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ORIGINALS

The impact of endothelin-1 on the efficacy of anti-VEGF therapy: A rationale for dual antagonism

Mohamed MSA

Urokinase-type plasminogen activator and related microRNAs in hepatocellular carcinoma; a bioinformatic based study

Seydel GS, et al.

Tau protein expression and phosphorylation in a glucose-repressed yeast model: Insights into the cancer-alzheimer's disease link

Yilmazer M, et al.

Adaptive mitochondrial modules: Going with the flow of cancer-specific metabolic rewiring

Yildiz MT

Can cinnamon reduce endoplasmic reticulum stress in diabetic nephropathy?: An experimental rat model

Oztas B, et al.

Platelet-normalized biomarkers as diagnostic and prognostic indicators in crimean-congo hemorrhagic fever

Bolat S, et al.

Determination of analytical performances of NT-proBNP and aPTT tests with three methods

Yeğın D

Adult references intervals for thyroid hormones using beckman coulter from Türkiye

Sahin I, et al.

Evaluation of the analytical performance of the access vitamin B12 II assay with the new calibrator

Madenci OC, et al.



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CONTENTS VOLUME VIII ISSUE 4 YEAR 2025

EDITORIAL

From analytical precision to molecular insight: Integrating biomarker science across the clinical continuum Konukoglu D.....	XIII
--	------

ORIGINAL ARTICLES

The impact of endothelin-I on the efficacy of anti-VEGF therapy: A rationale for dual antagonism Mohamed MSA.....	251
Urokinase-type plasminogen activator and related microRNAs in hepatocellular carcinoma; a bioinformatic based study Seydel GS, Ayan D.....	261
Tau protein expression and phosphorylation in a glucose-repressed yeast model: Insights into the cancer-alzheimer's disease link Yilmazer M, Uzuner SK.....	272
Adaptive mitochondrial modules: Going with the flow of cancer-specific metabolic rewiring Yildiz MT.....	282
Can cinnamon reduce endoplasmic reticulum stress in diabetic nephropathy?: An experimental rat model Oztas B, Eraldemir FC, Akbal S, Acar E, Hunc F, Yardimoglu Yilmaz M.....	292
Platelet-normalized biomarkers as diagnostic and prognostic indicators in crimean-congo hemorrhagic fever Bolat S, Buyuktuna SA.....	300
Determination of analytical performances of NT-proBNP and aPTT tests with three methods Yeğin D.....	306
Adult references intervals for thyroid hormones using beckman coulter from Türkiye Sahin I, Eraldemir FC, Oztas B, Yildirim Şik B, Kir HM.....	312
Evaluation of the analytical performance of the access vitamin B12 II assay with the new calibrator Madenci OC, Kutukcu A.....	318



EDITORIAL

From analytical precision to molecular insight: Integrating biomarker science across the clinical continuum

The current issue of the International Journal of Medical Biochemistry exemplifies the journal's mission to connect analytical rigor with molecular understanding in laboratory medicine. The studies in this collection range from the discovery of biomarkers and the exploration of mechanisms to the validation of analyses and the establishment of reference intervals. Together, they show the diverse and connected nature of today's clinical biochemistry.

Mohamed MSA provides a thought-provoking perspective on endothelin-I and anti-VEGF therapy, presenting a rationale for dual antagonism to overcome therapeutic resistance in angiogenesis-driven diseases. In the field of infectious disease diagnostics, Bolat and Buyuktuna demonstrate that platelet-normalized biomarkers can serve as sensitive diagnostic and prognostic indicators in Crimean-Congo hemorrhagic fever, a valuable contribution to hematology-based prognostication.

At the interface of cancer biology and bioinformatics, Seydel and Ayan investigate the urokinase-type plasminogen activator system and related microRNAs in hepatocellular carcinoma at the interface of cancer biology and bioinformatics, identifying potential regulatory nodes for molecular intervention.

A series of analytical and reference interval studies reinforce the journal's foundation in clinical laboratory standardization and metrology. The analytical performance of NT-proBNP and aPTT is assessed by Yeğin across three methods. In another study, Sahin et al. establish adult reference intervals for thyroid hormones using Beckman Coulter analyzers in the Turkish population. Madenci and Kutukcu further evaluate the Access Vitamin B12 II assay with a new calibrator, emphasizing harmonization and quality assurance across analytical systems.

In the field of fundamental molecular and metabolic research, Yilmazer and Uzuner examine tau protein expression and phosphorylation in a glucose-repressed yeast model. Yildiz discusses how cancer cells adapt through dynamic metabolic rewiring via mitochondrial modules. Finally, Oztas and colleagues report that cinnamon supplementation attenuates endoplasmic reticulum stress in an experimental diabetic nephropathy model, offering insight into natural compound interventions targeting cellular stress pathways.

