per 100,000 women globally, with an estimated 324,603 new cases reported in that year. In India, the incidence rate is around 6.6 cases per 100,000 women, totalling approximately 47,333 new cases. A major contributor to OC's high mortality is the lack of early, recognisable symptoms, resulting in delayed diagnosis [4]. However, vaginal ultrasound and serum Cancer Antigen 125 (CA125) testing are commonly used diagnostic tools; their low sensitivity and specificity limit early-stage detection [5]. The current standard of care involves cytoreductive surgery followed by platinum-based chemotherapy, often in combination with taxane agents. While these regimens initially induce remission in most patients, recurrence is frequent, and many eventually develop platinum-resistant ovarian cancer [6,7]. This therapeutic resistance underscores the urgent need for novel strategies that can overcome chemoresistance and improve long-term outcomes. Recent research efforts have focused on molecular targets, immune modulation, and receptor-mediated pathways, including the low-density lipoprotein receptor-related protein 1 (LRP1), which has emerged as a promising candidate for therapeutic intervention.

Initially identified as a receptor involved in lipid metabolism, LRP1 was subsequently shown to be a receptor for active a2-macroglobulin [8]. *LRP1* was once thought to function as a scavenger receptor. Still, an increasing amount of evidence points to the possibility that it may also control the activity of other membrane receptors, such as adhesion and tyrosine kinase receptors, and facilitate intracellular signalling [9]. *LRP1* influences tumour cell invasion and migration by regulating the expression of matrix metalloproteinases MMP2 and MMP9, which degrade the extracellular matrix and facilitate cancer cell movement. Additionally, *LRP1*-mediated activation of ERK signalling enhances tumour cell adhesion and motility, while its suppression of JNK signalling prevents apoptosis, further supporting invasive behaviour [9,10].

Among emerging molecular targets, *LRP1* has garnered significant interest due to its multifaceted role in cancer biology. *LRP1* is a transmembrane receptor involved in endocytosis and signal transduction, and its dysregulation has been associated with malignant behaviours such as enhanced cell motility, extracellular matrix remodelling, and reduced treatment efficacy. Notably, *LRP1* plays a key role in lipoprotein metabolism, facilitating the uptake of lipid-rich particles into cells. Tumours, including OC, often exploit these lipoprotein metabolic pathways to support rapid proliferation, with increased lipid intake and storage accelerating tumour growth and contributing to chemoresistance [11]. The viable therapeutic strategy for ovarian cancer involves inhibiting *LRP1* [12]. Inhibitors targeting LRP1 and related proteins are being researched as potential therapeutic agents since they have demonstrated promise in preclinical trials. It may be possible to prevent tumour growth and metastasis, as well as improve the efficiency of currently available ovarian cancer treatments, by impairing the functions of *LRP1* [12-15]. While *LRP1* shows potential as a therapeutic and diagnostic biomarker in ovarian cancer, its functional specificity and clinical utility remain to be fully elucidated.

Structure and Function of *LRP1*

LRP1 is a large endocytic receptor. During biosynthesis, the 600 kDa protein *LRP1* is cut into two polypeptides, which are permanently associated with the N-terminal extracellular domain (~515 kDa) and a transmembrane C-terminal fragment (~85 kDa) [13]. *LRP1* has a variety of biological functions that include lipid metabolism, cell proliferation, migration, inflammation, and death. *LRP1* functions as a key regulatory receptor in multiple physiological processes, including blood-brain barrier permeability, vascular tone modulation, and platelet-derived growth factor (PDGF) receptor signalling, which collectively

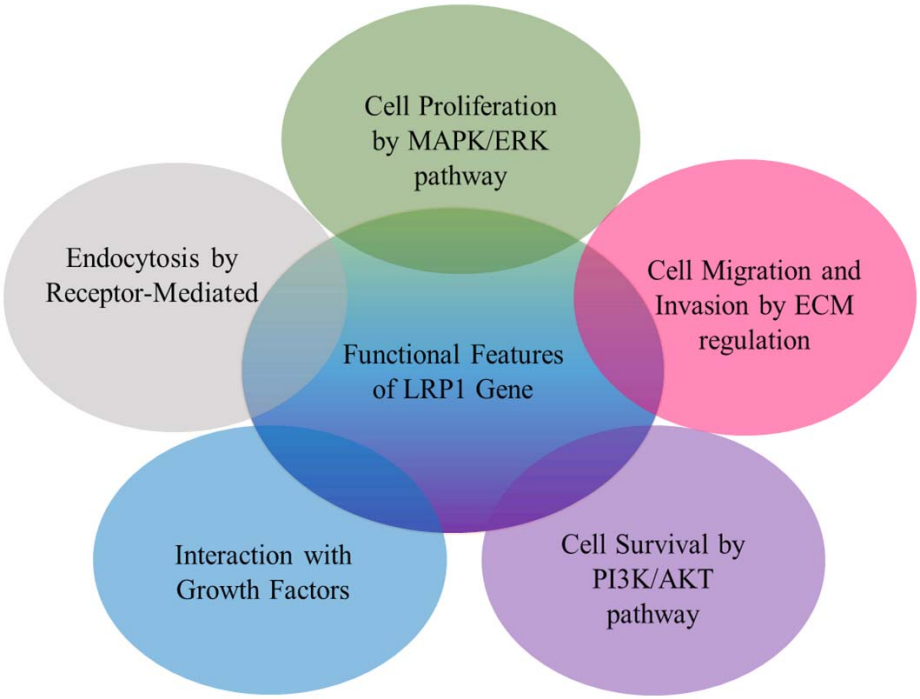


Figure 1. Low-density lipoprotein receptor-related protein 1 *(LRP1)* increases cell division by activating the Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase (MAPK/ERK) pathway, and it improves cell survival by blocking the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, which prevents apoptosis, or programmed cell death. The alteration of extracellular matrix (ECM) elements promotes the invasion and migration of cancer cells, aiding in the internalisation and degradation of signalling molecules, thereby inhibiting their access and activity. Interacting with several growth factors also affects the development and spread of cancer cells. Image created using Microsoft PowerPoint.

influence cellular migration and tissue remodelling. *LRP1* is also connected to diseases such as cancer, atherosclerosis, and neurological disorders [14,15]. *LRP1* may also affect several physiological processes by regulating cellular signalling and functioning as an endocytic receptor, according to earlier research. *LRP1* has been implicated in several cancers, including glioblastoma, breast cancer, and ovarian cancer, where it influences tumour progression through pathways involving vascular endothelial growth factor (VEGF). Studies suggest that *LRP1* can regulate VEGF expression, contributing to angiogenesis, tumour proliferation, and metastasis [8–10].

The function of this receptor is to regulate cell signalling pathways, such as the MAPK pathway (see **Figure.1**), which includes enzymes implicated in cancer invasion. It also participates in the metabolism of several external ligands, such as PDGF and MMP. By activating the MAPK/ERK pathway, *LRP1* promotes cell division. Several studies have examined *LRP1* gene expression and its function in various tumours. Additionally, LRP1 modulates the TGF-β signalling axis, which plays a dual role in OC, suppressing early tumorigenesis but promoting epithelial to mesenchymal transition (EMT) and metastasis in advanced stages. Through its interaction with urokinase-type plasminogen activator (uPA) and MMPs, LRP1 contributes to extracellular matrix (ECM) remodelling, facilitating tumour cell migration and invasion. The modification of ECM components facilitates the migration and invasion of tumour cells and helps internalise and degrade signalling molecules to regulate their exposure and activity. Tumours activate lipoprotein metabolic pathways, leading to increased lipid uptake and storage in various malignancies. This enhanced lipid metabolism supports rapid tumour growth and progression [16,17]. Several studies were conducted for the *LRP1* gene expression and functions in other tumours; however, its specific

involvement in the development of OC has not been thoroughly characterised [18].

Expression and localisation of *LRP1*

The *LRP1* gene is located on chromosome 12q13.14. There are 89 coding exons in the 85 kb gene. In addition, it has eight β-propeller domains, 22 EGF repeats, and 31 ligand-binding repeats. The endoplasmic reticulum synthesises *LRP1*, which is then cleaved into two subunits in the Golgi complex. *LRP1* is expressed in the cytoplasm of various cell types, including adipocytes, mesothelial cells, smooth muscle cells, fibroblasts, astrocytes, neurons, hepatocytes, macrophages, and malignant cells (see **Table 1**). It is essential for signalling pathways and endocytosis. It is found in the nucleoplasm and vesicles [19,20]. Amyloid-β (Aβ) peptide metabolism and lipid transport are regulated by *LRP1*, which is primarily present in the postsynaptic domain and the cell body of neurons [21].

The contribution of *LRP1* in ovarian cancer is complex, as both higher and lower expression have been implicated in tumour progression, depending on the cellular context. Elevated LRP1 expression has been associated with enhanced tumour invasion and metastasis, primarily through its regulation of MMP2/MMP9. Conversely, LRP1 downregulation has been linked to reduced tumour cell migration and proliferation, suggesting its involvement in maintaining tumour aggressiveness [22].

Role of *LRP1* in cancer progression and metastasis

In ovarian cancer, *LRP1* has been linked to both tumour growth and survival. It is capable of regulating several signalling pathways that are involved in cell survival, proliferation, and apoptosis resistance. The ability of ovarian tumour cells to spread is correlated with *LRP1* expression [22-24]. Through its modulation of processes such as extracellular matrix disintegration, cytoskeletal restructuring, and the EMT, it promotes invasion and metastasis [25,26].

Angiogenesis, which is essential for the development and spread of ovarian cancers, *LRP1* promotes angiogenesis, a key process in tumour growth and dissemination. It regulates the expression and activity of angiogenic mediators, including MMP and VEGF [27]. Chemotherapy resistance in OC has been linked to *LRP1* expression. It regulates the efflux of chemotherapeutic agents from cancer cells [28]. *LRP1* interacts with immune cells, extracellular matrix proteins, and stromal cells in the tumour microenvironment. This interaction affects immune suppression, inflammation, and the response to therapy, among other aspects of tumour growth [22,29].

Growth components, extracellular matrix proteins, toxins, protease inhibitor complexes, and viral proteins are examples of *ligands* for LRP1. *LRP1* maintains the integrity of the extracellular matrix and regulates the homeostasis of several secreted proteins by clearing proteases such as MMPs and extracellular proteins like coagula-

Table 1. *LRP1* plays distinct roles depending on its cellular localisation.

Localization	Function
Neurons	Regulates Aβ metabolism, lipid transport, and synaptic plasticity. Implicated in Alzheimer's disease due to its role in Aβ clearance.
Liver	Facilitates lipoprotein metabolism, including uptake of chylomicron remnants and regulation of cholesterol homeostasis.
Macrophages	Modulates inflammatory responses, phagocytosis, and clearance of apoptotic cells. Plays a role in atherosclerosis by influencing lipid uptake.
Cancer cells	Regulates tumor invasion and metastasis by regulating MMP2/MMP9 expression and interacting with PI3K/Akt and MAPK/ERK pathways.
Vascular Smooth Muscle Cells	Influences vascular remodeling, migration, and proliferation, contributing to atherosclerosis and vascular diseases.

Amyloid-β (Aβ), Extracellular signal-regulated kinase (ERK), Matrix Metalloproteinase (MMP), Mitogen-activated protein kinase (MAPK), Phosphatidylinositol 3-kinase (PI3K), Protein kinase B (Akt).

tion factor VIII [20]. *LRP1* may influence tumour growth by regulating the attachment and detachment of malignant cells. LRP1 promotes invasion and metastasis, as demonstrated by in vitro migration assays and in vivo mouse models showing enhanced metastatic potential via ERK-mediated MMP regulation [30]. It modulates focal adhesion dynamics through interactions with key proteins such as paxillin and focal adhesion kinase (FAK), which are involved in cell migration and invasion. This regulates integrin stimulation and the turnover of focal adhesions. The intricate nature of *LRP1*'s function in tumour cell invasion and migration is probably influenced by the type of tumour cell as well as the makeup and structure of the surrounding environment [31].

Researchers used short hairpin RNA (shRNA) to suppress *LRP1* expression in CL16 cells. This knockdown led to reduced VEGF expression and increased cell mortality under hypoxic conditions in vitro. In Severe Combined Immunodeficiency mice, *LRP1*-silenced cells created tumours and spread to the lungs, but the metastases did not expand, indicating a problem with cell proliferation or survival. These findings are supported by both in vitro and in vivo studies, which collectively validate the functional role of LRP1 in ovarian cancer progression, angiogenesis, and chemoresistance [32].

LRP1-Mediated tumour survival mechanisms in ovarian cancer

LRP1 significantly influences the regulation of cell apoptosis through its interactions with signalling pathways and apoptotic mediators. Through regulation of the expression of Caspase-3, the insulin receptor, the serine/threonine kinase signalling pathway, and *LRP1* has been shown to prevent cell apoptosis. The activation of caspase-3, an essential enzyme in cell death, is stimulated by *LRP1* [32]. AKT phosphorylation, insulin receptor signalling, apoptosis, and Caspase-3 activation were all markedly reduced in neurons upon *LRP1* knockdown. This implies that *LRP1* may inhibit Caspase-3 activation, hence preventing cancer cells from undergoing apoptosis [33]. The forebrain of mice lacking *LRP1* showed increased cell apoptosis, supporting LRP1's facilitation of insulin receptor signalling and AKT pathway activation, which regulates cell survival and metabolic regulation. The function of *LRP1* in apoptosis and its potential as a therapeutic target and its viability as a therapeutic target in the treatment of cancer require more investigation [34]. Apoptosis is orchestrated by a complex interplay of intracellular signalling pathways, including the insulin receptor, ERK, AKT, and JNK cascades. The insulin receptor activates downstream PI3K/AKT and MAPK/ERK pathways, which promote cell survival by inhibiting pro-apoptotic mediators such as BAD and caspase-9. AKT phosphorylation is particularly critical for suppressing apoptosis through modulation of transcription factors and metabolic regulators. ERK signalling, while primarily associated with proliferation, can exert anti-apoptotic effects depending on cellular context. In contrast, JNK activation is typically related to stress-induced apoptosis through c-Jun phosphorylation and the upregulation of death receptors [35].

Mechanisms of *LRP1* involvement in ovarian cancer

LRP1 was initially thought to have a tumour-suppressive function when multiple research groups reported lower *LRP1* expression in various tumour cell lines and tissues. It was recently demonstrated that *LRP1* acts as an internal suppressor of the melanoma aggressive phenotype in response to ApoE [36]. Nevertheless, conflicting data point to *LRP1*'s potential contribution to breast and ovarian cancer cell invasion and metastasis. Furthermore, it was discovered that elevated *LRP1* expression in endometrial carcinomas was linked to a higher histological grade and indicative of a more aggressive tumour behaviour. Since the extracellular portion of *LRP1* was initially discovered to be soluble in human plasma, *LRP1* shedders have been recognised as proteolytic enzymes belonging to various classes. These include BACE-1 and the serine proteinase tPA, MMP2 and MMP14, and others. The intracytoplasmic region of *LRP1* is capable of exiting the cytoplasmic membrane by γ-secretes after *LRP1* is shed, and this could serve as a mediator for signalling [36,37].

Early research on *LRP1*'s potential connection to cancer primarily used tumour cell lines

and hypothesised that the development of cancer is linked to a decrease in *LRP1* expression or, possibly, the gene's total deletion [38]. However, another study showed that hypoxic environments, which are typical of in vivo cancers, significantly boost *LRP1* expression. Therefore, the level of *LRP1* expression in cancer may not accurately reflect the expression of the protein in cancer cells grown under ambient conditions with abundant oxygen [39]. The involvement of *LRP1* in cancer, acting as both a promoter and suppressor of tumour progression, is regulated in a cellular context. On one hand, *LRP1* enhances tumour invasion and metastasis by regulating MMP2 and MMP9 and activating ERK signalling, which supports cancer cell migration.

On the other hand, *LRP1* has been shown to suppress tumour growth in specific conditions by modulating apoptotic pathways and immune responses, potentially limiting cancer cell survival [31,40]. Understanding these opposing functions is crucial for determining *the* therapeutic potential of LRP1 in ovarian cancer. It has been found that *LRP1* is increased in triple-negative breast cancer, malignant gliomas, and endometrial carcinomas. It is also associated with poor prognosis and tumour spread. *LRP1* is also an unfavourable prognostic factor for renal and urothelial cancers. It's interesting to note that tumour cell lines from ovarian, breast, and melanoma cancers all express *LRP1*, albeit to varying degrees, inhibiting the complexity of the link between *LRP1* expression and the development of cancer [41,42].

Proteomics research has demonstrated that the serum of OC patients contains higher levels of exosomal *LRP1* than that of healthy individuals [43]. Its function in the development of cancer has recently come into focus. OC progression is influenced by angiogenesis regulation, matrix metalloproteinase activity, and interactions with ERK signalling. These factors contribute to tumour invasion, metastasis, and resistance to apoptosis, highlighting key molecular mechanisms involved in OC pathophysiology. However, *LRP1* expression levels both low and high have been linked to worse prognosis in various cancer types (see **Figure 2**) [17]. Silencing *LRP1* disrupts cancer cell migration in a three-dimensional matrix by inhibiting FAK activation and increasing myosin light chain-2 (MLC-2) phosphorylation. This alteration in cytoskeletal dynamics reduces cell protrusion and motility, highlighting *LRP1*'s regulation in tumour invasion. In fact, despite a significant increase in pericellular proteolytic activity, *LRP1* knockdown limits cancer cell invasiveness. *LRP1* promotes cell invasion by precisely regulating the structure and adhesive properties of the actin network, facilitating cytoskeletal remodelling and dynamic cell movement. *LRP1* facilitates ovarian cancer cell invasion by regulating focal adhesion disassembly and activating key signalling cascades, notably the MAPK/JNK and ERK pathways [44]. It also modulates phosphatidylinositol 3-kinase signalling through adaptor protein interactions, contributing to enhanced cell survival [45]. Bioinformatics analyses have identified *LRP1* as a central node within the Notch signalling pathway's network, implicated in the dysregulation associated with various malignancies [46]. In both in vitro and in vivo models, *LRP1* has been shown to influence ovarian cancer migration through multiple pathways, including p-ERK/MMP2/MMP9 and Wnt signalling [47, 48].

LRP1 gene polymorphism

Cancer specimens were found to have mutations in the *LRP1* gene (see **Table 2**). The C766T polymorphism has been linked to an increased risk of breast carcinoma in Caucasian females. Despite being a silent variation that does not result in an amino acid change, it has also been associated with Alzheimer's disease and coronary artery disease [49]. The polymorphism 663 C>T (rs1800127) affects exon six and is linked to an increased likelihood of recurrent venous thromboembolism and coronary heart disease. Although the polymorphism's whole effect on the *LRP1* gene is unknown, it most certainly affects ligand binding. The exon 8 polymorphism rs1800137 results in a frameshift in exon 9, leading to a premature stop codon and potential mRNA instability and potential disruption of protein function [50].

Clinical implications of the *LRP1* gene as a key therapeutic target

A novel biomarker of OC cancer is necessary to enable early detection. While elevated carcinoembryonic antigen levels are considered a poor prog-

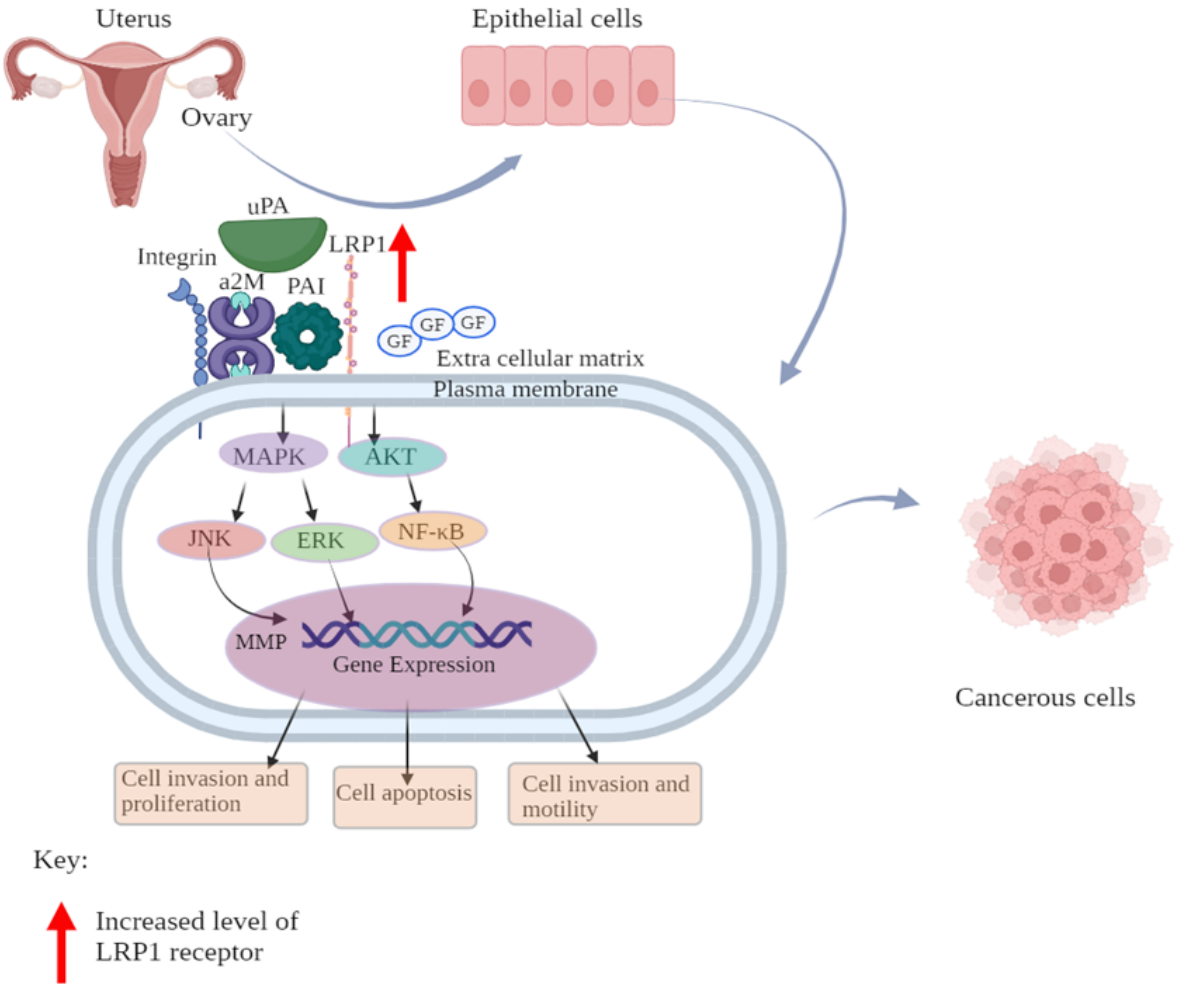


Figure 2. *LRP1*-mediated signalling pathways are involved in regulating various cellular processes, particularly those related to tumorigenesis and progression. *LRP1* modulates multiple signalling pathways in a manner regulated by phosphorylation. One such pathway is activated when *LRP1* binds to the growth factor (GF) receptor, triggering the mitogen-activated protein kinase (MAPK) signalling cascade. This activation eventually promotes the invasion and proliferation of cancer cells by activating the extracellular signal-regulated kinase (ERK) pathway and inhibiting c-jun N-terminal kinase (JNK). Furthermore, ERK promotes MMP2 and MMP9 gene expression patterns, which aid in the invasion of cancer cells. This figure was created using Microsoft PowerPoint.

Table 2. *LRP1* gene polymorphism and disease [23].

Known SNPs	Disease Association	Affect
rs1800127	Cardiovascular disease, obesity, cancer	*LRP1* expression and function
rs715948	Cancer	*LRP1* function
rs1799986	Hyperlipidemia and dyslipidemia, cancer, diabetes and metabolic syndrome	*LRP1* structure
rs138854007	Coronary atherosclerosis, familial hypercholesterolemia	*LRP1* expression
rs1800137	Obesity, hypertension, cancer	*LRP1* expression
rs1799986	Metabolic syndrome, cardiovascular disease, Alzheimer's disease	*LRP1* function
rs1800194	Cardiovascular disease, cancer, Alzheimer's disease	*LRP1* function
rs12814239	Unknown	*LRP1* function
rs34577247	Unknown	*LRP1* structure
rs7397167	Unknown	*LRP1* structure

c-Jun N-terminal Kinase (JNK), Extracellular Signal-Regulated Kinase (ERK), Low-Density Lipoprotein Receptor-Related Protein 1 (*LRP1*), Matrix Metalloproteinase (MMP), Mitogen-Activated Protein Kinase (MAPK).

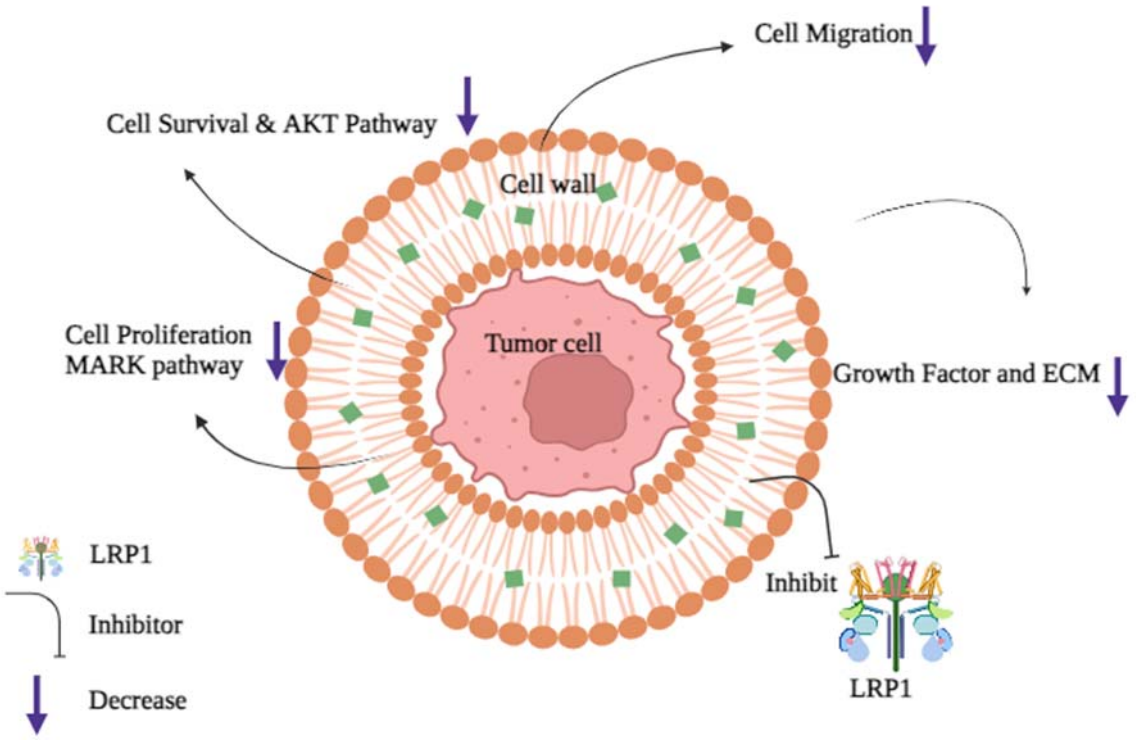


Figure 3. One particular inhibitor targets the ovarian cancer cell's surface *LRP1* receptor explicitly and blocks it. Signalling pathways like MAPK/ERK, which are essential for cell division and development, are disrupted by *LRP1*. The cancer cell's capacity to multiply is therefore reduced. Additionally, *LRP1* inhibition impacts the PI3K/AKT pathway, which often enables cancer cells to evade apoptosis. Inhibiting *LRP1* limits the cell's capacity for interaction with ECM, which in turn restricts the cell's ability to move around and invade other tissues—two essential processes in the metastasis of cancer—created using Microsoft PowerPoint.

nostic indicator in early-stage ovarian cancer, the favourable detection rates of α-fetoprotein and CA19-9 are comparatively low. However, CA125 and HE4 remain the most reliable biomarkers for ovarian cancer detection, with HE4 demonstrating higher specificity in differentiating malignant from benign pelvic masses. In OC tissues with downregulated *LRP1*, there was a decrease in the levels of MMP2, MMP9, and p-ERK (see **Figure 3**). Meanwhile, following *LRP1* knockdown, the MMP agonist 4-aminophenylmercuric acetate restored MMP2 and MMP9 expression. OC cells exposed to exosomes from healthy volunteers exhibited significantly higher levels of MMP9, MMP2, *LRP1*, and phosphorylated ERK (p-ERK) protein compared to OC cells treated with siRNA-mediated *LRP1* knockdown (SI-*LRP1*-Exos). Both exosomal and secreted forms of *LRP1* have been shown to influence ovarian cancer cell motility, primarily through activation of downstream signalling cascades such as p-ERK and matrix metalloproteinases [MMP2/MMP9]. The secreted *LRP1* and exosomal *LRP1* had a comparable mechanism influencing OC migration. Exosomes produced from tumour cells have been shown in accumulating data to facilitate cancer spread potentially. It was also demonstrated that the serum exosomes of OC patients encouraged the migration of OC cells.

Recent studies (see **Table 3**) have explored various strategies to inhibit LRP1, including receptor-associated protein, monoclonal antibodies, and siRNA-mediated knockdown. These approaches primarily function by blocking ligand binding, disrupting endocytic trafficking, or attenuating downstream signalling cascades such as ERK/MAPK and PI3K/AKT pathways known to regulate cell survival, migration, and chemoresistance [3,56]. Inhibiting the proliferation of tumour cells was observed in pancreatic cancer PANC-1 cells with the knockdown of *LRP1*. In pancreatic cancer, *LRP1* overexpression is linked to both cell invasion and a poor prognosis. *LDLR* is upregulated in tumour cells, and cholesterol levels rise as a result of the lipoprotein metabolic pathway in pancreatic cancer. High-grade gliomas, such as glioblastoma multiforme, are distinguished from low-grade astrocytomas by significantly

Table 3. Overview of in vitro and in vivo approaches targeting *LRP1* inhibition.

Author Name and reference	Place and study year	Study type	Sample Size	Description of the study	Finding
Wei Zhou et al. [3]	China, 2023	Experimental studies	Case-5 Control-5	Serum exosome proteomics analysis. Comparison of exosomal *LRP1* levels between ovarian cancer patients and healthy individuals. In-vitro and in-vivo migration assays to assess *LRP1*'s role in MMP2/MMP9 regulation via ERK signaling.	Exosomal *LRP1* levels were significantly higher in ovarian cancer patients compared to healthy individuals. *LRP1* influenced MMP2/MMP9 production via ERK signaling, affecting cell migration
Mengying Zhu et al. [24]	China, 2023	Experimental studies	Cell line	Bioinformatics analysis of *LRP1* expression in GI cancer. Western blot validation of *LRP1* protein presence in HepG2, BxPC-3, and HGC-27 cells. Lentivirus-mediated shRNA knockdown of *LRP1*. Functional assays to evaluate migration, invasion, and proliferation.	*LRP1* knockdown in gastrointestinal cancer cells reduced CD36 expression, inhibiting migration, invasion, and proliferation.
Aline Appert--Collin et al. [20]	France, 2017	Experimental studies	3D Cell line	FTC-133 thyroid cancer cell model. 3D collagen type I matrix experiments. Analysis of morphological changes, actin-cytoskeleton reorganization, and cell-matrix interactions. Assessment of FAK activation, RhoA activity, and MLC-2 phosphorylation.	*LRP1* suppression altered cell morphology, inhibited FAK activation, and increased RhoA activity, leading to reduced migration.
Océane Campion et al. [23]	France, 2021	Experimental studies	Cell line	RNA interference to silence *LRP1* in MDA-MB-231 cells. In-vivo tumor growth assessment using angiogenic assays and orthotopic xenograft models. DCE-MRI, FMT, and IHC for vascular structure and function analysis. Proteomic analysis of *LRP1*-regulating signaling pathways.	*LRP1* suppression in TNBC models delayed tumor growth by 60%, disrupted vascular structures, and inhibited angiogenesis via plasminogen/ TGF signaling
Cao Cuong Le et al. [58]	France, 2020	Experimental studies	Cell line	3D collagen matrix experiments. Analysis of *LRP1*-DDR1 molecular interactions at the plasma membrane. Endocytosis studies to assess DDR1 expression regulation. Cell cycle progression and apoptosis assays.	*LRP1*-mediated DDR1 endocytosis enhanced colon cancer cell proliferation, reduced apoptosis, and regulated tumor microenvironment interactions

Cluster of Differentiation 36 (CD36), Discoidin Domain Receptor 1 (DDR1), Dynamic contrast enhanced MRI (DCE-MRI), Extracellular Signal-Regulated Kinase (ERK), Faecal Microbiota Transplantation (FMT), Focal Adhesion Kinase (FAK), Gastrointestinal Cancer (GI cancer), Immunohistochemistry (ICH), Low Density Lipoprotein Receptor-related Protein 1 (*LRP1*), Matrix Metalloproteinase (MMP), Myosin Light Chain 2 (MLC-2), Ras homolog gene A (RhoA), Triple-Negative Breast Cancer (TNBC)

elevated levels of *LRP1* protein and mRNA. Low hepatocellular carcinoma metastatic potential is associated with high *LRP1* expression.

In contrast to similar normal tissues, *LRP1* was found at increased levels in cancer cells, such as pancreatic, ovarian, renal, and breast malignancies, among others [51,52]. The effect of *LRP1* inhibition is a marked decrease in the aggressive characteristics of ovarian cancer cells, including their capacity for unchecked proliferation, survival in harsh environments, and metastasis to other bodily regions. This highlights the potential therapeutic benefits of targeting *LRP1* in the treatment of ovarian cancer.

Challenges and future directions

Exploring how *LRP1* contributes to ovarian cancer remains challenging due to its complex involvement in tumour initiation, progression, and therapy response. Overcoming these obstacles is

essential to elucidating its specific functions in cancer biology fully. Scientists are attempting to determine if mutations in or levels of *LRP1* expression can act as predictive or prognostic indicators for ovarian cancer. Patient classification and personalised treatment plans may benefit from this. Another crucial avenue to pursue is the investigation of *LRP1* targeting's therapeutic potential in ovarian cancer. This involves creating antibodies, small-molecule inhibitors, or other targeted treatments that can successfully block *LRP1*-mediated pathways linked to tumour growth and metastasis [53,54].

There are several obstacles to overcome to comprehend the function of *LRP1* in ovarian cancer, especially when considering tumour genesis, development, and treatment resistance. Their unintentional contribution to drug resistance may compromise the effectiveness of treatments targeting LRP1-related pathways. Essential processes that inhibit *LRP1* expression and affect cancer cell survival include epigenetic changes, such as DNA methylation and histone alterations, as well as noncoding RNA activity. Additionally, microRNAs (miRNAs) modulate *LRP1* levels, influencing response to therapy. Beyond genetic regulation, *LRP1*-expressing ovarian cancer cells interact with extracellular matrix components, immune cells, and stromal cells, shaping tumour progression and resistance pathways. Investigating these interactions is crucial for predicting disease trajectory and therapeutic response. Translating preclinical findings into clinical trials is another challenge, as assessing the safety and efficacy of *LRP1*-targeted therapies requires extensive validation. Furthermore, identifying *LRP1* as a biomarker for therapy prediction could enhance precision medicine approaches, improving patient outcomes. Addressing these complexities will be essential for advancing ovarian cancer research and treatment strategies [55-57].

Conclusion

Although bioinformatics analyses have suggested that *LRP1* may serve as a prognostic indicator in OC, direct clinical validation remains limited, and further studies are required to establish its diagnostic significance. High *LRP1* expression correlates with advanced disease stage, poor differentiation, and worse clinical outcomes. *LRP1* is being explored as a therapeutic target in ovarian cancer. Strategies targeting *LRP1* signalling or its downstream effectors have shown encouraging outcomes in preclinical research, including the reversal of chemotherapy resistance and the prevention of tumour growth and metastasis. Prior research indicates that *LRP1* inhibited MMP2 and MMP9 expression via ERK signalling pathways. Enhancing the prognosis and survival of individuals with OC is contingent upon the timely identification of OC genesis. Although vaginal ultrasonography and blood CA125 testing are widely used for ovarian cancer diagnosis, their low sensitivity and specificity limit early-stage detection. Given these challenges, recent studies have explored alternative biomarkers, including *LRP1*, which has been found at elevated levels in ovarian cancer patients. This review highlights the role of *LRP1* in modulating the p-ERK/MMP2/MMP9 signalling axis, which facilitates ovarian cancer cell motility and invasion. Based on current evidence, we propose that LRP1 expression holds potential as a diagnostic and prognostic biomarker in ovarian cancer. However, its clinical relevance remains to be fully elucidated, and further validation through comprehensive in vitro and in vivo studies is warranted.

Glossary

OC: Ovarian Cancer, LRP1: Low-density lipoprotein receptor-related protein-1, MMP: Matrix metalloproteinases, LDL-R: Low-density lipoprotein receptor, ECM: Extracellular matrix, EMT: Epithelial-to-mesenchymal transition, VEGF: Vascular endothelial growth factor, shRNA: Short hairpin RNA, PDGF: Platelet-derived growth factor, FAK: Focal adhesion kinase.

Disclosures

Acknowledgements
We are grateful to our department for the facilities and to our Biochemical Genetics lab mates and staff for their support and encouragement.

Conflict of interest
The Authors do not have any competing interests.

Funding
This study received no external funding.

Authors' contributions
Devi Krishna RB was responsible for conceptualisation, methodology design, data analysis, and preparation of the original draft, including figures and tables. Nandini Krishnamurthy and Sanjana Murali contributed to data curation and literature investigation. Dr. Andrea Mary F and Dr. Preet Agarwal provided supervision and project administration throughout the study. Dr. Elizabeth Rani Junieus, Dr. Leena Joseph, and Dr. Banukeerthana participated in manuscript review, editing, and validation of the final content. All authors contributed to the development of the manuscript and approved its final version for submission.

Availability of data and materials
Not applicable

Ethics approval and consent to participate
Not applicable

Patient consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

References

1. Moch H. Female genital tumours: WHO Classification of Tumours, Volume 4. WHO classification of tumours. 2020.
2. Committee on the State of the Science in Ovarian Cancer Research; Board on Health Care Services; Institute of Medicine; National Academies of Sciences, Engineering, and Medicine. Ovarian Cancers: Evolving Paradigms in Research and Care. Washington (DC): National Academies Press (US); 2016 Apr 25.
3. Zhou W, Ma J, Zhao H, Wang Q, Guo X, Chen L, Cao Z, Xu J, Zhang B, Zhou X. Serum exosomes from epithelial ovarian cancer patients contain LRP1, which promotes the migration of epithelial ovarian cancer cells. Mol. Cell. Proteom. 2023 Apr 1;22(4):100520. https://doi.org/10.1016/j.mcpro.2023.100520.
4. International Agency for Research on Cancer. Global Cancer Observatory: Cancer Today. Lyon, France: IARC. Retrieved from GLOBOCAN 2022.
5. Bian W, Tang M, Jiang H, Xu W, Hao W, Sui Y, Hou Y, Nie L, Zhang H, Wang C, Li N. Low-density-lipoprotein-receptor-related protein 1 mediates Notch pathway activation. Dev. Cell. 2021 Oct 25;56(20):2902-19. https://doi.org/10.1016/j.devcel.2021.09.015.
6. Ortiz M, Wabel E, Mitchell K, Horibata S. Mechanisms of chemotherapy resistance in ovarian cancer. Cancer Drug Resist. 2022 Apr 3;5(2):304. https://doi.org/10.20517/cdr.2021.147.
7. Wang L, Wang X, Zhu X, Zhong L, Jiang Q, Wang Y, Tang Q, Li Q, Zhang C, Wang H, Zou D. Drug resistance in ovarian cancer: from mechanism to clinical trial. Mol. Cancer. 2024 Mar 28;23(1):66. https://doi.org/10.1186/s12943-024-01967-3.
8. Guo X, Yang F, Liu T, Chen A, Liu D, Pu J, Jia C, Wu Y, Yuan J, Ouyang N, Herz J. Loss of LRP1 Promotes Hepatocellular Carcinoma Progression via UFL1-Mediated Activation of NF-κB Signaling. Adv. Sci. 2024 Dec;11(45):2401672. https://doi.org/10.1002/advs.202401672.
9. Herz J, Strickland DK. LRP: a multifunctional scavenger and signaling receptor. J. Clin. Invest. 2001 Sep 15;108(6):779-84. https://doi.org/10.1172/JCI13992.
10. Vormittag R, Bencur P, Ay C, Tengler T, Vukovich T, Quehenberger P, Mannhalter C, Pabinger I. Low-density lipoprotein receptor-related protein 1 polymorphism 663 C> T affects clotting factor VIII activity and increases the risk of venous thromboembolism. J. Thromb. Haemost. 2007 Mar;5(3):497-502. https://doi.org/10.1111/j.1538-7836.2007.02337.x.
11. Vitols S, Peterson C, Larsson O, Holm P, Åberg B. Elevated uptake of low density lipoproteins by human lung cancer tissue in vivo. Cancer Res. 1992 Nov 15;52(22):6244-6247.
12. Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H, Stanley KK. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. EMBO J. 1988 Dec 20;7(13):4119-27. https://doi.org/10.1002/j.1460-2075.1988.tb03306.x.
13. Kang HS, Kim J, Lee HJ, Kwon BM, Lee DK, Hong SH. LRP1-dependent pepsin clearance induced by 2'-hydroxycinnamaldehyde attenuates breast cancer cell invasion. Int. J. Biochem. Cell Biol. 2014 Aug 1;53:15-23. https://doi.org/10.1016/j.biocel.2014.04.021.
14. Coleman RL, Monk BJ, Sood AK, Herzog TJ. Latest research and treatment of advanced-stage epithelial ovarian cancer. Nat. Rev. Clin. Oncol. 2013 Apr;10(4):211-24. https://doi.org/10.1038/nrclinonc.2013.5.
15. Zhang W, Ou X, Wu X. Proteomics profiling of plasma exosomes in epithelial ovarian cancer: A potential role in the coagulation cascade, diagnosis and prognosis. Int. J. Oncol. 2019 Mar 7;54(5):1719-33. https://doi.org/10.3892/ijo.2019.4742.
16. Spuch C, Ortolano S, Navarro C. LRP-1 and LRP-2 receptors function in the membrane neuron. Trafficking mechanisms and proteolytic processing in Alzheimer's disease. Front. Physiol. 2012 Jul 16;3:269. https://doi.org/10.3389/fphys.2012.00269.
17. Czekay RP, Wilkins-Port CE, Higgins SP, Freytag J, Overstreet JM, Klein RM, Higgins CE, Samarakoon R, Higgins PJ. PAI-1: an integrator of cell signaling and migration. Int. J. Cell Biol. 2011;2011(1):562481. https://doi.org/10.1155/2011/562481.
18. Vos MC, van der Wurff AA, van Kuppevelt TH, Massuger LF. The role of MMP-14 in ovarian cancer: a systematic review. J. Ovarian Res. 2021 Dec;14:1-3. doi.org/10.1186/s13048-021-00852-7.
19. Boucher P, Herz J. Signaling through LRP1: Protection from atherosclerosis and beyond. Biochem. Pharma-

col. 2011 Jan 1;81(1):1-5. https://doi.org/10.1016/j.bcp.2010.09.018.
20. Appert-Collin A, Bennasroune A, Jeannesson P, Terryn C, Fuhrmann G, Morjani H, Dedieu S. Role of LRP-1 in cancer cell migration in 3-dimensional collagen matrix. Cell Adhes. Migr. 2017 Jul 4;11(4):316-26. https://doi.org/10.1080/19336918.2016.1215788.
21. Feng C, Ding G, Ding Q, Wen H. Overexpression of low-density lipoprotein receptor-related protein 1 (LRP1) is associated with worsened prognosis and decreased cancer immunity in clear-cell renal cell carcinoma. Biochem. Biophys. Res. Commun. 2018 Sep 10;503(3):1537-1543. https://doi.org/10.1016/j.bbrc.2018.07.076.
22. Montel V, Gaultier A, Lester RD, Campana WM, Gonias SL. The low-density lipoprotein receptor–related protein regulates cancer cell survival and metastasis development. Cancer Res. 2007 Oct 15;67(20):9817-9824. https://doi.org/10.1158/0008-5472.CAN-07-0683.
23. Campion O, Al Khalifa T, Langlois B, Thevenard-Devy J, Salesse S, Savary K, Schneider C, Etique N, Dedieu S, Devy J. Contribution of the low-density lipoprotein receptor family to breast cancer progression. Front. Oncol. 2020 Jul 30;10:882. https://doi.org/10.3389/fonc.2020.00882.
24. Zhu M, Shen H, Wang B, He Y, Chen J, Ren J, Zhang Z, Jian X. LRP1 as a promising therapeutic target for gastrointestinal tumors: Inhibiting proliferation, invasion and migration of cancer cells. Oncol. Lett. 2023 Aug 2;26(4):1-12. https://doi.org/10.3892/ol.2023.14019.
25. May P, Woldt E, Matz RL, Boucher P. The LDL receptor-related protein (LRP) family: An old family of proteins with new physiological functions. Ann. Med. 2007 Jan 1;39(3):219-28. doi.org/10.1080/07853890701214881.
26. Jusić A, Avdić A, Hadžiavdić V, Junuzović I, Kurtčehajić D. Identification of 677C> T Polymorphism of LRP1 Gene by PCR-RFLP Method. TEM J. 2018 Nov;7(4):803-807. https://doi.org/10.18421/TEM74-17.
27. Fields BN. Genetic Polymorphisms in LRP1, a Newly Identified Receptor for Emerging Bunyaviruses. Doctoral dissertation. University of Pittsburgh, 2023.
28. Vučinić N, Stokić E, Djan I, Obreht D, Veličković N, Stankov K, Djan M. The gene polymorphism is associated with increased risk of metabolic syndrome prevalence in the Serbian population. Balkan J. Med. Genet. 2017 Jun 30;20(1):51-58. https://doi.org/10.1515/bjmg-2017-0004.
29. Shinohara M, Tachibana M, Kanekiyo T, Bu G. Role of LRP1 in the pathogenesis of Alzheimer's disease: evidence from clinical and preclinical studies. J. Lipid Res. 2017 Jul;58(7):1267. https://doi.org/10.1194/jlr.R075796.
30. Lee JD, Hsiao KM, Chang PJ, Chen CC, Kuo YW, Huang YC, Hsu HL, Lin YH, Wu CY, Huang YC, Lee M, Hsu CY, Pan YT, Kuo CY, Lin CH. A common polymorphism decreases LRP1 mRNA stability and is associated with increased plasma factor VIII levels. Biochim. Biophys. Acta Mol. Basis Dis. 2017 Jun;1863(6):1690-1698. https://doi.org/10.1016/j.bbadis.2017.04.015.
31. Xing P, Liao Z, Ren Z, Zhao J, Song F, Wang G, Chen K, Yang J. Roles of low-density lipoprotein receptor-related protein 1 in tumors. Chin. J. Cancer. 2016 Jan 6;35:6. https://doi.org/10.1186/s40880-015-0064-0.
32. Actis Dato V, Chiabrando GA. The role of low-density lipoprotein receptor-related protein 1 in lipid metabolism, glucose homeostasis and inflammation. Int. J. Mol. Sci. 2018 Jun 15;19(6):1780. https://doi.org/10.3390/ijms19061780.
33. Gan M, Jiang P, McLean P, Kanekiyo T, Bu G. Low-density lipoprotein receptor-related protein 1 (LRP1) regulates the stability and function of GluA1 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor in neurons. PloS One. 2014 Dec 12;9(12):e113237. https://doi.org/10.1371/journal.pone.0113237.
34. Fuentealba RA, Liu Q, Kanekiyo T, Zhang J, Bu G. Low density lipoprotein receptor-related protein 1 promotes anti-apoptotic signaling in neurons by activating Akt survival pathway. J. Biol. Chem. 2009 Dec 4;284(49):34045-34053. https://doi.org/10.1074/jbc.M109.021030.
35. Mantuano E, Inoue G, Li X, Takahashi K, Gaultier A, Gonias SL, Campana WM. The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. J. Neurosci. 2008 Nov 5;28(45):11571-11582. https://doi.org/10.1523/JNEUROSCI.3053-08.2008.
36. Yancey PG, Blakemore J, Ding L, Fan D, Overton CD, Zhang Y, Linton MF, Fazio S. Macrophage LRP-1 controls plaque cellularity by regulating efferocytosis and Akt activation. Arterioscler. Thromb. Vasc. Biol. 2010 Apr;30(4):787-795. https://doi.org/10.1161/ATVBAHA.109.202051.
37. Van Leuven F, Cassiman JJ, Van Den Berghe H. Demonstration of an alpha2-macroglobulin receptor in human fibroblasts, absent in tumor-derived cell lines. J. Biol. Chem. 1979 Jun 25;254(12):5155-5160.
38. Gonias SL, Campana WM. LDL receptor–related protein-1: a regulator of inflammation in atherosclerosis, cancer, and injury to the nervous system. Am. J. Pathol. 2014 Jan;184(1):18-27. https://doi.org/10.1016/j.ajpath.2013.08.029.
39. Pires LA, Hegg R, Freitas FR, Tavares ER, Almeida C, Baracat EC, Maranhão RC. Effect of neoadjuvant chemotherapy on low-density lipoprotein (LDL) receptor and LDL receptor-related protein 1 (LRP-1) receptor in locally advanced breast cancer. Braz. J. Med. Biol. Res. 2012 Jun;45:557-564. https://doi.org/10.1590/S0100-879X2012007500068.
40. Sizova O, John LS, Ma Q, Molldrem JJ. Multi-faceted role of LRP1 in the immune system. Front. Immunol. 2023 Mar 20;14:1166189. https://doi.org/10.3389/fimmu.2023.1166189.
41. Jin HR, Wang J, Wang ZJ, Xi MJ, Xia BH, Deng K, Yang JL. Lipid metabolic reprogramming in tumor microenvironment: From mechanisms to therapeutics. J. Hematol. Oncol. 2023 Sep 12;16(103). https://doi.org/10.1186/s13045-023-01498-2.

42. Mathews SG, Krishna RD, Lavanya M, Nandini K, Murali S, Agarwal P, Rani E. The Role of the Plasminogen Activator Inhibitor 1 (PAI1) in Ovarian Cancer: Mechanisms and Therapeutic Implications. Glob. Med. Genet. 2024 Dec;11(04):358-65. https://doi.org/10.1055/s-0044-1791734.
43. Bourazopoulou E, Kapsogeorgou EK, Routsias JG, Manoussakis MN, Moutsopoulos HM, Tzioufas AG. Functional expression of the alpha 2-macroglobulin receptor CD91 in salivary gland epithelial cells. J. Autoimmun. 2009 Sep 1;33(2):141-6. https://doi.org/10.1016/j.jaut.2009.06.004.
44. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. Physiol. Rev. 2008 Jul;88(3):887-918. https://doi.org/10.1152/physrev.00033.2007.
45. Timilsina S, Amara AR, Abu R, Spring BQ. Identification of potential cell surface targets in patient-derived cultures toward photoimmunotherapy of high-grade serous ovarian cancer. Photochem. Photobiol. 2025 Apr 9. https://doi.org/10.1111/php.14091.
46. Datta A, Deng S, Gopal V, Yap KC, Halim CE, Lye ML, Ong MS, Tan TZ, Sethi G, Hooi SC, Kumar AP. Cytoskeletal dynamics in epithelial-mesenchymal transition: insights into therapeutic targets for cancer metastasis. Cancers. 2021 Apr 14;13(8):1882. https://doi.org/10.3390/cancers13081882.
47. Duffy AM, Bouchier-Hayes DJ, Harmey JH. Vascular endothelial growth factor (VEGF) and its role in non-endothelial cells: autocrine signalling by VEGF. In: Madame Curie Bioscience Database. Austin (TX): Landes Bioscience; 2000-2013.
48. Seager RJ, Hajal C, Spill F, Kamm RD, Zaman MH. Dynamic interplay between tumour, stroma and immune system can drive or prevent tumour progression. Converg. Sci. Phys. Oncol. 2017 Jul 28;3(3):034002. https://doi.org/10.1088/2057-1739/aa7e86.
49. Van Gool B, Dedieu S, Emonard H, Roebroek AJ. The matricellular receptor LRP1 forms an interface for signaling and endocytosis in modulation of the extracellular tumor environment. Front. Pharmacol. 2015 Nov 10;6:271. https://doi.org/10.3389/fphar.2015.00271.
50. Qu L, Wang F, Wang Y, Li Z. The regulation of LRPs by miRNAs in cancer: influencing cancer characteristics and responses to treatment. Cancer Cell Int. 2025 Dec;25(1):1-22. https://doi.org/10.1186/s12935-025-03804-z.
51. Xing P, Liu H, Xiao W, Zhang G, Zhang C, Liao Z, Li T, Yang J. The fusion gene LRP1–SNRNP25 drives invasion and migration by activating the pJNK/37LRP/MMP2 signaling pathway in osteosarcoma. Cell Death Discov. 2024 Apr 27;10(1):198. https://doi.org/10.1038/s41420-024-01962-z.
52. NR S, Behera MM, Naik SK, Das SK, Gopan S, Ghosh A, Sahu RN, Patra S, Purkait S. Elevated expression of cholesterol transporter LRP-1 is crucially implicated in the pathobiology of glioblastoma. Front. Neurol. 2022 Oct 4;13:1003730. https://doi.org/10.3389/fneur.2022.1003730.
53. Pencheva N, Tran H, Buss C, Huh D, Drobnjak M, Busam K, Tavazoie SF. Convergent multi-miRNA targeting of ApoE drives LRP1/LRP8-dependent melanoma metastasis and angiogenesis. Cell. 2012 Nov 21;151(5):1068-82. https://doi.org/10.1016/j.cell.2012.10.028.
54. Dai W, Zhou J, Chen T. Unraveling the extracellular vesicle network: insights into ovarian cancer metastasis and chemoresistance. Mol. Cancer. 2024 Sep 16;23(1):201. https://doi.org/10.1186/s12943-024-02103-x.
55. Wang Y, Zhu N, Liu J, Chen F, Song Y, Ma Y, Yang Z, Wang D. Role of tumor microenvironment in ovarian cancer metastasis and clinical advancements. J. Transl. Med. 2025 Dec;23(1):1-63. doi.org/10.1186/s12967-025-06508-0.
56. Xie W, Sun H, Li X, Lin F, Wang Z, Wang X. Ovarian cancer: epigenetics, drug resistance, and progression. Cancer Cell Int. 2021 Dec;21:1-6. https://doi.org/10.1186/s12935-021-02136-y.
57. Fu R, Hu R, Li W, Lv X, Zhao H, Li F. Unveiling drug resistance pathways in high-grade serous ovarian cancer (HGSOC): recent advances and future perspectives. Front. Immunol. 2025 Apr 30;16:1556377. https://doi.org/10.3389/fimmu.2025.1556377.
58. Le CC, Bennasroune A, Collin G, Hachet C, Lehrter V, Rioult D, Dedieu S, Morjani H, Appert-Collin A. LRP-1 promotes colon cancer cell proliferation in 3D collagen matrices by mediating DDR1 endocytosis. Front. Cell Dev. Biol. 2020 Jun 3;8:412. https://doi.org/10.3389/fcell.2020.00412.

REVIEW PAPER

JMS Journal of Medical Science

The Impact of Digital Screens on Eye and Nervous System Health

Witold Gajewski*
University Hospital in Poznań, Poznań, Poland
https://orcid.org/0009-0000-3900-236X
Corresponding author: witold.r.gajewski@gmail.com

Jan Górski*
University Hospital in Poznań, Poznań, Poland
https://orcid.org/0009-0001-9504-8482

Julia Janecka*
University Hospital in Poznań, Poznań, Poland
https://orcid.org/0009-0005-9011-817X

Michał Hofman*
HCP Medical Center in Poznań, Poznań, Poland
https://orcid.org/0000-0001-9365-4537

Weronika Skoczek*
Raszeja Hospital in Poznań, Poznań, Poland
https://orcid.org/0000-0003-3810-3956

Wiktor Gałęcki*
Józef Struś Multispecialist Municipal Hospital, Poznań, Poland
https://orcid.org/0009-0003-1269-8442

* All authors contributed equally to the manuscript

https://doi.org/10.20883/medical.e1283

Keywords: eye health, mental health, screen addiction, preventive strategies, screen time management

Received 2025-05-09
Accepted 2025-08-14
Published 2025-09-19

How to Cite: Gajewski Witold, Górski J, Janecka J, Hofman M, Skoczek W, Gałęcki W. The Impact of Digital Screens on Eye and Nervous System Health. Journal of Medical Science. 2025 September;94(3);e. doi:10.20883/medical.e1283

ABSTRACT

Introduction. The increasing reliance on digital technology has led to a significant rise in daily screen exposure, raising concerns about its potential impact on eye health and the nervous system. Prolonged screen use is associated with conditions such as dry eye disease (DED), computer vision syndrome (CVS), progressive myopia, sleep disturbances, mental fatigue, and screen addiction - especially among children and adolescents.

Material and methods. A narrative review of peer-reviewed articles, systematic reviews, and meta-analyses indexed in PubMed and Google Scholar was conducted. Boolean search strategies combined terms related to digital screens, ocular health, neurocognitive effects, and preventive interventions. Studies published up to 2025, with emphasis on data from 2020-2025, were included. The review focused on both ophthalmological and neurological consequences of screen exposure.

Results. Current evidence indicates a strong correlation between prolonged screen use and the incidence of DED, CVS, and myopia. Neurocognitive impacts include circadian rhythm disruption due to blue light exposure, sensory overload, mental fatigue, and early signs of attentional deficits – especially in younger populations. Screen addiction amplifies these risks. Although some mitigation strategies, such as the 20-20-20 rule and digital detox programmes, have demonstrated effectiveness, others, like blue light filters, show inconsistent results.

Conclusions. Prolonged use of digital screens has been shown to affect both visual and neurological health adversely. While complete avoidance is unrealistic, adopting healthy screen habits and evidence-based preventive strategies is critical. Further longitudinal research is essential to clarify long-term effects and support informed public health recommendations.

Introduction

The modern world is increasingly reliant on digital technology, with screens of electronic devices such as smartphones, computers, tablets, and televisions becoming an integral part of everyday life. These devices are used for work, education, entertainment, and communication, resulting in a steadily increasing amount of screen time [1,2]. According to research, the average user may spend several hours per day in front of digital screens, with this number rising significantly among individuals working remotely or engaging in online learning [3]. This issue was further exacerbated by the Coronavirus Disease 2019 (COVID-19) pandemic, during which people confined to their homes reported an even greater increase in digital screen use [4]. While modern technologies offer many advantages, excessive use has raised growing concerns regarding potential health consequences, particularly in relation to visual health and the nervous system.

Scientific literature increasingly highlights a link between prolonged screen exposure and a variety of health disorders, such as diminished visual quality, chronic fatigue, sleep disturbances, sensory overload, and even neurobiological changes in brain structure and function [5]. A growing concern is also digital device addiction, which may intensify these symptoms and hinder their management. These issues are especially alarming in children and adolescents, whose visual and nervous systems are in a critical stage of development and are therefore particularly vulnerable to environmental stressors [6].

The purpose of this review is to systematically summarise and critically assess the current evidence regarding the effects of prolonged digital screen use on visual and neurological health. Additionally, the review aims to identify key risk factors, highlight gaps in existing research, and present practical, evidence-based strategies to mitigate the adverse health outcomes associated with excessive screen exposure.

Methods

Study design

This narrative review aimed to provide an in-depth overview of the current scientific evidence on the impact of digital screen use on ocular health and the nervous system. The review focused on primary screen-related conditions such as Dry Eye Disease, Computer Vision Syndrome, myopia, circadian rhythm disturbances, sensory overload, and screen addiction.

A literature search was conducted using PubMed and Google Scholar, covering publications up to 2025, with particular emphasis on studies from 2020 to 2025. Older studies were included when recent data were limited or when they provided foundational insights into the studied phenomena.

The inclusion criteria encompassed peer-reviewed original research articles, systematic reviews, meta-analyses, and clinical studies focused on the ocular or neurocognitive effects of digital screen use in both children and adults. Studies addressing interventions and preventive strategies were also included.

Exclusion criteria involved studies unrelated to screen use (e.g., general visual impairment causes, non-digital media exposure), animal studies not directly translatable to human health, and reports lacking primary data (e.g., editorials, non-systematic opinion pieces).

Additionally, reference lists of key publications were manually reviewed to identify further relevant studies. Given the heterogeneity of study designs, outcome measures, and populations, this review did not include a meta-analytic synthesis. Instead, it focused on summarising recurring findings, highlighting contradictory results, and identifying research gaps to inform future investigations.

Risk of bias

As a narrative review, this work lacks a formal systematic review protocol or standardised quality assessment of included studies, which introduces a potential risk of selection bias. Study inclusion was based on the authors' critical judgement rather than predefined methodological scoring systems.

Although efforts were made to ensure comprehensive literature coverage using Boolean strategies across major databases, reliance on English-language publications and selected databases may have resulted in the omission of relevant studies.

The heterogeneity of clinical endpoints, study populations, and methodological approaches limited the possibility of direct comparisons between studies. Therefore, this review provides a qualitative synthesis rather than a quantitative assessment.

Furthermore, the possibility of publication bias cannot be excluded – studies reporting significant or positive findings are more likely to be published, potentially skewing the evidence base. While priority was given to recent research, the exclusion of older studies may have led to under-representation of important historical data.

The authors aimed to maintain a balanced and objective perspective; however, subjective interpretation of study outcomes and emphasis on specific findings may have influenced the narrative. The conclusions presented should be considered as a foundation for further research rather than definitive clinical guidance.

Effects of screens on eye health

This chapter provides an in-depth analysis of the effects of digital screen exposure on ocular health, with a particular emphasis on the prevalence and clinical implications of Computer Vision Syndrome, Dry Eye Syndrome and the rising incidence of myopia.

Dry Eye Syndrome and Dry Eye Disease

Dry Eye Syndrome (DES), or more broadly referred to as Dry Eye Disease (DED), is one of the most common causes of ophthalmologic consultations. It is characterised by insufficient ocular surface lubrication, most commonly due to reduced tear production or excessive tear evaporation. The most frequently reported symptoms include dryness and burning sensations, a foreign body or "gritty" sensation under the eyelids, red and fatigued eyes, blurred vision, and photophobia [7].

According to the Tear Film and Ocular Surface Society, the prevalence of DED ranges from 5% to 50% of the population, occurring more frequently in women and individuals of Asian descent, with age being the most significant risk factor [8]. In addition to non-modifiable risk factors, modifiable contributors have also been identified, most notably excessive use of digital screens [9]. Prolonged screen exposure is associated with a decreased blink rate and an increased frequency of incomplete blinks. These factors contribute to tear film instability, increased evaporation, tear hyperosmolarity, and ocular surface inflammation and damage [10].

Fjaervoll et al. [11] conducted a systematic review in which, following predefined inclusion and exclusion criteria, they selected 57 studies investigating the relationship between digital screen use and the occurrence of DED. Their analysis demonstrated a strong correlation between excessive screen time and the incidence of DED. Notably, they found that even 1–2 hours of screen use per day may contribute to DED-related symptoms [11].

A recent study by Jadeja et al. [12] focused on the paediatric population to assess the impact of screen exposure on the development of DED. Among 462 children examined, 90.5% were diagnosed with DED. The study revealed that moderate and severe DED were significantly associated with higher digital screen use compared to mild DED ($P = 0.001$). Furthermore, screen time exceeding three hours per day markedly increased the risk of DED in children. Additional factors, such as every 30-minute increment of computer use and being in higher school grades (which are associated with more screen-based academic work), were found to significantly increase the likelihood of moderate to severe DED [12].

DED represents a prevalent ophthalmological condition strongly linked to modern lifestyle factors. The studies indicate that even short daily exposures to screens can substantially elevate the risk of DED, especially in children. The rising prevalence in this age group underscores the urgent need for greater awareness and preventative measures, such as limiting screen time and promoting healthy visual habits.

Computer Vision Syndrome

Computer Vision Syndrome (CVS), also known as digital eye strain, refers to a collection of visual and ocular symptoms associated with prolonged use of electronic screens, such as computer monitors, smartphones, and tablets. Contemporary work and lifestyle patterns have led to a significant increase in daily screen time, often resulting in visual strain and a range of related com-

plaints. CVS encompasses symptoms including eye fatigue, dryness, burning sensations, headaches, double vision, blurred vision, and difficulty with accommodation. The primary contributing factors include extended screen exposure under suboptimal lighting conditions, reduced blink rate, excessive exposure to blue light, and poor ergonomic design of the workspace [13,14].

According to a meta-analysis by Anbesu and Lema (2023) [15], the overall prevalence of CVS, based on 45 studies, was found to be 66% in the studied populations [15]. Given the increasing number of individuals affected, CVS has become a significant concern in both ophthalmology and optometry, with potential consequences not only for visual comfort but also for work efficiency and general well-being.

Alamri et al. [16] conducted a questionnaire-based study involving 400 participants to assess symptoms, risk factors, and other aspects related to CVS. Their findings revealed that 9% of respondents reported isolated eye pain, 8% experienced DES, 6% reported tearing and eye redness, 20% exhibited multiple symptoms simultaneously, and 9% were asymptomatic. Notably, 69% of participants reported a worsening of symptoms following the onset of COVID-19 lockdowns [16].

A comparable investigation was carried out by Abudawood et al. [17], aiming to assess the prevalence of CVS symptoms and their associated risk factors among 651 medical students at King Abdulaziz University in Jeddah, Saudi Arabia. The study reported that 95% of respondents (558 individuals) experienced at least one CVS-related symptom while working on a computer. Key risk factors identified included prolonged screen time, close head positioning to the screen, and high screen brightness. The most common ocular symptoms were excessive tearing and dryness, while non-ocular symptoms included neck, back, shoulder, and head pain [17].

CVS represents a growing public health concern affecting a substantial portion of digital screen users. Studies consistently report a high prevalence of CVS and emphasise the role of significant risk factors, such as extended screen exposure, poor ergonomics, and intense screen light. The observed exacerbation of symptoms during pandemic-related lockdowns further underscores the relevance of this condition. Continued research into effective preventive and therapeutic strategies for CVS is crucial for enhancing visual comfort and overall quality of life for individuals exposed to prolonged screen use.

Myopia and its association with digital screen exposure

Myopia, or nearsightedness, is a refractive error in which light rays focus in front of the retina rather than directly on it. This condition results in blurred distance vision while near objects remain clear. Although genetic predisposition plays a role, environmental factors – particularly prolonged near work involving digital screens – may significantly accelerate the onset and progression of myopia [18].

Extended periods of near work with digital devices require sustained accommodation, wherein the eye continually focuses on nearby objects (e.g., a smartphone or computer screen). This persistent engagement of the ciliary muscle can lead to accommodative spasm [19]. Over time, the eye may adapt to near vision at the expense of distance clarity. This phenomenon is especially concerning in children and adolescents whose visual systems are still developing, thereby increasing the likelihood of permanent myopic changes.

Numerous studies have investigated the relationship between digital screen use and the development of myopia. A meta-analysis by Ha et al. [20] found that each additional hour of daily screen time increases the risk of developing myopia by 21%. When analysing the correlation between screen exposure duration and refractive risk, a substantial increase in risk was observed with increasing screen time. The study indicated that exposure of less than one hour per day is associated with low risk, whereas exposure ranging from 1 to 4 hours is linked to a sharp increase in risk. Beyond 4 hours, the risk continues to rise but at a slower, more stable rate [20].

Similarly, a meta-analysis by Zong et al. [21] confirmed a significant correlation between more prolonged digital screen exposure and the risk of developing myopia, compared to shorter durations. The authors reported a 7% increase in myopia risk for every additional hour of daily screen use. Notably, a subgroup analysis examined the influence of specific device types on myopia development. Results indicated that extended use of computers and televisions was significant-

ly associated with increased risk, whereas smartphone use did not show a similar correlation [21]. This discrepancy may stem from typical smartphone usage patterns, which often involve shorter, more intermittent sessions interspersed with rest periods, potentially reducing their cumulative impact on ocular development.

In response to increased screen time among children and adolescents during the COVID-19 pandemic, mainly due to remote learning, AlShamlan et al. [22] conducted a retrospective study demonstrating that the progression of myopia accelerated significantly in these age groups during the lockdown period [22].

In summary, available evidence clearly demonstrates a significant association between prolonged digital screen exposure and an elevated risk of myopia. Moreover, intensive use of digital devices during early life stages – especially during periods of active eyeball growth – may accelerate both the onset and progression of this refractive error. Given the increasing prevalence of screen use among children and adolescents, further research and the implementation of effective preventive strategies are essential.

The impact of screens on the nervous system

This chapter examines the effects of prolonged digital screen exposure on the functioning of the nervous system. Drawing on current scientific research, it discusses potential associations between screen use and sleep disturbances, impaired concentration, increased stress levels, and symptoms of anxiety and depression. Evidence from the literature is presented to support the existence of correlations between the intensity of screen use and alterations in neuropsychological functioning.

Disruption of circadian rhythm and sleep problems

Melatonin is a key hormone involved in maintaining the body's homeostasis, particularly in regulating the circadian sleep-wake cycle. It is primarily synthesised and secreted by the pineal gland and exerts its effects through MT1 and MT2 receptors, which facilitate sleep onset and inhibit arousal-promoting signals. The rhythm of melatonin secretion is regulated by the interaction between the suprachiasmatic nucleus and the retina; secretion increases in response to low-light conditions [23,24].

A growing body of evidence suggests that evening use of screen-based electronic devices—such as smartphones, computers, tablets, and televisions—may impair sleep quality and lead to increased daytime sleepiness [25]. This effect is believed to be associated with the high levels of blue light emitted by modern LED-based digital screens. Blue light, particularly in the wavelength range of 446 to 477 nm, has been shown to exert the most potent suppressive effect on melatonin secretion, thereby disrupting circadian rhythms [26,27].

In a study conducted by Heo et al. [28], the effects of blue light emitted from conventional smartphone LED screens were evaluated in relation to plasma melatonin and cortisol levels, core body temperature, and outcomes on standardised psychiatric tests. The control group used smartphones equipped with blue light-blocking filters. Participants were exposed to the respective device from 19:30 to 22:00, with blood samples and temperature measurements collected before, during, and after the experiment. Results showed that participants using conventional smartphones reported lower subjective sleepiness, greater confusion-bewilderment, and a higher number of commission errors on cognitive performance tasks (as assessed by the Profile of Mood States, Epworth Sleepiness Scale, Fatigue Severity Scale, and auditory and visual Continuous Performance Tests). Additionally, the onset of melatonin secretion under dim light conditions was delayed in these individuals. Although increases in melatonin, cortisol, and body temperature were observed, these changes were not statistically significant. The findings suggest that evening smartphone use can impair sleep onset and increase cognitive errors, particularly commission-type errors [28].

A similar study by Chinoy et al. [29] compared the use of screen-based devices versus traditional reading before bedtime. Consistent with prior findings, participants who used screen-emitting devices reported reduced sleepiness, opted for later bedtimes, and experienced longer sleep latency. Laboratory assessments showed lower evening melatonin levels and delayed onset

of melatonin secretion. Moreover, these individuals demonstrated reduced alertness the following day compared to those who read from non-light-emitting sources before sleep [29].

Melatonin plays a crucial role in regulating circadian rhythms through interactions with the MT1 and MT2 receptors. Its secretion is highly sensitive to lighting conditions and particularly susceptible to suppression by blue light. Studies have shown that evening exposure to blue light from LED-based screen devices delays melatonin onset, disrupts sleep patterns, reduces subjective sleepiness, and increases the occurrence of cognitive errors such as commission mistakes. These findings collectively support the conclusion that exposure to blue light negatively affects sleep-wake homeostasis and cognitive functioning.

Sensory overload and mental fatigue

In addition to sleep disturbances caused by excessive blue light exposure and the subsequent suppression of melatonin secretion, the use of screen-based digital devices contributes to sensory overload and mental fatigue. Continuous stimulation from digital cues (e.g., notifications, messages, and rapid visual transitions) may induce a state of mental fatigue, characterised by cognitive exhaustion, lack of energy for mental tasks, and difficulty maintaining attention. Research suggests that sustained digital device use without adequate breaks lowers overall alertness and may trigger symptoms resembling sensory overstimulation, as observed in anxiety disorders [30].

Excessive and frequent use of screens can lead to a phenomenon referred to as 'digital burnout' – a condition of physical and mental exhaustion caused by chronic digital overstimulation [31]. Individuals experiencing digital burnout commonly report persistent fatigue, sleep disturbances, headaches, and emotional symptoms such as apathy, irritability, and heightened anxiety. Mental health professionals have observed a growing number of patients experiencing media overload, including symptoms of information stress related to compulsive monitoring of online news content [32].

An illustrative phenomenon emerged during the COVID-19 pandemic, when social isolation led to increased use of video conferencing platforms, resulting in what is now known as “Zoom fatigue”. Ahn et al. [33] conducted a survey study which revealed that prolonged participation in virtual meetings contributed to fatigue, reduced motivation, and elevated stress levels. The causes included cognitive overload (e.g., constant eye contact, self-view monitoring, sustained attention) and the absence of natural breaks and physical movement [33].

Lastly, a study by Nagata et al. [34] demonstrated a correlation between screen time and the prevalence of various behavioural disturbances. It was found that higher overall screen exposure was associated with a small but statistically significant increase in symptoms of depression and Attention-Deficit/Hyperactivity Disorder [34].

Screen use can have adverse effects on mental well-being and cognitive performance. Neural overload may manifest as fatigue, irritability, difficulty concentrating, and reduced efficiency in academic or professional tasks. Modern lifestyles, characterised by continuous technological engagement, are increasingly linked to digital burnout and heightened levels of information-induced stress. These effects were especially pronounced during the COVID-19 pandemic, when daily life shifted even more heavily into the digital realm.

Neurocognitive effects of digital screen exposure

With the growing accessibility and pervasive use of digital devices, increasing scientific interest has been directed toward the impact of screen exposure not only on physical health but also on neurocognitive functioning. The use of screen-based technologies – whether passive (e.g., video viewing) or interactive (e.g., gaming, social media) – has been associated with alterations in neuronal activity, brain structure, and cognitive processing. Recent research efforts have focused on identifying correlations between digital media use and neural patterns observed via electroencephalography (EEG) and magnetic resonance imaging (MRI).

A substantial body of evidence suggests a relationship between excessive screen time and attentional deficits, particularly among children and adolescents during critical stages of neurodevelopment. A longitudinal study demonstrated that children with increased screen exposure at age one exhibited significantly more attention

and executive function difficulties by age nine, as assessed through standardised neuropsychological tests and parent-teacher reports. Notably, EEG data revealed early neurophysiological changes as soon as 18 months of age in high screen-time infants, including elevated theta/beta ratios – an established biomarker of hypoarousal and attentional vulnerability [35]. These findings imply that excessive screen-mediated stimulation during early childhood may disrupt normative patterns of attentional development.

In a 2023 EEG study, children with high levels of screen use showed reduced cortical activation during tasks involving attentional control. Specifically, decreased amplitudes of the P2 and P3 event-related potential components were recorded during Go/No-Go paradigms, suggesting diminished efficiency in processing inhibitory cues. This occurred despite no significant differences in response times or accuracy, indicating potential subclinical cognitive alterations [36].

Among young adults, functional MRI studies have identified subtle yet statistically significant differences in intrinsic brain connectivity associated with problematic smartphone use. In one investigation, individuals with elevated scores on smartphone addiction scales exhibited increased static functional connectivity within the frontoparietal control network (implicated in executive functions) and decreased dynamic variability within attentional networks. These patterns may reflect reduced cognitive flexibility and impaired attentional switching mechanisms potentially underlying compulsive engagement with digital devices [37]. Although intergroup behavioural differences were modest, the neuroimaging data suggest that excessive smartphone use is associated with functional reorganisation of brain networks.

Collectively, the current literature indicates that intensive exposure to screen-based media may alter neurocognitive function, particularly in domains related to attention, executive control, and cognitive flexibility. These effects appear to be most pronounced during neurodevelopment windows in childhood and adolescence. While such changes may not be overtly observable in behaviour, advanced neuroimaging methodologies provide robust evidence of disrupted neural processes and information integration mechanisms as a consequence of excessive digital stimulation.

Screen addiction

The rapid development of digital technologies and the widespread availability of screen-based devices have significantly altered the daily functioning of modern individuals. Increasingly, excessive and difficult-to-control screen use is being recognised as exhibiting features characteristic of behavioural addiction. This trend has intensified notably since 2010, coinciding with the proliferation of communication technologies, and accelerated even further after 2020, following the global shift to remote interaction during the COVID-19 pandemic [38]. A central role in this process is played by the brain's reward system, particularly dopamine, a key neurotransmitter involved in the experience of pleasure, motivation, and learning [39]. This subsection aims to explore the impact of screen use on the risk of addiction and the intensification of screen-related behavioural symptoms.

A significant risk factor for screen addiction is high exposure to digital screens during early developmental periods. The American Academy of Paediatrics recommended that children under the age of two should not be exposed to screens at all, children between the ages of two and five should be limited to a maximum of one hour per day, and those over five years of age should not exceed two hours of daily screen time [40]. Similar limitations were included in the 2019 World Health Organisation (WHO) guidelines on physical activity, sedentary behaviour and sleep for children under 5 years of age [41]. Tekeci et al. [42] investigated the impact of increased screen exposure on digital screen addiction, measured using the Problematic Media Use Scale, as well as behavioural disorders in children aged 6 to 10. Children were divided into groups based on daily screen time: more than 2 hours versus less than 2 hours. The high-exposure group demonstrated significantly greater rates of screen addiction, attentional difficulties, and sedentary behaviour. These findings underscore the need to limit screen exposure in developing children.

Screen addiction also exacerbates other adverse effects associated with excessive screen use, as individuals with addictive tendencies spend more time in front of screens, par-

ticularly in the evening. This was confirmed in a large-scale Norwegian survey involving 49,051 university students. The study found that participants exhibiting signs of screen addiction were more likely to engage with digital devices in the evening and experienced greater disruptions in both the quantity and quality of sleep compared to non-addicted individuals. Notably, the severity of these disruptions was directly proportional to the degree of addiction. The findings suggest that screen addiction negatively affects psychophysical health, with evening use being especially detrimental to sleep regulation, more so than excessive daytime use. Digital screen use exerts multifaceted adverse effects, and addiction further amplifies its impact on health [43].

Screen addiction is a complex process underpinned by neurobiological mechanisms, particularly those involving dopaminergic signalling. This issue is increasingly prevalent among children and adolescents and is associated with sleep disturbances, attentional deficits, and the adoption of sedentary behaviours. Evening exposure to screens further exacerbates these adverse outcomes. Given the growing prevalence of this phenomenon, the implementation of preventative and educational interventions is critical to mitigating the risk of addiction and its long-term consequences for mental health and the functioning of the nervous system.

Minimising the negative effects of screen use

In light of the growing body of evidence documenting the negative impact of digital screen use on human health, preventive measures and mitigation strategies have become increasingly important. While complete avoidance of screen-based devices is unrealistic in modern society, adopting healthy digital habits is both feasible and strongly recommended. Such practices can help reduce the risk of visual disturbances, sleep disorders, mental fatigue, and behavioural addiction.

This subsection presents evidence-based, practical approaches to minimising the adverse outcomes associated with prolonged screen exposure. These include digital detox protocols, the use of blue light filters, making proper ergonomic adjustments in the workspace, and incorporating regular breaks during screen use. The implementation of these strategies aims to support physical and mental well-being in a digitally saturated environment.

20–20–20 rule

One of the simplest and most effective strategies for mitigating the adverse visual effects of screen exposure is the 20–20–20 rule (see **Figure 1**). Initially proposed by Anshel [44] in the late 1990s, the rule recommends that every 20 min-

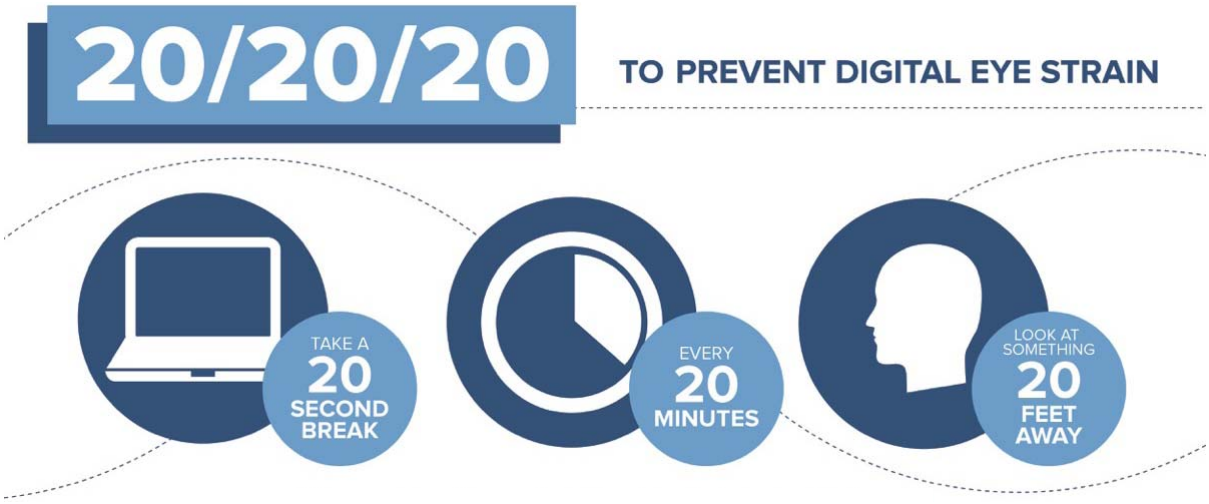


Figure 1. The 20–20–20 rule, as recommended by the American Optometric Association, is to reduce digital eye strain. Source: American Optometric Association, www.aoa.org

utes of screen use be followed by a 20-second break, during which the user focuses on an object at least 20 feet (approximately 6 metres) away [44,45]. This method has gained widespread recognition among ophthalmologists and occupational health professionals as an effective preventive tool for CVS.

The efficacy of the 20–20–20 rule has been investigated in multiple studies. For example, Talens-Estarelles et al. [46] conducted a study in which participants (n = 29) used a software application that provided reminders to take breaks according to the 20–20–20 guideline. Results demonstrated a reduction in symptoms of CVS and DES during the intervention period. Notably, these symptoms returned within two weeks after discontinuation of the method, suggesting a direct link between adherence and symptom control [46].

In another study, researchers divided participants diagnosed with CVS into two groups: one was instructed to follow the 20–20–20 rule, while the control group received no such intervention. The experimental group showed significant improvement in both subjective symptoms and tear film quality, further supporting the clinical relevance of this strategy [47].

Regular implementation of the 20–20–20 rule helps alleviate ocular muscle tension, prevent corneal dryness, and support the eye's natural accommodative mechanisms, thereby reducing the risk of long-term visual impairment associated with prolonged digital device use.

Blue light filters and night modes

Modern digital screens emit a considerable amount of blue light (wavelengths between 400–490 nm), which has been shown to disrupt circadian rhythms, impair sleep quality, and negatively affect visual comfort, particularly during prolonged screen use in the evening hours. In response to health concerns related to blue light exposure, a growing number of mitigation strategies have been introduced. These include both physical blue light filters, such as specially coated lenses, and digital solutions like Night mode available on most electronic devices. However, the actual effectiveness of these interventions remains under scrutiny.

A systematic review by Singh et al. [48] investigated the efficacy of blue light-filtering lenses. The results raised questions about their clinical relevance. Specifically, no significant differences were found in the reduction of CVS symptoms when compared to standard lenses. Similarly, the impact on sleep quality was inconclusive – approximately half of the included studies reported subjective improvement, while the other half found no statistically significant effects [48].

The use of night-mode features on digital devices has also yielded mixed findings. Two separate studies involving smartphone and tablet users demonstrated that activating night mode alone, without concurrently reducing screen brightness, had no measurable effect compared to everyday device use. Only when screen brightness was decreased in conjunction with night mode did researchers observe a positive impact on endogenous melatonin production [49, 50].

Currently, there is insufficient evidence to support the widespread assumption that blue light-filtering lenses or built-in night-mode features provide significant benefits in terms of reducing eye strain, improving visual acuity, enhancing sleep quality, or optimising other visual parameters. Further high-quality, controlled studies are needed to determine the actual effectiveness of these technologies.

Ergonomic practices for digital screen use

The ergonomics of digital screen use play a critical role during prolonged interaction with electronic devices. A well-designed workstation can significantly reduce the risk of discomfort and disorders related to visual strain and musculoskeletal overload. In Poland, the first regulations concerning occupational safety and health for screen-based workstations were introduced in 1998, and in 2023, these regulations were updated to reflect the current realities of computer-based work environments [51,52].

Key ergonomic recommendations include using appropriate ambient lighting, positioning the monitor at eye level and a comfortable viewing distance, and allowing for adjustable screen tilt angles. Equally important is maintaining a proper seated posture, supported by an adjustable ergonomic chair, along with a workstation layout that allows for natural arm positioning while using a keyboard and mouse. For individuals using a laptop for extended periods, the addition of an external monitor, keyboard, and mouse becomes essential to maintain ergonomic integrity.

Another crucial element of preventing visual fatigue and physical strain is the incorporation of regular work breaks. According to occupational health guidelines, at least a five-minute break should be taken after each hour of computer use. Additionally, implementing the 20–20–20 rule offers a practical and time-efficient strategy for preserving visual health.

Adherence to ergonomic principles not only minimises the adverse effects of prolonged screen exposure but also promotes overall well-being and supports both physical and mental health in screen-intensive environments.

Reducing exposure to digital devices

While numerous strategies exist to mitigate the adverse effects of digital screen use, the most effective, yet arguably the most challenging to implement, remains a reduction in overall screen exposure. Although the modern lifestyle makes complete avoidance of screen-based technologies unrealistic, even partial reductions, particularly during critical times of day, can yield measurable benefits for health and well-being.

One practical example involves minimising digital device use during evening hours. A study conducted at the University of Oxford demonstrated that restricting screen use among adolescents after 9:00 p.m. led to earlier sleep onset and increased total sleep duration, which in turn improved alertness and daily functioning [53]. Similarly, a study involving adult participants found that avoiding screen use for 30 minutes before bedtime resulted in shorter sleep latency and longer and higher-quality sleep, as well as improved mood and working memory performance compared to a control group [54].

Another promising approach is the practice of digital detox, defined as intentional and scheduled breaks from the use of electronic devices and digital media [55]. Studies have shown that such digital abstinence can reduce symptoms of anxiety and depression [56], as well as lower levels of digital media dependency [57].

Although total avoidance of digital screens is virtually unattainable in today's world, even modest reductions in screen time, especially before bedtime, and periodic disconnection from electronic devices may significantly enhance sleep quality, psychological well-being, and overall cognitive functioning.

Limitations

Despite the growing body of research on the effects of digital screen exposure on visual and neurological health, key limitations persist. Most studies are observational or cross-sectional, limiting causal inference. While associations with dry eye disease, myopia, or sleep disturbances are well-documented, causation remains speculative, and potential confounders—such as genetics, outdoor activity, or lifestyle—are often inadequately controlled.

Existing research also focuses mainly on short- to medium-term effects, with a notable lack of longitudinal studies assessing the lasting impact of chronic screen exposure on ocular health and neurodevelopment.

Moreover, screen use is frequently measured by total daily time, neglecting qualitative factors like content type, interaction mode, or context, all of which may influence health outcomes.

Generalisability is further limited by narrow study populations, often confined to specific regions or age groups. Older adults, patients with comorbidities, and diverse cultural contexts remain under-represented.

Addressing these gaps through robust, prospective studies will be crucial in informing evidence-based guidelines. Notably, the potential link between prolonged screen exposure and neurodegeneration remains an open area for future research.

Conclusions

The ongoing digitalisation of daily life, education, and professional activity has rendered exposure to electronic screens virtually unavoidable. While technology offers numerous benefits, its excessive and unregulated use is increasingly associated with significant health risks. This in-depth review of the scientific literature suggests that prolonged screen exposure has adverse effects on both the visual and nervous systems.

From an ophthalmological perspective, Dry Eye Disease, Computer Vision Syndrome, and progressive myopia remain the most significant screen-associated disorders. Evidence confirms that even short-term daily screen use may disrupt tear film stability, strain accommodative mecha-

nisms, and increase the risk of refractive changes – particularly in children and adolescents.

Regarding the nervous system, prolonged exposure to screen-emitted blue light impairs circadian rhythms and sleep quality. Furthermore, continuous sensory and cognitive stimulation from digital content can contribute to mental fatigue and attention deficits and may also influence brain activity patterns. These effects appear particularly pronounced among younger populations. The growing phenomenon of screen addiction further exacerbates these risks, potentially leading to emotional dysregulation and behavioural disturbances.

Although complete avoidance of digital devices is neither realistic nor necessary, it is both possible and imperative to implement preventive strategies aimed at mitigating these adverse health effects. This review highlights practical, evidence-based measures, including ergonomic interventions, visual hygiene practices such as the 20–20–20 rule, limiting screen time before bedtime, and promoting digital detox habits.

Given the interdisciplinary impact of digital screen exposure, healthcare professionals, including ophthalmologists, neurologists, psychiatrists, and primary care physicians, should be aware of its multifaceted health implications. Incorporating preventive counselling on screen time management and digital hygiene into routine clinical practice may offer tangible benefits for patient well-being.

Future research should explore how different types of screen content and modes of interaction – such as passive viewing versus active engagement – affect visual and neurological health. Equally important is the need for longitudinal studies evaluating the long-term impact of chronic screen exposure across various populations and age groups. Such evidence is crucial for guiding public health strategies and informing evidence-based policy in an increasingly digital world.

Glossary

Accommodation – The eye's ability to adjust focus between near and distant objects.
Accommodative spasm – A temporary focusing problem after prolonged near work.
Blue light – High-energy light from screens that can interfere with sleep and cause eye strain.
Blue light filter – A screen setting or lens coating that reduces blue light exposure, especially in the evening.
Circadian rhythm – The body's internal 24-hour cycle that regulates sleep, alertness, and hormone release.
Digital burnout – a state of mental and physical exhaustion resulting from prolonged screen use and constant connectivity.
Digital detox – A planned break from digital devices to reduce stress and improve well-being.
Dopamine – A brain chemical involved in motivation and reward, linked to habit formation and screen addiction.
Electroencephalography (EEG) – A method for recording brain activity using electrodes on the scalp.
Ergonomics – The science of designing comfortable and health-supportive work environments, especially during screen use.
Functional MRI (fMRI) – A brain scan that detects active areas based on blood flow during tasks or rest.
Melatonin – A hormone that promotes sleep, often suppressed by evening exposure to screen-emitted blue light.
Myopia – A vision condition (nearsightedness) where distant objects appear blurry.
Photophobia – Sensitivity or discomfort when exposed to bright light.
Reward system – A group of brain structures that regulate pleasure, motivation, and reinforcement behaviour.
Screen addiction – Excessive and compulsive screen use that negatively affects health and daily functioning.
Sensory overload – Feeling overwhelmed by too much visual, auditory, or informational input.
Zoom fatigue – Tiredness and concentration difficulties caused by prolonged video conferencing.

Disclosures

Author contribution statement
All authors contributed equally to every aspect of the development of this research paper, including conceptualisation, investigation, project administration, visualisation, writing – original draft, and writing – review & editing. All authors have read and agreed with the published version of the manuscript.

Funding statement
This research did not receive any external funding.

Institutional review board statement
Ethical review and approval were waived due to the review nature of this study.

Informed consent statement: not applicable.

Data availability statement: not applicable.

Acknowledgements
The authors do not have anyone to acknowledge.

Conflict of interest statement
The authors declare that they have no conflicts of interest.

Declaration of the use of generative AI and AI-assisted technologies
In the preparation of this work, the authors used OpenAI for the purpose of improving language and readability, text formatting, and verification. After using this tool/service, the authors have reviewed and edited the content as needed and accept full responsibility for the substantive content of the publication.

References

1. Bai H. Role of digital technology in transforming organizational competencies influencing green economy: moderating role of product knowledge hiding. Front Psychol. 2021 Dec;12:792550. https://doi.org/10.3389/fpsyg.2021.792550.
2. Haleem A, Javaid M, Qadri MA, Suman R. Understanding the role of digital technologies in education: a review. Sustain Oper Comput. 2022;3:275-85. https://doi.org/10.1016/j.susoc.2022.05.004.
3. Mora-Monteros M, Suris JC, Chok L, Siwiak A, Stadelmann S, Barrense-Dias Y. Evolution of screen use among youth between 2012 and 2020 in Switzerland. Arch Pediatr. 2023 Nov;30(8):563-6. https://doi.org/10.1016/j.arcped.2023.09.001.
4. Ratan ZA, Zaman SB, Islam SMS, Hosseinzadeh H. Smartphone overuse: a hidden crisis in COVID-19. Health Policy Technol. 2021 Mar;10(1):21-2. https://doi.org/10.1016/j.hlpt.2021.01.002.
5. Small GW, Lee J, Kaufman A, Jalil J, Siddarth P, Gaddipati H, et al. Brain health consequences of digital technology use. Dialogues Clin Neurosci. 2020 Jun;22(2):179-87. https://doi.org/10.31887/DCNS.2020.22.2/gsmall.
6. Konrad K, Firk C, Uhlhaas PJ. Brain development during adolescence: neuroscientific insights into this developmental period. Dtsch Arztebl Int. 2013 Jun;110(25):425-31. https://doi.org/10.3238/arztebl.2013.0425.
7. Golden MI, Meyer JJ, Zeppieri M, et al. Dry eye syndrome. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan–. Updated 2024 Feb 29. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470411/
8. Stapleton F, Alves M, Bunya VY, Jalbert I, Lekhanont K, Malet F, et al. TFOS DEWS II epidemiology report. Ocul Surf. 2017 Jul;15(3):334-65. https://doi.org/10.1016/j.jtos.2017.05.003.
9. Talens-Estarelles C, Garcia-Marques JV, Cerviño A, Garcia-Lazaro S. Dry eye-related risk factors for digital eye strain. Eye Contact Lens. 2022 Oct;48(10):410-5. https://doi.org/10.1097/ICL.0000000000000923.
10. Bron AJ, de Paiva CS, Chauhan SK, Bonini S, Gabison EE, Jain S, et al. TFOS DEWS II pathophysiology report. Ocul Surf. 2017 Jul;15(3):438-510. https://doi.org/10.1016/j.jtos.2017.05.011.
11. Fjaervoll H, Fjaervoll K, Magno M, Moschowits E, Vehof J, Dartt DA, et al. The association between visual display terminal use and dry eye: a review. Acta Ophthalmol. 2022 Jun;100(4):357-75. https://doi.org/10.1111/aos.15049.
12. Jadeja JN, Shroff KV, Shah A, Pandey A, Dubey S. Association of digital device usage and dry eye disease in school children. Indian J Ophthalmol. 2024 Jul;72(7):1031-6. https://doi.org/10.4103/IJO.IJO_703_23.
13. Rosenfield M. Computer vision syndrome: a review of ocular causes and potential treatments. Ophthalmic Physiol Opt. 2011 Sep;31(5):502-15. https://doi.org/10.1111/j.1475-1313.2011.00834.x.
14. Gowrisankaran S, Sheedy JE. Computer vision syndrome: a review. Work. 2015;52(2):303-14. https://doi.org/10.3233/WOR-152162.
15. Anbesu EW, Lema AK. Prevalence of computer vision syndrome: a systematic review and meta-analysis. Sci Rep. 2023 Jan;13(1):1801. https://doi.org/10.1038/s41598-023-28750-6.
16. Alamri A, Amer KA, Aldosari AA, Althubait BMS, Alqahtani MS, Al Mudawi AAM, et al. Computer vision syndrome: symptoms, risk factors, and practices. J Family Med Prim Care. 2022 Sep;11(9):5110-5. https://doi.org/10.4103/jfmpc.jfmpc_1627_21.
17. Abudawood GA, Ashi HM, Almarzouki NK. Computer vision syndrome among undergraduate medical students in King Abdulaziz University, Jeddah, Saudi Arabia. J Ophthalmol. 2020 Apr;2020:2789376. https://doi.org/10.1155/2020/2789376.
18. Baird PN, Saw SM, Lanca C, Guggenheim JA, Smith EL 3rd, Zhou X, et al. Myopia. Nat Rev Dis Primers. 2020 Dec;6(1):99. https://doi.org/10.1038/s41572-020-00231-4.
19. Lindberg L. [Spasm of accommodation]. Duodecim. 2014;130(2):168-73.
20. Ha A, Lee YJ, Lee M, Shim SR, Kim YK. Digital screen time and myopia: a systematic review and dose-response meta-analysis. JAMA Netw Open. 2025 Feb;8(2):e2460026. https://doi.org/10.1001/jamanetworkopen.2024.60026.
21. Zong Z, Zhang Y, Qiao J, Tian Y, Xu S. The association between screen time exposure and myopia in children and adolescents: a meta-analysis. BMC Public Health. 2024 Jun;24(1):1625. https://doi.org/10.1186/s12889-024-19113-5.
22. AlShamlan FT, Bubshait LK, AlAhmad EA, AlOtaibi BS, AlShakhs AA, AlHammad FA. Myopia progression in school children with prolonged screen time during the coronavirus disease confinement. Med Hypothesis Discov Innov Ophthalmol. 2023 Dec;12(2):90-7. https://doi.org/10.51329/mehdiophthal1474.

23. Savage RA, Zafar N, Yohannan S, Miller JMM. Melatonin. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan–. Updated 2024 Feb 9. PMID:30521244.
24. Claustrat B, Leston J. Melatonin: physiological effects in humans. Neurochirurgie. 2015 Apr-Jun;61(2-3):77-84. https://doi.org/10.1016/j.neuchi.2015.03.002.
25. AlShareef SM. The impact of bedtime technology use on sleep quality and excessive daytime sleepiness in adults. Sleep Sci. 2022 Apr-Jun;15(Spec 2):318-27. https://doi.org/10.5935/1984-0063.20200128.
26. West KE, Jablonski MR, Warfield B, Cecil KS, James M, Ayers MA, et al. Blue light from light-emitting diodes elicits a dose-dependent suppression of melatonin in humans. J Appl Physiol (1985). 2011 Mar;110(3):619-26. https://doi.org/10.1152/japplphysiol.01413.2009.
27. Sroykham W, Wongsawat Y. Effects of LED-backlit computer screen and emotional self-regulation on human melatonin production. Conf Proc IEEE Eng Med Biol Soc. 2013;2013:1704-7. https://doi.org/10.1109/EMBC.2013.6609847.
28. Heo JY, Kim K, Fava M, Mischoulon D, Papakostas GI, Kim MJ, et al. Effects of smartphone use with and without blue light at night in healthy adults: a randomized, double-blind, cross-over, placebo-controlled comparison. J Psychiatr Res. 2017 Apr;87:61-70. https://doi.org/10.1016/j.jpsychires.2016.12.010.
29. Chinoy ED, Duffy JF, Czeisler CA. Unrestricted evening use of light-emitting tablet computers delays self-selected bedtime and disrupts circadian timing and alertness. Physiol Rep. 2018 May;6(10):e13692. https://doi.org/10.14814/phy2.13692.
30. Li K, Jiang S, Yan X, Li J. Mechanism study of social media overload on health self-efficacy and anxiety. Heliyon. 2023 Dec;10(1):e23326. https://doi.org/10.1016/j.heliyon.2023.e23326.
31. Savić D. Information fatigue syndrome and digital burnout. Grey J. 2023;19:95-101.
32. Huff C. Media overload is hurting our mental health. Here are ways to manage headline stress. Monit Psychol. 2022;53. Available from: https://www.apa.org/monitor/2022/11/strain-media-overload
33. Anh LET, Eoin W, Umair A. "You're still on mute". A study of video conferencing fatigue during the COVID-19 pandemic from a technostress perspective. Behav Inf Technol. 2023;42(11):1758-72. https://doi.org/10.1080/0144929X.2022.2095304.
34. Nagata JM, Al-Shoaibi AAA, Leong AW, Zamora G, Testa A, Ganson KT, et al. Screen time and mental health: a prospective analysis of the Adolescent Brain Cognitive Development (ABCD) study. BMC Public Health. 2024 Oct;24(1):2686. https://doi.org/10.1186/s12889-024-20102-x.
35. Law EC, Han MX, Lai Z, Lim S, Ong ZY, Ng V, et al. Associations between infant screen use, electroencephalography markers, and cognitive outcomes. JAMA Pediatr. 2023 Mar;177(3):311-8. https://doi.org/10.1001/jamapediatrics.2022.5674.
36. Lewin KM, Meshi D, Aladé F, Lescht E, Herring C, Devaraju DS, et al. Children's screen time is associated with reduced brain activation during an inhibitory control task: a pilot EEG study. Front Cogn. 2023 Jan;2:1018096. https://doi.org/10.3389/fcogn.2023.1018096.
37. Liu D, Liu X, Long Y, Xiang Z, Wu Z, Liu Z, et al. Problematic smartphone use is associated with differences in static and dynamic brain functional connectivity in young adults. Front Neurosci. 2022 Oct;16:1010488. https://doi.org/10.3389/fnins.2022.1010488.
38. Aragay N, Valles V, Ramos-Grille I, Garrido G, Grimalt EG, Miranda Ruiz E, et al. Differences in screen addiction in the past 15 years. Int J Environ Res Public Health. 2023 Dex;21(1):1. https://doi.org/10.3390/ijerph21010001.
39. Klein MO, Battagello DS, Cardoso AR, Hauser DN, Bittencourt JC, Correa RG. Dopamine: functions, signaling, and association with neurological diseases. Cell Mol Neurobiol. 2019 Jan;39(1):31-59. https://doi.org/10.1007/s10571-018-0632-3.
40. American Academy of Pediatrics, Committee on Public Education. Children, adolescents, and television. Pediatrics. 2001 Feb;107(2):423-6. https://doi.org/10.1542/peds.107.2.423.
41. World Health Organization. Guidelines on physical activity, sedentary behaviour and sleep for children under 5 years of age [Internet]. Geneva: World Health Organization; 2019. Available from: https://iris.who.int/handle/10665/311664.
42. Tekeci Y, Torpil B, Altuntas O. The impact of screen exposure on screen addiction and sensory processing in typically developing children aged 6-10 years. Children (Basel). 2024 Apr;11(4):464. https://doi.org/10.3390/children11040464.
43. Hjetland GJ, Skogen JC, Hysing M, Sivertsen B. The association between self-reported screen time, social media addiction, and sleep among Norwegian university students. Front Public Health. 2021 Dec;9:794307. https://doi.org/10.3389/fpubh.2021.794307.
44. Anshel JR. Visual ergonomics in the workplace. AAOHN J. 2007 Oct;55(10):414-20; quiz 421-2. https://doi.org/10.1177/216507990705501004.
45. Anshel J. Letter to the editor: 20-20-20 rule: are these numbers justified? Optom Vis Sci. 2023 Apr;100(4):296. https://doi.org/10.1097/OPX.0000000000002006.
46. Talens-Estarelles C, Cerviño A, Garcia-Lazaro S, Fogelton A, Sheppard A, Wolffsohn JS. The effects of breaks on digital eye strain, dry eye and binocular vision: testing the 20-20-20 rule. Cont Lens Anterior Eye. 2023 Apr;46(2):101744. https://doi.org/10.1016/j.clae.2022.101744.
47. Alrasheed SH, Alghamdi WM. Impact of an educational intervention using the 20/20/20 rule on computer vision syndrome. Afr Vis Eye Health. 2020 Sep;79(1):1-6. https://doi.org/10.4102/aveh.v79i1.554.
48. Singh S, Keller PR, Busija L, McMillan P, Makrai E, Lawrenson JG, et al. Blue-light filtering spectacle lenses for visual performance, sleep, and macular health in adults. Cochrane Database Syst Rev. 2023 Aug;8:CD013244. https://doi.org/10.1002/14651858.CD013244.pub2.
49. Duraccio KM, Zaugg KK, Blackburn RC, Jensen CD. Does iPhone night shift mitigate negative effects

of smartphone use on sleep outcomes in emerging adults? Sleep Health. 2021 Aug;7(4):478-84. https://doi.org/10.1016/j.sleh.2021.03.005.
50. Nagare R, Plitnick B, Figueiro MG. Does the iPad night shift mode reduce melatonin suppression? Light Res Technol. 2019 May;51(3):373-83. https://doi.org/10.1177/1477153517748189.
51. Ministerstwo Pracy i Polityki Społecznej. Rozporządzenie Ministra Pracy i Polityki Społecznej z dnia 1 grudnia 1998 r. w sprawie bezpieczeństwa i higieny pracy na stanowiskach wyposażonych w monitory ekranowe. Dz U. 1998;148. Available from: https://isap.sejm.gov.pl/isap.nsf/DocDetails.xsp?id=wdu19981480973.
52. Ministerstwo Rodziny i Polityki Społecznej. Rozporządzenie Ministra Rodziny i Polityki Społecznej z dnia 18 października 2023 r. zmieniające rozporządzenie w sprawie bezpieczeństwa i higieny pracy na stanowiskach wyposażonych w monitory ekranowe. Dz U. 2023;2367. Available from: https://dziennikustaw.gov.pl/DU/2023/2367.
53. Perrault AA, Bayer L, Peuvrier M, Afyouni A, Ghisletta P, Brockmann C, et al. Reducing the use of screen electronic devices in the evening is associated with improved sleep and daytime vigilance in adolescents. Sleep. 2019 Sep;42(9):zsz125. https://doi.org/10.1093/sleep/zsz125.
54. He JW, Tu ZH, Xiao L, Su T, Tang YX. Effect of restricting bedtime mobile phone use on sleep, arousal, mood, and working memory: a randomized pilot trial. PLoS One. 2020 Feb;15(2):e0228756. https://doi.org/10.1371/journal.pone.0228756.
55. Syvertsen T, Enli G. Digital detox: media resistance and the promise of authenticity. Convergence. 2020;26(5-6):1269-83. https://doi.org/10.1177/1354856519847325.
56. Alanzi TM, Arif W, Aqeeli R, Alnafisi A, Qumosani T, Alreshidi A, et al. Examining the impact of digital detox interventions on anxiety and depression levels among young adults. Cureus. 2024 Dec;16(12):e75625. https://doi.org/10.7759/cureus.75625.
57. Mohamed SM, Abdallah LS, Ali FNK. Effect of digital detox program on electronic screen syndrome among preparatory school students. Nurs Open. 2023 Apr;10(4):2222-8. https://doi.org/10.1002/nop2.1472.

REVIEW PAPER

JMS Journal of Medical Science

After 20 Years on the Market: The Current Outlook on E-Cigarette Use Among Youth

Katarzyna Drewnowska
National Medical Institute of the Ministry of the Interior and Administration, Warsaw, Poland
https://orcid.org/0009-0007-9388-3533
Corresponding author: kat.dre.99@gmail.com

Jakub Modrzewski
National Medical Institute of the Ministry of the Interior and Administration, Warsaw, Poland
https://orcid.org/0009-0003-0208-2046

Julia Radziszewska
National Medical Institute of the Ministry of the Interior and Administration, Warsaw, Poland
https://orcid.org/0009-0005-5112-0381

Agata Drozd
Medical University of Gdańsk, Poland
https://orcid.org/0009-0004-7083-3718

Agata Kościelniak
Independent Public Healthcare Center in Pruszków, Poland
https://orcid.org/0009-0005-8088-4452

Klaudia Danielewicz
The District Medical Center in Grójec, Poland
https://orcid.org/0009-0006-6767-3906

Agata Rypińska
National Medical Institute of the Ministry of the Interior and Administration, Warsaw, Poland
https://orcid.org/0009-0000-6117-7676

Zofia Kupczyk
Independent Public Healthcare Institution in Mińsk Mazowiecki, Poland
https://orcid.org/0009-0006-7945-8605

Julia Kuźmiuk
Independent Public Healthcare Institution in Mińsk Mazowiecki, Poland
https://orcid.org/0009-0008-7474-4666

Klaudia Piskorowska
5th Military Hospital with Polyclinic in Cracow, Poland
https://orcid.org/0009-0000-9134-0745

Łukasz Kreft
Military Institute of Medicine – National Research Institute, Warsaw, Poland
https://orcid.org/0009-0006-9865-6906

https://doi.org/10.20883/medical.e1306

Keywords: electronic cigarettes, vaping, electronic nicotine delivery systems, e-liquids, adolescents, addiction

Received 2025-06-05
Accepted 2025-07-22
Published 2025-09-26

How to Cite: Drewnowska K, Modrzewski J, Radziszewska J, Drozd A, Kościelniak A, Danielewicz K, Rypińska A, Kupczyk Z, Kuźmiuk J, Piskorowska K, Kreft Ł. After 20 Years on the Market: The Current Outlook on E-Cigarette Use Among Youth. Journal of Medical Science. 2025 September;94(3);e1306. https://doi.org/10.20883/medical.e1306.

ABSTRACT

Introduction. Electronic nicotine dispensing systems, commonly known as e-cigarettes, remain the most widely used tobacco product among the youth, with current worldwide use estimated at approximately 5 to 7%. Nevertheless, several aspects of knowledge on their sustained consumption are based on suppositions, such as their role as a "gateway" into cigarette smoking for new generations and the potential subsequent renormalisation of tobacco use. Moreover, recent clinical trials and toxicological assessments have yielded noteworthy findings, revealing their potential to cause tissue damage in the lungs, heart, and oral cavity.

Material and methods. PubMed searches yielded 253 studies on e-cigarette use among youth, of which 78 met inclusion criteria (original data, published between 2000 and July 1, 2025). Keywords covered "electronic cigarette", "e-cigarette", "ENDS", "electronic nicotine delivery system", "electronic nicotine delivery device", and "EVALI".
Results. This narrative review offers a synthesis of the current state of knowledge on e-cigarette consumption patterns and their influencing factors, the public health implications of sustained use, and recent policy developments, along with their presumed effectiveness.
Conclusions. We aim to inform clinicians and youth caregivers about the high prevalence of e-cigarette use among adolescents and present clinically relevant information derived from the latest empirical evidence.

Introduction

Electronic cigarettes continue to be used by millions around the world, particularly among the younger population. A total of 1.63 million (5.9%) American students currently use e-cigarettes, including 410,000 middle schoolers and 1.21 million high schoolers [1]. Since e-cigarettes are combustion-free, there is a widespread assumption that their use is much safer than that of conventional cigarettes [2]. Similar messages are conveyed in e-cigarette advertisements, often featuring celebrities, which are widely present throughout social media to reach their audience most effectively. Along with the ability to bypass smoke-free laws by enabling users to "smoke anywhere" [3], e-cigarettes have become a step toward the normalisation of smoking behaviour. All of this occurs in the context of the widespread and continued availability of conventional cigarettes and other tobacco products, with high levels of dual use [4]. Despite evidence that the toxins present in e-cigarette aerosol can compromise both heart and lung function [5], long-term health risks associated with their use are not yet fully established.

This paper provides a contemporary overview of e-cigarette use patterns among adolescents, critically examines the current evidence regarding their health impacts, and discusses prospective public health implications associated with their sustained use.

Material and methods

Initial searches conducted via PubMed yielded 253 studies. Keywords included "electronic cigarette", "e-cigarette", "ENDS", "electronic nicotine delivery system", "electronic nicotine delivery device", and "EVALI." Articles or abstracts presenting original data on any topic relevant to e-cigarette use among youth, published between 2000 and July 1, 2025, were included. Articles that were not relevant, not in English, or were reviews or commentaries without original data were excluded; however, some were cited for background and context. We also reviewed technical reports prepared by health organisations, news articles, and relevant websites. The final reference list was determined based on relevance to the main topic, resulting in a total of 78 articles forming the basis for this review. Given the scope and heterogeneity of the available literature, a narrative review format was chosen to allow for a more flexible and interpretive synthesis.

E-Cigarette use and device characteristics

Electronic nicotine dispensing systems (ENDS), commonly known as electronic cigarettes or e-cigarettes, were invented in their current form by Chinese pharmacist Hon Lik in the early 2000s [6]. They first appeared on the market over a decade ago, becoming widely available around 2013. In 2024, e-cigarettes were the most commonly used tobacco product among middle and high school students in the United States. A total of 1.63 million (5.9%) students currently use e-cigarettes, with 26.3% of them using an e-cigarette every day [1]. The current use rate was higher among females than among males. Prevalence has mostly remained stable in recent years, following a peak in 2019 when it exceeded 20% [7].

Device evolution

The evolution of ENDS spans four generations. First-generation devices, known as "cigalikes,"

are designed for single use. Second-generation e-cigarettes feature rechargeable batteries and replaceable pre-filled pods, which may contain traditional e-liquid or tetrahydrocannabinol (THC) oil. Third-generation devices, such as tanks or mods, are larger, more customizable, and capable of producing higher aerosol volumes. "Sub-ohm" models, featuring low-resistance coils, provide greater nicotine delivery through increased heat and vapour production. Fourth-generation e-cigarettes use nicotine salts, enabling smoother inhalation of high nicotine concentrations with fewer side effects; compatible pods may also contain cannabidiol.

Consumer preference

Several studies have examined consumer preferences for e-cigarette attributes. Starting with device type, a pooled analysis by Barrington-Trimis et al. [8] involving 2,166 adolescent and young adult e-cigarette users found that fewer than 15% primarily used disposable/cigalike devices in the past month. In contrast, 77% reported using later-generation devices. More recent data from an online survey of 636 Australian users aged 12 and older showed that 82% used nicotine-containing e-liquids, 60% used non-nicotine variants, and disposable e-cigarettes were most common among those under 25 [9].

Findings show that flavour is the primary reason adolescents try e-cigarettes [9]. In a U.S. national survey of 2,253 individuals aged 14–20, 92% of past 30-day e-cigarette users reported using at least one non-tobacco flavour – most commonly sweet (76%) or menthol/fruit-ice (70%) [10]. Latent class analysis identified four flavour preference groups: mint, no preference, fruit/sweet, and flavour aversion. Compared to the no preference group, those favouring fruit/sweet or mint flavours were more likely to have used e-cigarettes ≥50 times. Notably, fruit/sweet preference was negatively associated with combustible tobacco use.

Marketing and promotion of e-cigarettes

E-cigarettes entered the U.S. market around 2006–2007, and since then, the promotion and distribution channels for these products have undergone significant evolution. Marketing expenditures can be traced back to 2008 for approximately 130 e-cigarette brands [11]. The minimal spending through 2010 was followed by an acceleration from $12 million in 2011 to $125 million in 2014. The trajectory for spending was consistent with the pattern for product sales.

It is noteworthy that the product class took hold when e-commerce was rapidly expanding in the United States, and major social media platforms – such as Facebook (founded in 2004), YouTube (2005), and Twitter (2006) – were emerging [12]. In this environment, information about new products like e-cigarettes can spread rapidly across regions, facilitating swift adoption. To assess the magnitude of this effect, Peak et al. [13] conducted a content analysis of 365 e-cigarette–related videos on YouTube, published between June 2007 and June 2011. They estimated that more than 1.2 million youth and approximately 15.5 million individuals worldwide were exposed to these clips. Only 16% of the videos were formal advertisements or news segments, while 79.2% were coded as user-generated content. E-cigarette companies or their affiliates sponsored the majority (85.2%) of the videos.

As of July 2022, the Institute for Global Tobacco Control identified 109 countries or jurisdictions that regulate or ban ENDS, counting 31 distinct e-cigarette regulatory policy approaches [14]. However, the effectiveness of these regulatory measures remains a subject of investigation. The most recent cross-sectional study on this topic analysed data from 165,299 respondents across 48 countries, with a mean participant age of 14, following the 2016/2018 World Health Organisation Framework Convention on Tobacco Control implementation reports. Approximately one in ten respondents reported current e-cigarette use [15]. Only internet tobacco advertising, promotion, and sponsorship (TAPS) bans were found to be effective across all countries. Additionally, in lower-middle-income and low-income countries, bans on displaying tobacco products at the point of sale, bans on product placement, and the strength of additional TAPS measures were associated with a lower prevalence of e-cigarette use among students, as well as being taught about the dangers of tobacco use in school. Surprisingly, no significant differences in e-cigarette

use were observed across TAPS policy types in high-income countries.

Some practitioners argue that the limited effectiveness of e-cigarette regulatory measures may stem from significant delays in their implementation, which allowed marketing materials to spread widely across jurisdictions and capitalise on the most profitable strategies for reaching potential consumers. A primary example is the case of the United States Food and Drug Administration (FDA), which repeatedly postponed the enforcement of its first major regulatory policy – the premarket tobacco product application – until August 2022. It was only after a lawsuit was filed against the FDA by various public health advocacy groups, citing the delays surrounding e-cigarette reviews, that the deadline was ultimately moved forward to May 2020. Despite this adjustment, the U.S. e-cigarette market remained largely unregulated in terms of distribution and marketing for approximately 14 years.

The ensuing backlash accompanying these events was directed primarily at the company JUUL, which was widely criticised for its central role in the youth e-cigarette epidemic. Considerable attention was focused on cartridge-based e-cigarettes with fruity and sweet flavours – products that significantly contributed to JUUL's sales growth and were found to be particularly appealing to youth – ultimately leading to pressure on the company to withdraw them from the market [16]. In light of these concerns, in April 2020, the FDA issued a final enforcement guidance with the intention to implement stricter controls over the marketing of ENDS flavoured products. By September 2021, the FDA had issued approximately 946,000 Marketing Denial Orders, and as of January 2024, not a single flavoured ENDS manufacturer had received authorisation to market their product [17].

Studies aimed to illustrate the impact of flavoured e-cigarette regulations report mixed findings. A cross-sectional study conducted by Ali et al. [18] found that statewide restrictions on non-tobacco-flavoured e-cigarette sales were associated with a 25.01% to 31.26% reduction in total e-cigarette unit sales compared to states without such restrictions. Conversely, other research has identified a marginally significant shift of 0.7 to 1.9 percentage points from e-cigarette use to combustible cigarette smoking, particularly among individuals aged 18 to 20 [19]. Additional studies indicate that users often circumvent flavour bans by obtaining products through in-state stores (45.1%), out-of-state retailers (31.2%), online sources (25.5%), or informal channels [20]. Moreover, some users transitioned from restricted cartridge-based products to flavoured disposable e-cigarettes, the sales of which surged from 29.9% to 49.6% [21].

Neurobiological mechanisms of dependence

The National Institute on Drug Abuse reports that tobacco use is primarily established during adolescence, with nearly 90% of adult smokers starting before the age of 18 [22]. Youth are particularly vulnerable to nicotine addiction, with even infrequent use significantly increasing the risk of dependence. Adolescent smokers are also the most likely to relapse and are more vulnerable to peer pressure, which makes them more susceptible to smoking relapse after cessation [23].

During adolescence, nicotinic acetylcholine receptors remain functionally immature. These receptors are widely distributed across neuroanatomical regions associated with tobacco addiction [24], and their activation regulates monoaminergic neurotransmitter systems, particularly dopamine, which plays a key role in reward processing and drug reinforcement. In rodent studies, the expression and binding of specific nicotinic acetylcholine receptor subtypes are higher in many brain regions during adolescence compared to adulthood [25].

Furthermore, nicotine more robustly enhances neuronal activity in adolescents than in adults, as indicated by increased c-fos mRNA expression in several reward-related brain regions, including the nucleus accumbens shell, basolateral amygdala, and ventral tegmental area [26].

The altered neuronal sensitivity to nicotine during adolescence is paralleled by behavioural responses [27]. Following nicotine exposure, adolescent rodents exhibited increased locomotor activity and reduced anxiety [28]. They also associated nicotine with greater rewarding effects and self-administered higher amounts of nicotine compared to adults [29]. In contrast, adolescent rodents demonstrated lower aversion to

nicotine and experienced less pronounced withdrawal symptoms than their adult counterparts [30]. This shift in the balance between nicotine's positive and negative effects during adolescence may contribute to increased vulnerability to nicotine dependence.

The heightened vulnerability, coupled with the increased likelihood of relapse, constitutes alarming evidence that strongly supports the implementation of stricter age restrictions on the purchase of e-cigarettes. In parallel, the development of comprehensive school-based interventions focused on early education is essential to increase legal awareness, heighten harm perception, and reduce the likelihood of current or future e-cigarette use among youth.

E-Cigarettes as a gateway to nicotine addiction

Over recent years, the proportion of U.S. youth using electronic cigarettes has remained high. This trend has raised concerns that e-cigarettes may contribute to the renormalisation of tobacco use and initiate new generations of youth into cigarette smoking, potentially reversing decades of progress in reducing tobacco-related disease and mortality [31].

A range of research approaches has been employed to evaluate this theory. Barrington-Trimis et al. [32] conducted a prospective cohort study of approximately 300 11th–12th graders (mean age 17.4), comparing never-smoking e-cigarette users (n = 146) to never-smoking, never-e-cigarette users (n = 152). Participants reported their use of e-cigarettes, cigarettes, cigars, pipes, and hookah at baseline and follow-up (~16 months). Cigarette initiation occurred in 40.4% of e-cigarette users versus 10.5% of never users, with e-cigarette users having 6.17 times the odds of initiating cigarette use. This association was stronger among those initially reporting no intention to smoke. E-cigarette users were also more likely to begin any combustible tobacco use.

Berry et al. [33] conducted a prospective cohort study using Waves 1–3 (2013–2016) of the Population Assessment of Tobacco and Health Study, including 6,123 youth aged 12–15. By Wave 3, cigarette use was reported by 20.5% of prior e-cigarette users, compared to 3.8% of those with no prior tobacco use. Prior e-cigarette use was linked to over four times the odds of ever smoking and nearly three times the odds of current smoking. Between 2013 and 2016, it was estimated that 21.8% new cases of ever cigarette use (178,850 youth) and 15.3% of current cigarette use (43,446 youth) could be attributed to prior e-cigarette use. These findings suggest that e-cigarette use may contribute to cigarette smoking initiation at the population level.

Additionally, a meta-analysis of 25 longitudinal studies conducted by Baenziger et al. [34] found evidence that young never-smokers and non-smokers who use e-cigarettes are about three times as likely as non-users to start smoking tobacco and to become regular smokers. All included studies identified elevations in risk.

On the contrary, in their paper exploring the gateway theory in the context of e-cigarettes, Bell and Keane [35] suggest that the theory itself, often treated as a straightforward concept, in the case of e-cigarettes, is rather a retroactively assembled notion. Since the "gateway" in question is from nicotine to nicotine, the same substance is portrayed as both innocuous and harmful. Etter [36] offers a similar perspective and argues that the experiments used to assess the gateway theory cannot account for all the variance in smoking propensity, as most are observational studies that solely adjust for confounders. He warns that policies based on this theory could have adverse consequences on smoking rates, particularly if common liabilities better explain the association between vaping and smoking. If access to less harmful alternatives to combustible cigarettes is restricted, more young people may resort to smoking rather than adopting new alternative technologies.

A significant new concern has recently emerged alongside evidence that the nicotine content of e-cigarettes has increased over time [37]. At present, a substantial proportion of e-cigarettes contain nicotine salts, which allow users to consume high levels of nicotine without experiencing the harshness associated with free-base nicotine [38]. In fact, high-nicotine products dominate U.S. e-cigarette unit sales. As of March 2022, products with a nicotine strength of 5% or more accounted for 81% of total e-cigarette unit sales. Moreover, in recent years, the price of high-nicotine products has decreased

or remained stable, while the cost of low-nicotine products has increased [39]. Even if gateway effects are not currently substantial, this could change in the future if newer e-cigarette models prove significantly more addictive than current ones.

Considering the evidence presented above, the gateway theory cannot be confidently accepted at this time. Clearly determining whether the relationship between e-cigarette use and combustible cigarette smoking is causal or merely correlational remains a critical priority.

Health Outcomes of e-cigarette use

Understanding the harms of prolonged e-cigarette use requires examining e-liquid composition, which typically includes propylene glycol, vegetable glycerin, nicotine, flavourings, and other additives [40]. While propylene glycol and vegetable glycerin are considered safe for ingestion, their long-term inhalation effects remain unclear. Similarly, flavourings deemed "generally recognised as safe" by the FDA apply to ingestion, not inhalation [41]. Margham et al. [42] investigated the chemical composition of e-cigarette aerosols and discovered that they encompass a diverse array of volatile organic chemicals, including aldehydes, ketones, and hydrocarbons. Additionally, in the case of cannabis-containing e-liquids, a commonly used thickening agent is vitamin E acetate, which has been strongly linked to e-cigarette or vaping product use-associated lung injury (EVALI) [40].

Physical health problems associated with the use

The current state of research on the adverse effects of e-cigarette use on physical health indicates that, although e-cigarettes may be considered a safer alternative to combustible cigarettes, their chronic use is not free of health risks. The chemicals and oxidant metals present in aerosols from e-cigarette use have the potential to cause damage to tissues in the lungs, heart, and mouth [5]. There is evidence of nicotine-containing e-cigarettes causing poisoning and immediate inhalation toxicity (including seizures, dizziness, nausea, and vomiting), particularly in children and adolescents [43].

Around the turn of the millennium, concerns emerged regarding the safety of diacetyl, a flavouring agent commonly used in e-liquids to produce a buttery flavour [44]. This compound was associated with the onset of bronchiolitis obliterans, a severe pulmonary condition colloquially known as "popcorn lung", in workers at a microwave popcorn factory. The disease is characterised by inflammation and scarring of the lung tissue, leading to airway obstruction [45]. Propylene glycol, another frequently used e-liquid component that facilitates the mixing of other ingredients, has been shown to damage the epithelial lining of the airways and impair cellular repair mechanisms [46]. This may pose risks for e-cigarette users with chronic obstructive pulmonary disease. In addition, vegetable glycerin has been found to interfere with normal nasal function, potentially leading to the production of thicker mucus and an increased risk of inflammation and compromised airway function [47].

According to research conducted by the Center for Tobacco Research and Education at the University of California, San Francisco, daily use of e-cigarettes can also double the risk of heart attack [48]. This study, which involved nearly 70,000 participants, found that the elevated risk of heart attack among e-cigarette users is comparable to that of combustible cigarette users. Among individuals who use both combustible cigarettes and e-cigarettes daily, the risk increases five times.

The oral cavity is the first site of exposure to nicotine and other chemicals in e-cigarette aerosol. Pushalkar et al. [49] found that e-cigarette users exhibited significantly altered oral microbiome beta-diversity and elevated levels of interleukins IL-6 and IL-1β, indicating an inflammatory immune response. E-cigarette aerosol also induced hypoxia and oxidative stress, increasing epithelial susceptibility to infection.

The adverse effects of e-cigarette use may further compromise oral health through different pathways. Propylene glycol degrades into compounds toxic to enamel and soft tissue, contributes to xerostomia, and promotes caries and gum disease. Vegetable glycerin enhances microbial adhesion and biofilm formation while also reducing enamel hardness [43]. Additionally, nicotine's vasoconstrictive effects impair gingival blood flow and immune responses and increase the risk of periodontal disease and tooth loss [49].

To enable a general comparison, it is worth recalling the adverse health risks connected to other forms of nicotine consumption, including nicotine replacement therapy. Smoking of combustible cigarettes was linked to elevated risks of multiple systemic diseases – including cardiovascular, digestive, musculoskeletal, endocrine, metabolic, and eye disorders – and a range of cancers, such as lung, head and neck, oesophageal or pancreatic cancer. Evidence linking nicotine replacement therapy use to serious adverse health effects is limited, with one study suggesting a possible association with respiratory congenital abnormalities, while no clear links were found for cardiovascular, reproductive, cancer, or stroke-related outcomes [50]. Dual use of both e-cigarettes and combustible cigarettes appears to be associated with greater health risks than the use of either product alone. A recent meta-analysis reported significantly higher odds ratios for various disease outcomes – including chronic obstructive pulmonary disease, cardiovascular events, and asthma – among dual users [51]. Another study found that dual users had significantly higher odds of experiencing incident respiratory symptoms within the past 12 months [52]. To date, this issue remains a subject of ongoing investigation, with the available evidence too limited to permit definitive conclusions.

Mental health problems associated with e-cigarette use

Depression and depressive symptoms seem to emerge more often in adolescent e-cigarette users than non-users [53]. This theory was explored in a study conducted by Dunbar et al. [54], which included 2,039 youths. Participants completed three web-based surveys over the course of three years, beginning at age 16 and continuing until age 20. The subsequently created model assessed the correlation between e-cigarette use and mental health symptoms over time and revealed no associations between the two.

Leventhal et al. [55] investigated psychiatric comorbidity among 3,310 ninth-grade students (mean age 14) in Los Angeles. Participants completed self-reports on e-cigarette/conventional cigarette use, emotional disorders, substance use, and transdiagnostic psychiatric phenotypes. E-cigarette-only users reported lower levels of internalizing symptoms and transdiagnostic traits (e.g., distress intolerance, anxiety sensitivity) compared to conventional cigarette users. However, depression, panic disorder, and anhedonia were higher among e-cigarette users than non-users. An ordered pattern of externalizing outcomes (e.g., mania, substance use) and anhedonia was observed: lowest in non-users, moderate in single-product users, and highest in dual users.

Research findings suggest that use of e-cigarettes could be associated with higher suicidality. This hypothesis was assessed by a study conducted by Lee and Lee [56], in which they examined the results of the 2017 Korean Youth Risk Behavior Web-based Survey of 62,276 students. The statistical analysis of the association between suicidality and patterns of cigarette use revealed that, for lifetime use, e-cigarette-only users were three times more likely to have engaged in suicide planning and five times more likely to have made a suicide attempt than non-users. Additionally, current e-cigarette-only users were six times more likely to have made a suicide attempt than non-users.

Although these studies present promising findings, they are subject to several limitations and should be interpreted with caution. First, the data were self-reported, introducing the possibility of information and/or recall bias. Neither the type nor the nicotine content of e-cigarettes was assessed, limiting the ability to determine whether a higher e-cigarette use profile is genuinely associated with an increased prevalence of depressive and suicidal behaviors. Moreover, it is possible that the causal relationship between e-cigarette use and depressive symptoms operates in the opposite direction from what was assumed in the studies. Previous research has demonstrated that adolescents who use e-cigarettes are more likely to engage in risk-prone behaviors than non-users, and that adolescents with problem behaviors are more prone to depression [56]. To address this issue, future research should include externalizing behaviors as potential confounding variables in the analysis.

EVALI

EVALI is a syndrome, with no specific diagnostic test that defines the condition. According to the CDC criteria, confirmed cases are defined as the onset of pulmonary infiltrates on chest X-ray

or computed tomography that occur within 90 days of e-cigarette use, with no alternative cause found after medical assessment [57].

It was officially identified and named in 2019 because of its outbreak in March 2019, when a cluster of cases emerged in the USA of patients who had developed lung injuries associated with using e-cigarettes. As of February 2020, more than 2,800 patients had been admitted to various hospitals in the US due to an EVALI, with 68 deaths reported so far [58]. It has been reported in a broad age range but was most common in young males between the ages of 18 and 24 years [59].

EVALI was primarily linked to the inclusion of vitamin E acetate in e-liquids, mainly from THC-containing e-cigarettes, largely, but not exclusively, from informal sources [60]. Vitamin E acetate was often used as a thickening agent, likely to dilute THC oil without significantly altering its viscosity. Studies show that it can impair breathing and, when heated, decomposes into harmful compounds such as ketene, alkenes, and benzene, all of which can damage lung tissue [61]. In fact, the CDC found vitamin E acetate present in the bronchoalveolar lavage fluid of most EVALI patients and in product samples associated with the outbreak.

Furthermore, research indicates that vitamin E acetate interferes with the normal function of lung surfactant. It can disrupt the performance of the surfactant system by interfering with the kinetic lipid processes that stabilize the monolayer under compression, contributing to the respiratory distress associated with EVALI [62].

EVALI typically presents as an acute or subacute respiratory illness characterized by nonspecific symptoms, including dyspnea, cough, chest pain, and/or hemoptysis [63]. The majority of patients also exhibit gastrointestinal manifestations (such as nausea, vomiting, and/or diarrhea) and/or constitutional symptoms (including fever, chills, fatigue, and/or weight loss), which tend to develop over a period of days to weeks. On physical examination, patients commonly present with fever (33%), tachycardia (63%), and tachypnea (43%). Additionally, impaired oxygenation is frequently observed, with approximately one in four patients demonstrating a pulse oxygen saturation of ≤89%. Laboratory findings are generally nonspecific and may include leukocytosis and an elevated erythrocyte sedimentation rate.

In certain cases, the clinical presentation of EVALI associated with vitamin E acetate in adolescents appears to differ from that observed in adults. One case series reported that adolescents with EVALI experienced significant weight loss secondary to gastrointestinal symptoms, necessitating hospitalization [64]. Another series described the use of venovenous extracorporeal membrane oxygenation to manage EVALI in adolescents with preexisting asthma [65]. Although the pathophysiology of EVALI in adolescents remains not fully understood, it is reasonable to suspect a distinct clinical presentation in terms of symptomatology, severity, or both.

No randomized clinical trials have evaluated specific therapies for EVALI, and long-term outcome data remain limited. Cessation of e-cigarette use is essential, as continued vaping has been linked to recurrent EVALI and respiratory failure [66]. Supportive care is the primary treatment, typically involving supplemental oxygen (target saturation 88–92%) via nasal cannula, high-flow oxygen, or high-flow nasal cannula. Mechanical ventilation, required in ~26% of cases, follows lung-protective strategies used in acute respiratory distress syndrome [63]; extracorporeal membrane oxygenation is rarely needed.

Infectious causes must be ruled out, with testing for influenza and empiric antiviral/antimicrobial therapy recommended. Patients with severe lung injury and suspected EVALI have shown favourable responses to systemic corticosteroids [67], though their efficacy has not been formally studied. Given the risk of worsening undiagnosed infections, pulmonologist consultation is advised before initiating corticosteroids.

The Future Perspectives

Emerging novel technologies offer promising solutions to combat current challenges in e-cigarette cessation. A variety of digital health interventions help establish a more inclusive and readily accessible healthcare environment [68], offering innovations such as interactive text-messaging programs that remotely deliver cognitive-behavioural coping strategies and peer support. The capabilities of artificial intelligence keep extending across various domains – machine learning enables predictive modelling of

relapse risk and tobacco initiation patterns, while reinforcement learning optimises personalised cessation interventions based on user engagement data [69]. Novel pharmacological options are also being studied – the first U.S. placebo-controlled randomized trial of varenicline for e-cigarette cessation showed promising results, revealing a 15% higher quit rate in the medication group (45%) compared to the control group [70].

Concerning future regulatory policy, beginning at the federal level, agencies responsible for protecting public health should assert their regulatory authority by requiring e-cigarette manufacturers to register their products, disclose ingredient lists, and comply with good manufacturing practices. In addition, manufacturers should be obligated to address the presence of impure or untested additives, resolve issues related to misbranding, and adhere to strict regulations on marketing and sales. It is essential to accelerate the implementation of evidence-based interventions, such as reducing youth exposure to smoking imagery in media, conducting robust counter-marketing campaigns, and ensuring equitable access to tobacco dependence treatment for all who are seeking to quit. Further state policy measures may include increasing the cost of e-cigarettes and restricting their sale exclusively to adult consumers. Additionally, school-based policies, such as banning the use of e-cigarettes on school grounds and/or implementing prevention and cessation programs, represent an important context for shaping youth tobacco use behavior. Equally important is the pursuit of novel solutions and maintaining openness to innovative approaches in responding to emerging public health challenges. However, it is crucial to proceed cautiously, as overly restrictive policies may support the established tobacco industry, encourage the poly-use of tobacco products, and, as a result, perpetuate sales of conventional cigarettes well into future decades rather than hurry their disappearance.

Applied to tobacco, the most effective strategies for harm reduction are those that promote cessation among current users and prevent initiation among non-users. In this context, the role of e-cigarettes remains a complex public health issue due to their dual influence on population health outcomes. On one hand, e-cigarettes can serve as a harm reduction tool for current smokers, for whom switching completely to e-cigarettes may significantly reduce exposure to harmful combustion-related toxins. On the other hand, the concern that e-cigarette use among adolescents introduces nicotine dependence cannot be ruled out.

Carefully framing public health messaging may be one of the keys to navigating this complex issue. One example would be avoiding binary messaging that might discourage combustible cigarette smokers from switching. Another is crafting messages that support cessation without inadvertently attracting youth or downplaying risks for non-users. A complementary strategy should focus on removing features that make e-cigarettes disproportionately appealing to youth, such as by promoting adult-only sales environments or limiting flavor profiles and marketing tactics known to attract adolescents. To ensure the effectiveness of such efforts, continuous monitoring of relevant data is essential, including current trends in youth e-cigarette use and updates on the efficacy of e-cigarettes in smoking cessation. New policies should evolve accordingly and adapt in the shortest possible time to avoid negative public health consequences resulting from legislative delays.

Conclusions

This review aimed to summarise and systematise the current state of knowledge on various aspects of e-cigarette use among adolescents. Extensive research has demonstrated that, in this particular age group, the central nervous system is especially sensitive to the effects of nicotine, making it more likely for nicotine addiction to develop and persist over time. Nevertheless, to date, definitive evidence supporting the "gateway theory" is lacking. Current research findings conclude that e-cigarettes are responsible for the development of EVALI and suggest that they may cause lung injury; many of their chemical components have been linked to adverse health effects. Several aspects of e-cigarette consumption among youth remain poorly understood. Future research should focus on the chemical safety of e-liquids, examine the stability of their ingredients when heated, and identify potential by-products resulting from thermal degradation. It is essential to determine

the long-term health outcomes of e-cigarette use and evaluate their effectiveness as a smoking cessation tool. The "gateway theory" requires confirmation through further high-quality trials. Based on such clinical evidence, effective new cessation strategies targeted at youth should be developed. Rigorous enforcement of regulations governing e-cigarette production and marketing remains an urgent priority.

Disclosures

Acknowledgements
We would like to thank Mr Luigi Mercurio for contributing an insightful outlook on the topic and providing valuable writing assistance.

Author contributions
Katarzyna Drewnowska: Conceptualization (lead); Writing – original draft (lead); Formal analysis (lead). Jakub Modrzewski: Writing – original draft (supporting); Methodology (lead); Data curation (equal). Łukasz Kreft, Julia Radziszewska: Investigation (lead); Data curation (equal). Agata Drozd, Klaudia Piskorowska: Conceptualization (supporting); Writing – review and editing (lead). All authors have read the final version of the manuscript, approved it, and take responsibility for its content.

Conflict of interest statement
The authors declare no conflict of interest.

Funding sources
There are no sources of funding to declare.

References

1. Centers for Disease Control and Prevention. Youth and tobacco use [Internet]. Atlanta (GA): CDC; 2023 [cited 2025 Jun 3]. Available from: https://www.cdc.gov/tobacco/e-cigarettes/youth.html.
2. Marques P, Piqueras L, Sanz MJ. An updated overview of e-cigarette impact on human health. Respir Res. 2021;22(1):151. https://doi.org/10.1186/s12931-021-01737-5.
3. Grana RA, Ling PM. "Smoking Revolution": A content analysis of electronic cigarette retail websites. Am J Prev Med. 2014;46(4):395–403. https://doi.org/10.1016/j.amepre.2013.12.010.
4. Dutra LM, Glantz SA. E-cigarettes and conventional cigarette use among US adolescents: a cross-sectional study. JAMA Pediatr. 2014;168(7):610–7. https://doi.org/10.1001/jamapediatrics.2013.5488.
5. National Academies of Sciences, Engineering, and Medicine. Public health consequences of e-cigarettes. Washington (DC): National Academies Press; 2018. 774 p. https://doi.org/ 10.17226/24952.
6. Grana R, Benowitz N, Glantz SA. Background paper on e-cigarettes (electronic nicotine delivery systems). San Francisco (CA): Center for Tobacco Control Research and Education, University of California, San Francisco; 2013. Prepared for the World Health Organization. https://doi.org/10.13140/RG.2.1.4010.0484.
7. Wang TW, Gentzke AS, Creamer MR, Cullen KA, Holder-Hayes E, Sawdey MD, et al. Tobacco product use and associated factors among middle and high school students—United States, 2019. MMWR Surveill Summ. 2019;68(12):1–22. https://doi.org/10.15585/mmwr.ss6812a1.
8. Barrington-Trimis JL, Gibson LA, Halpern-Felsher B, Harrell MB, Kong G, Krishnan-Sarin S, et al. Type of e-cigarette device used among adolescents and young adults: findings from a pooled analysis of eight studies of 2166 vapers. Nicotine Tob Res. 2018;20(2):271–4. https://doi.org/10.1093/ntr/ntx069.
9. Zare S, Nemati M, Zheng Y. A systematic review of consumer preference for ecigarette attributes: flavor, nicotine strength, and type. PLoS One. 2018;13(3):e0194145. https://doi.org/10.1371/journal.pone.0194145.
10. Chaffee BW, Halpern-Felsher B, Croker JA, Werts M, Couch ET, Cheng J. Preferences, use, and perceived access to flavored e-cigarettes among United States adolescents and young adults. Drug Alcohol Depend Rep. 2022;3:100068. https://doi.org/10.1016/j.dadr.2022.100068.
11. Kornfield R, Huang J, Vera L, Emery SL. Industry watch: rapidly increasing promotional expenditures for e-cigarettes. Tob Control. 2015;24(2):110–111. https://doi.org/10.1136/tobaccocontrol-2014-051580.
12. U.S. Department of Health and Human Services. E-cigarette use among youth and young adults: a report of the Surgeon General. Atlanta (GA): U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 2016.
13. Paek HJ, Kim S, Hove T, Huh JY. Reduced harm or another gateway to smoking? Source, message, and information characteristics of e-cigarette videos on YouTube. J Health Commun. 2014;19(5):545-60. https://doi.org/10.1080/10810730.2013.821560.
14. Yan D, Wang Z, Laestadius L, Mosalpuria K, Wilson FA, Yan A, et al. A systematic review for the impacts of global approaches to regulating electronic nicotine products. J Glob Health. 2023;13:04071. https://doi.org/10.7189/jogh.13.04071.
15. Ylitörmänen T, Tarasenko Y, Hiilamo H, Ruokolainen O, Puska P, Ollila H. Crosssectional study of the associations between the implementation of the WHO FCTC tobacco advertising, promotion and sponsorship bans and current e-cigarette use among youth from countries with different income levels. Tob Control. 2024;0:1–9. https://doi.org/10.1136/tc-2023-058160.
16. Liber A, Cahn Z, Larsen A, Drope J. Flavored e-cigarette sales in the United States under self-regulation from January 2015 through October 2019. Am J Public Health. 2020;110(6):785–787. https://doi.org/10.2105/AJPH.2020.305667.
17. O'Leary A. Too JUUL for School: Evaluating the FDA's Regulation of Flavored Electronic Nicotine Deliv-

ery Systems. NYU Moot Court Proceedings. 20 Mar 2025 [cited 2025 Jun 26]. Available from: https://proceedings.nyumootcourt.org/2025/03/too-juul-for-school-evaluating-thefdas-regulation-of-flavored-electronic-nicotine-delivery-systems/#_ftn19
18. Ali FRM, Vallone D, Seaman EL, Cordova J, Diaz MC, Tynan MA, et al. Evaluation of statewide restrictions on flavored e-cigarette sales in the US from 2014 to 2020. JAMA Netw Open. 2022;5(2):e2147813. https://doi.org/10.1001/jamanetworkopen.2021.47813.
19. Cotti CD, Courtemanche CJ, Liang Y, Maclean JC, Nesson ET, Sabia JJ. The effect of e-cigarette flavor bans on tobacco use. NBER Working Paper Series. 2024;No. 32535. https://doi.org/10.3386/w32535.
20. Yang Y, Lindblom EN, Ward KD, Salloum RG. The impact of flavored e-cigarette bans on e-cigarette use in three US states. medRxiv [Preprint]. 2023 May 21. https://doi.org/ 10.1101/2023.05.19.23290249.
21. CDC Foundation. Monitoring U.S. E-Cigarette Sales: National Trends. Data Brief. July 2022. Available from: https://www.cdcfoundation.org/sites/default/files/2022-07/CDC-Foundation-Monitoring-US-E-Cigarette-Sales-July2022.pdf.
22. Substance Abuse and Mental Health Services Administration. Results from the 2011 National Survey on Drug Use and Health: Summary of National Findings. Rockville (MD): Substance Abuse and Mental Health Services Administration; 2012. NSDUH Series H-44, HHS Publication No. (SMA) 12-4713.
23. Collins SE, Witkiewitz K, Kirouac M, Marlatt GA. Preventing Relapse Following Smoking Cessation. Curr Cardiovasc Risk Rep. 2010;4:421–428. https://doi.org/10.1007/s12170-010-0124-6.
24. Gotti C, Clementi F. Neuronal nicotinic receptors: from structure to pathology. Progress in Neurobiology. 2004;74(5):363-396. https://doi.org/10.1016/j.pneurobio.2004.09.006.
25. Doura MB, Gold AB, Keller AB, Perry DC. Adult and periadolescent rats differ in expression of nicotinic cholinergic receptor subtypes and in the response of these subtypes to chronic nicotine exposure. Brain Res. 2008 Jun 18;1215:40-52. https://doi.org/10.1016/j.brainres.2008.03.056.
26. Dao JM, McQuown SC, Loughlin SE, Belluzzi JD, Leslie FM. Nicotine Alters Limbic Function in Adolescent Rat by a 5-HT1A Receptor Mechanism. Neuropsychopharmacology. 2011;36:1319-1331. https://doi.org/10.1038/npp.2011.8.
27. Yuan M, Cross SJ, Loughlin SE, Leslie FM. Nicotine and the adolescent brain. J Physiol. 2015;593(16):3397-3412. https://doi.org/10.1113/JP270492.
28. Cao J, Belluzzi JD, Loughlin SE, Dao JM, Chen Y, Leslie FM. Locomotor and Stress Responses to Nicotine Differ in Adolescent and Adult Rats. Pharmacol Biochem Behav. 2010;96(1):82-90. https://doi.org/10.1016/j.pbb.2010.04.010.
29. Natividad LA, Torres OV, Friedman TC, O'Dell LE. Adolescence is a period of development characterized by short- and long-term vulnerability to the rewarding effects of nicotine and reduced sensitivity to the anorectic effects of this drug. Behav Brain Res. 2013;257:275-285. https://doi.org/10.1016/j.bbr.2013.10.003.
30. Shram MJ, Siu ECK, Li Z, Tyndale RF, Lê AD. Interactions between age and the aversive effects of nicotine withdrawal under mecamylamine-precipitated and spontaneous conditions in male Wistar rats. Psychopharmacology. 2008;198(2):181- 190. https://doi.org/10.1007/s00213-008-1115-x.
31. U.S. Department of Health and Human Services. The Health Consequences of Smoking: 50 Years of Progress. A Report of the Surgeon General. Atlanta (GA): U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; 2014.
32. Barrington-Trimis JL, Urman R, Berhane K, Unger JB, Cruz TB, Pentz MA, et al. ECigarettes and Future Cigarette Use. Pediatrics. 2016;138(1):e20160379. https://doi.org/10.1542/peds.2016-0379.
33. Berry KM, Fetterman JL, Benjamin EJ, Bhatnagar A, Barrington-Trimis JL, Leventhal AM, et al. Association of Electronic Cigarette Use With Subsequent Initiation of Tobacco Cigarettes in US Youths. JAMA Netw Open. 2019;2(2):e187794. https://doi.org/10.1001/jamanetworkopen.2018.7794.
34. Baenziger ON, Ford L, Yazidjoglou A, Joshy G, Banks E. E-cigarette use and combustible tobacco cigarette smoking uptake among non-smokers, including relapse in former smokers: umbrella review, systematic review and meta-analysis. BMJ Open. 2021;11(3):e045603. https://doi.org/10.1136/bmjopen-2020-045603.
35. Bell K, Keane H. The 'gateway theory', e-cigarettes and the remaking of nicotine. Soc Sci Med. 2014;119:45-52. https://doi.org/10.1016/j.socscimed.2014.08.016.
36. Etter JF. Gateway effects and electronic cigarettes. Addiction. 2017. https://doi.org/10.1111/add.13924.
37. Romberg AR, Miller Lo EJ, Cuccia AF, Willett JG, Xiao H, Hair EC, Vallone DM, Marynak K, King BA. Patterns of nicotine concentrations in electronic cigarettes sold in the United States, 2013–2018. Drug Alcohol Depend. 2019 Oct 1;203:1-7. https://doi.org/10.1016/j.drugalcdep.2019.05.029.
38. Leventhal AM, Madden DR, Peraza N, Schiff SJ, Lebovitz L, Whitted L, et al. Effect of Exposure to e-Cigarettes With Salt vs Free-Base Nicotine on the Appeal and Sensory Experience of Vaping. JAMA Netw Open. 2021;4(1):e2032757. https://doi.org/10.1001/jamanetworkopen.2020.32757.
39. Ali FRM, Seaman EL, Crane E, Schillo B, King BA. Trends in US E-cigarette Sales and Prices by Nicotine Strength, Overall and by Product and Flavor Type, 2017-2022. Nicotine Tob Res. 2023;25:1052-1056. https://doi.org/10.1093/ntr/ntac284.
40. Amjad MA, Ocazionez Trujillo D, Estrada-Y-Martin RM, Cherian SV. E-Cigarette or Vaping Product Use-Associated Lung Injury: A Comprehensive Review. Int J Environ Res Public Health. 2025;22(5):792. https://doi.org/ 10.3390/ijerph22050792.
41. Gordon T, Karey E, Rebuli ME, Escobar YNH, Jaspers I, Chen LC. E-cigarette toxicology. Annu Rev

Pharmacol Toxicol. 2022;62:301–322. https://doi.org/10.1146/annurev-pharmtox-042921-084202.
42. Margham J, McAdam K, Forster M, Liu C, Wright C, Mariner D, Proctor C. Chemical composition of aerosol from an e-cigarette: a quantitative comparison with cigarette smoke. Chem Res Toxicol. 2016;29(10):1662–1678. https://doi.org/10.1021/acs.chemrestox.6b00188.
43. Sapru S, Vardhan M, Li Q, Guo Y, Li X, Saxena D. E-cigarettes use in the United States: reasons for use, perceptions, and effects on health. BMC Public Health. 2020 Oct 9;20(1):1518. https://doi.org/10.1186/s12889-020-09572-x.
44. White AV, Wambui DW, Pokhrel LR. Risk assessment of inhaled diacetyl from electronic cigarette use among teens and adults. Sci Total Environ. 2021;772:145486. https://doi.org/10.1016/j.scitotenv.2021.145486.
45. Kreiss K, Gomaa A, Kullman G, Fedan K, Simoes EJ, Enright PL. Clinical bronchiolitis obliterans in workers at a microwave-popcorn plant. N Engl J Med. 2002;347(5):330-8. https://doi.org/10.1056/NEJMoa020300.
46. Komura M, Sato T, Yoshikawa H, Nitta NA, Suzuki Y, Koike K, et al. Propylene glycol, a component of electronic cigarette liquid, damages epithelial cells in human small airways. Respir Res. 2022;23:216. https://doi.org/ 10.1186/s12931-022-02142-2.
47. Kim MD, Chung S, Dennis JS, Yoshida M, Aguiar C, Aller SP, Mendes ES, Schmid A, Sabater J, Baumlin N, Salathe M. Vegetable glycerin e-cigarette aerosols cause airway inflammation and ion channel dysfunction. Front Pharmacol. 2022;13:1012723. https://doi.org/10.3389/fphar.2022.1012723.
48. Fernandez E. Risk of heart attacks is double for daily e-cigarette users. University of California, San Francisco, UCSF News. 2018 Aug 23. [cited 2025 Jun 4]. Available from: https://www.ucsf.edu/news/2018/08/411476/risk-heart-attacks-double-daily-ecigarette-users.
49. Pushalkar S, Paul B, Li Q, Yang J, Vasconcelos R, Makwana S, et al. Electronic Cigarette Aerosol Modulates the Oral Microbiome and Increases Risk of Infection. iScience. 2020;23:100884. https://doi.org/10.1016/j.isci.2020.100884.
50. Lee PN, Fariss MW. A systematic review of possible serious adverse health effects of nicotine replacement therapy. Arch Toxicol. 2017;91(4):1565–1594. https://doi.org/10.1007/s00204-016-1856-y.
51. Glantz SA, Nguyen N, Oliveira da Silva AL. Population-based disease odds for e-cigarettes and dual use versus cigarettes. NEJM Evid. 2024;3(3). https://doi.org/10.1056/EVIDoa2300229.
52. Reddy KP, Schwamm E, Kalkhoran S, Noubary F, Walensky RP, Rigotti NA. Respiratory symptom incidence among people using electronic cigarettes, combustible tobacco, or both. Am J Respir Crit Care Med. 2021 Jul 15;204(2):231–3. https://doi.org/10.1164/rccm.202012-4441LE.
53. Livingston JA, Chen CH, Kwon M, Park E. Physical and mental health outcomes associated with adolescent E-cigarette use. J Pediatr Nurs. 2022;64:1-17. https://doi.org/10.1016/j.pedn.2022.01.006.
54. Dunbar MS, Davis JP, Rodriguez A, Tucker JS, Seelam R, D'Amico EJ. Disentangling within- and between-person effects of shared risk factors on e-cigarette and cigarette use trajectories from late adolescence to young adulthood. Nicotine Tob Res. 2019;21(10):1414-22. https://doi.org/10.1093/ntr/nty179.
55. Leventhal AM, Strong DR, Sussman S, Kirkpatrick MG, Unger JB, BarringtonTrimis JL, et al. Psychiatric comorbidity in Adolescent Electronic and Conventional Cigarette Use. J Psychiatr Res. 2016 Feb;73:71-78. https://doi.org/10.1016/j.jpsychires.2015.11.008.
56. Lee Y, Lee K-S. Association of Depression and Suicidality with Electronic and Conventional Cigarette Use in South Korean Adolescents. Subst Use Misuse. 2019; https://doi.org/10.1080/10826084.2018.1552301.
57. Centers for Disease Control and Prevention. E-cigarette Use, or Vaping, Among Youth and Young Adults — A Report of the Surgeon General. MMWR Morb Mortal Wkly Rep [Internet]. 2019 Sep 13 [cited 2025 Jun 4];68(Early Release):1-3. Available from: https://www.cdc.gov/mmwr/volumes/68/wr/mm6836e2.htm.
58. Zulfiqar H, Rahman O. Vaping associated pulmonary injury. StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan– [cited 2025 Jun 5]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK560656.
59. Cecchini MJ, Mukhopadhyay S, Arrossi AV, Beasley MB, Butt YM, Jones KD, Pambuccian S, Mehrad M, Monaco SE, Saqi A, Smith ML, Tazelaar HD, Larsen BT. E-Cigarette or Vaping Product Use-Associated Lung Injury: A Review for Pathologists. Arch Pathol Lab Med. 2020;144(12):1490-500. https://doi.org/10.5858/arpa.2020- 0024-RA.
60. Rebuli ME, Rose JJ, Noël A, Croft DP, Benowitz NL, Cohen AH, et al. The Ecigarette or Vaping Product Use–Associated Lung Injury Epidemic: Pathogenesis, Management, and Future Directions. An Official American Thoracic Society Workshop Report. Ann Am Thorac Soc. 2023 Jan;20(1):1–17. https://doi.org/10.1513/AnnalsATS.202209-796ST.
61. Wu D, O'Shea DF. Potential for release of pulmonary toxic ketene from vaping pyrolysis of vitamin E acetate. Proc Natl Acad Sci U S A. 2020 Mar 24;117(12):6349–55. https://doi.org/10.1073/pnas.1920925117.
62. DiPasquale M, Dziura M, Gbadamosi O, Castillo SR, Fahim A, Roberto J, Atkinson J, Boccalon N, Campana M, Pingali SV, Chandrasekera PC, Zolnierczuk PA, Nagao M, Kelley EG, Marquardt D. Vitamin E acetate causes softening of pulmonary surfactant membrane models. Chem Res Toxicol. 2025;38(3):400–414. https://doi.org/10.1021/acs.chemrestox.4c00425.
63. Layden JE, Ghinai I, Pray I, Kimball A, Layer M, Tenforde MW, Navon L, Hoots B, Salvatore PP, Elderbrook M, Haupt T, Kanne J, Patel MT, Saathoff-Huber L, King BA, Schier JG, Mikosz CA, Meiman J. Pulmonary Illness Related to E-Cigarette Use in Illinois and Wisconsin: Final Report. N Engl J Med. 2020;382(10):903-16. https://doi.org/10.1056/NEJMoa1911614.
64. Nacca N, McGraw MD, Croft DP, Kalininskiy A. Weight loss in adolescents presenting with EVA-

LI. Pediatr Pulmonol. 2021;56(8):2785-6. https://doi.org/10.1002/ppul.25523.
65. Bradford LE, Rebuli ME, Ring BJ, Jaspers I, Clement KE, Loughlin CE. Danger in the vapor? ECMO for adolescents with status asthmaticus after vaping. J Asthma. 2019;56(1):1-6. https://doi.org/10.1080/02770903.2019.1643361.
66. Blagev DP, Harris D, Dunn AC, Guidry DW, Grissom CK, Lanspa M. Clinical presentation, treatment, and short-term outcomes of lung injury associated with ecigarettes or vaping: a prospective observational cohort study. Lancet Respir Med. 2019;7(12):1017-26. https://doi.org/10.1016/S2213-2600(19)30403-2.
67. Glynn TJ. E-cigarettes and the future of tobacco control. CA Cancer J Clin. 2014;64(3):164-8. https://doi.org/10.3322/caac.21226.
68. Graham AL, Amato MS, Cha S, Jacobs MA, Bottcher MM, Papandonatos GD, et al. Effectiveness of a vaping cessation text message program among young adult e-cigarette users: a randomized clinical trial. JAMA Intern Med. 2021 Jul;181(7):923–30. PMCID: PMC11307165.
69. Olawade DB, Aienobe-Asekharen CA. Artificial intelligence in tobacco control: A systematic scoping review of applications, challenges, and ethical implications. Int J Med Inform. 2025;202:105987. https://doi.org/10.1016/j.ijmedinf.2025.105987.
70. Fucito LM, Baldassarri SR, Baker NL, Palmer AM, O'Malley SS, Carpenter MJ, et al. Varenicline for e-cigarette cessation in adults: a preliminary placebo-controlled randomized trial. Am J Prev Med. 2024 Aug;67(2):296–298. https://doi.org/10.1016/j.amepre.2024.04.007.

Journal of Medical Science (JMS) is a PEER-REVIEWED, OPEN ACCESS journal that publishes original research articles and reviews which cover all aspects of clinical and basic science research. The journal particularly encourages submissions on the latest achievements of world medicine and related disciplines. JMS is published quarterly by Poznan University of Medical Sciences.

ONLINE SUBMISSION:
Manuscripts should be submitted to the Editorial Office by an e-mail attachment: nowinylekarskie@ump.edu.pl. You do not need to mail any paper copies of your manuscript.

All submissions should be prepared with the following files:
- Cover Letter
- Manuscript
- Tables
- Figures
- Supplementary Online Material

COVER LETTER: Manuscripts must be accompanied by a cover letter from the author who will be responsible for correspondence regarding the manuscript as well as for communications among authors regarding revisions and approval of proofs. The cover letter should contain the following elements: (1) the full title of the manuscript, (2) the category of the manuscript being submitted (e.g. Original Article, Brief Report), (3) the statement that the manuscript has not been published and is not under consideration for publication in any other journal, (4) the statement that all authors approved the manuscript and its submission to the journal, and (5) a list of at least two referees.

MANUSCRIPT: Journal of Medical Science publishes Original Articles, Brief Reports, Review articles, Mini-Reviews, Images in Clinical Medicine and The Rationale and Design and Methods of New Studies. From 2014, only articles in English will be considered for publication. They should be organized as follows: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Conflict of Interest, References and Figure Legends. All manuscripts should be typed in Arial or Times New Roman font and double spaced with a 2,5 cm (1 inch) margin on all sides. They should be saved in DOC, DOCX, ODT, RTF or TXT format. Pages should be numbered consecutively, beginning with the title page.

Ethical Guidelines
Authors should follow the principles outlined in the Declaration of Helsinki of the World Medical Association (www.wma.net). The manuscript should contain a statement that the work has been approved by the relevant institutional review boards or ethics committees and that all human participants gave informed consent to the work. This statement should appear in the Material and Methods section. Identifying information, including patients' names, initials, or hospital numbers, should not be published in written descriptions, illustrations, and pedigrees. Studies involving experiments with animals must be conducted with approval by the local animal care committee and state that their care was in accordance with institution and international guidelines.

Authorship
According to the International Committee on Medical Journal Ethics (ICMJE), an author is defined as one who has made substantial contributions to the conception and development of a manuscript. Authorship should be based on all of the following: 1) substantial contributions to conception and design, data analysis and interpretation; 2) article drafting or critical advice for important intellectual content; and 3) final approval of the version to be published. All other contributors should be listed as acknowledgments. All submissions are expected to comply with the above definition.

Conflict of Interest
The manuscript should contain a conflict of interest statement from each author. Authors should disclose all financial and personal relationships that could influence their work or declare the absence of any conflict of interest. Author's conflict of interest should be included under Acknowledgements section.

Abbreviations
Abbreviations should be defined at first mention, by putting abbreviation between brackets after the full text. Ensure consistency of abbreviations throughout the article. Avoid using them in the title and abstract. Abbreviations may be used in tables and figures if they are defined in the table footnotes and figure legends.

Trade names
For products used in experiments or methods (particularly those referred to by a trade name), give the manufacturer's full name and location (in parentheses). When possible, use generic names of drugs.

Title page
The first page of the manuscript should contain the title of the article, authors' full names without degrees or titles, authors' institutional affiliations including city and country and a running title, not exceeding 40 letters and spaces. The first page should also include the full postal address, e-mail address, and telephone and fax numbers of the corresponding author.

Abstract
The abstract should not exceed 250 words and should be structured into separate sections: Background, Methods, Results and Conclusions. It should concisely state the significant findings without reference to the rest of the paper. The abstract should be followed by a list of 3 to 6 Key words. They should reflect the central topic of the article (avoid words already used in the title).

The following categories of articles can be proposed to the Journal of Medical Science:

ORIGINAL RESEARCH

Original articles: Manuscripts in this category describe the results of original research conducted in the broad area of life science and medicine. The manuscript should be presented in the format of Abstract (250-word limit), Keywords, Introduction, Material and Methods, Results, Discussion, Perspectives, Acknowledgments and References. In the Discussion section, statements regarding the importance and novelty of the study should be presented. In addition, the limitations of the study should be articulated. The abstract must be structured and include: Objectives, Material and Methods, Results and Conclusions. Manuscripts cannot exceed 3500 words in length (excluding title page, abstract and references) and contain no more than a combination of 8 tables and/or figures. The number of references should not exceed 45.

Brief Reports: Manuscripts in this category may present results of studies involving small sample sizes, introduce new methodologies, describe preliminary findings or replication studies. The manuscript must follow the same format requirements as full length manuscripts. Brief reports should be up to 2000 words (excluding title page, abstract and references) and can include up to 3 tables and/or figures. The number of references should not exceed 25.

REVIEW ARTICLES

Review articles: These articles should describe recent advances in areas within the Journal's scope. Review articles cannot exceed 5000 words length (excluding title page, abstract and references) and contain no more than a combination of 10 tables and/or figures. Authors are encouraged to restrict figures and tables to essential data that cannot be described in the text. The number of references should not exceed 80.

A THOUSAND WORDS ABOUT... is a form of Mini-Reviews. Manuscripts in this category should focus on latest achievements of life science and medicine. Manuscripts should be up to 1000 words in length (excluding title page, abstract and references) and contain up to 5 tables and/or figures and up to 25 most relevant references. The number of authors is limited to no more than 3.

OTHER SUBMISSIONS

Invited Editorials: Editorials are authoritative commentaries on topics of current interest or that relate to articles published in the same issue. Manuscripts should be up to 1500 words in length. The number of references should not exceed 10. The number of authors is limited to no more than 2.

Images in Clinical Medicine: Manuscripts in this category should contain one distinct image from life science or medicine. Only original and high-quality images are considered for publication. The description of the image (up to 250 words) should present relevant information like short description of the patient's history, clinical findings and course, imaging techniques or molecular biology techniques (e.g. blotting techniques or immunostaining). All labeled structures in the image should be described and explained in the legend. The number of references should not exceed 5. The number of authors is limited to no more than 5.

The Rationale, Design and Methods of New Studies: Manuscripts in this category should provide information regarding the grants awarded by different founding agencies, e.g. National Health Institute, European Union, National Science Center or National Center for Research and Development. The manuscript should be presented in the format of Research Project Objectives, Research Plan and Basic Concept, Research Methodology, Measurable Effects and Expected Results. The article should also contain general information about the grant: grant title, keywords (up to five), name of the principal investigator and co-investigators, founding source with the grant number, Ethical Committee permission number, code in clinical trials (if applicable). Only grant projects in the amount over 100,000 Euro can be presented. Manuscripts should be up to 2000 words in length (excluding references) and can include up to 5 tables and/or figures. The abstract should not exceed 150 words. The number of authors is limited to the Principal Investigator and Co-investigators.

Acknowledgements

Under acknowledgements please specify contributors to the article other than the authors accredited. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.). Also acknowledge all sources of support (grants from government agencies, private foundations, etc.). The names of funding organizations should be written in full.

References

All manuscripts should use the 'Elsevier – Vancouver (no "et al.")' style for references. References should be numbered consecutively in the order in which they appear in the text and listed at the end of the paper**.** References cited only in Figures/Tables should be listed in the end. Reference citations in the text should be identified by Arabic numbers in square brackets. Some examples:

This result was later contradicted by Smith and Murray [3].
Smith [8] has argued that...
Multiple clinical trials [4–6, 9] show...

Journal names should be abbreviated according to Index Medicus. If available alwaysprovide Digital Object Identifier (DOI) or PubMed Identifier (PMID) for every reference.

Some examples

Standard journal articles

1. Petrova NV, Kashirskaya NY, Vasilyeva TA, Kondratyeva EI, Marakhonov AV, Macek Jr M, Ginter EK, Kutsev SI, Zinchenko RA. Characteristics of the L138ins (p.Leu138dup) mutation in Russian cystic fibrosis patients. JMS [Internet]. 2020 Mar 31;89(1):e383. doi: 10.20883/medical.383.

Books

Personal author(s)

1. Rang HP, Dale MM, Ritter JM, Moore PK. Pharmacology. 5th ed. Edinburgh: Churchill Livingstone; 2003.

Editor(s) or compiler(s) as authors

2. Beers MH, Porter RS, Jones TV, Kaplan JL, Berkwits M (editors). The Merck manual of diagnosis and therapy. 18th ed. Whitehouse Station (NJ): Merck Research Laboratories; 2006.

Chapter in the book

1. Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995. p. 465–478.

TABLES: Tables should be typed on sheets separate from the text (each table on a separate sheet). They should be numbered consecutively with Arabic numerals. Tables should always be cited in text (e.g. table 2) in consecutive numerical order. Each table should include a compulsory, concise explanatory title and an explanatory legend. Footnotes to tables should be typed below the table body and referred to by superscript lowercase letters. No vertical rules should be used. Tables should not duplicate results presented elsewhere in the manuscript (e.g. in figures).

FIGURES: All illustrations, graphs, drawings, or photographs are referred to as figures and must be uploaded as separate files when submitting a manuscript. Figures should be numbered in sequence with Arabic numerals. They should always be cited in text (e.g. figure 3) in consecutive numerical order. Figures for publication must only be submitted in high-resolution TIFF or EPS format (minimum 300 dpi resolution). Each figure should be self-explanatory without reference to the text and have a concise but descriptive legend. All symbols and abbreviations used in the figure must be defined, unless they are common abbreviations or have already been defined in the text. Figure Legends must be included after the reference section of the Main Text.

Color figures: Figures and photographs will be reproduced in full colour in the online edition of the journal. In the paper edition, all figures and photographs will be reproduced as black-and-white.

SUPPLEMENTARY ONLINE MATERIAL: Authors may submit supplementary material for their articles to be posted in the electronic version of the journal. To be accepted for posting, supplementary materials must be essential to the scientific integrity and excellence of the paper. The supplementary material is subject to the same editorial standards and peer-review procedures as the print publication.

Review Process

All manuscripts are reviewed by the Editor-in-Chief or one of the members of the Editorial Board, who may decide to reject the paper or send it for external peer review. Manuscripts accepted for peer review will be blind reviewed by at least two experts in the field. After peer review, the Editor-in-Chief will study the paper together with reviewer comments to make one of the following decisions: accept, accept pending minor revision, accept pending major revision, or reject. Authors will receive comments on the manuscript regardless of the decision. In the event that a manuscript is accepted pending revision, the author will be responsible for completing the revision within 60 days.

Copyright