Together, these contributions capture the interdisciplinary essence of clinical biochemistry, where analytical performance, molecular biology, and translational relevance converge. By integrating laboratory precision with mechanistic depth, this issue reflects the journal's continuing commitment to advancing biomarker-driven diagnostics, monitoring, and therapy for various human diseases.

Prof. Dildar Konukoglu, MD.

Editor-in-Chief



Research Article

The impact of endothelin-1 on the efficacy of anti-VEGF therapy: A rationale for dual antagonism

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Abstract

Objectives: Angiogenesis-associated disease conditions are often treated with anti-angiogenic therapy. Many of the anti-angiogenic agents approved as adjuvant cancer therapy target the vascular endothelial growth factor (VEGF) axis, as VEGF signaling is regarded as the primary angiogenesis promoter. These drugs are expected to enhance immunity, antagonizing the immunosuppressive functions of VEGF, and to control angiogenesis. Despite a mechanistic rationale that strongly supports their benefits, anti-VEGF agents have shown limited success rates in most cases, along with an association with hypertensive side effects. This article briefly reviews the approved anti-VEGF agents and offers a possible explanation for their limitations.

Methods: PubMed and Scopus databases were searched with the corresponding keywords (such as anti-VEGF), and the relevant knowledge was collected. The included studies were limited to these, which report indications, responses, and side effects. In addition to the review, HuH7 and HEK293T cells were subjected to chemical induction of hypoxia by means of treatment with cobalt chloride (CoCl₂). This treatment induced hypoxia inducible factor 1 alpha (HIF-1) under normoxic conditions. Target protein levels were then assessed with immunoblotting to confirm the review results.

Results: The results support the fact that both VEGF and endothelin-1 (ET-1) levels are elevated in response to hypoxia. Consequently, the modulation of the proangiogenic and vasodilatory effects of the VEGF axis by anti-VEGF agents is anticipated to have an incomplete impact on angiogenesis, while resulting in hypertensive complications due to the ongoing proangiogenic activity and unopposed vasoconstrictive effects of endothelin-1.

Conclusion: Given the uncertainty regarding the capacity of anti-VEGF therapy to concurrently inhibit ET-1, the dual antagonism of VEGF and ET-1 appears to be the preferred approach for effective management of angiogenesis-related pathologies. Additional studies are necessary to validate this conclusion.

Keywords: Adjuvant therapy, angiogenesis, anti-VEGF therapy, endothelin-1, vascular endothelial growth factor

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Angiogenesis refers to the formation of new blood vessels through the migration, growth, and differentiation of endothelial cells. This process is regulated by various chemical signals within the human body, with some, such as vascular endothelial growth factor (VEGF) signaling, acting as promoters, while others function as inhibitors [1]. Under normal physiological conditions, there is a balance between angiogenesis-stimulating and inhibiting signals, ensuring the formation of new blood vessels only when and where

necessary, such as during growth or tissue repair. However, disruptions in this balance can lead to pathological conditions or diseases, such as angiogenesis in cancer and metastasis or in age-related wet macular degeneration [2].

Vascular endothelial growth factor A (VEGF-A) is one of the most important and extensively studied stimulators of angiogenesis [3]. It has become a target for numerous angiogenesis inhibitors, many of which have been approved or are in advanced clinical trials for adjuvant cancer treatment. Examples of these inhibitors

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Table 1. Examples of anti-VEGF agents considered for clinical application

Drug	Mechanism of action
Axitinib (Inlyta®)	A tyrosine kinase inhibitor capable of inhibiting the angiogenic effects mediated by VEGF receptors 1–3, c-KIT, and PDGFR.
Bevacizumab (Avastin®)	Monoclonal antibody against VEGF-A.
Cabozantinib (Cometriq®)	Impedes MET (hepatocyte growth factor receptor protein), VEGFR, RET (receptor tyrosine kinase), GAS6 receptor (AXL), KIT, and Fms-like tyrosine kinase-3 (FLT-3).
Lenvatinib mesylate (Lenvima®)	A multi-kinase inhibitor targeting VEGFR1, VEGFR2, and VEGFR3 kinases.
Pazopanib (Votrient®)	A multi-kinase inhibitor that targets and inhibits the vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), c-KIT, and fibroblast growth factor receptor (FGFR).
Ramucirumab (Cyramza®)	A direct competitive inhibitor of VEGFR2, exhibiting a high affinity for binding to the extracellular domain of VEGFR2, thereby preventing its interaction with the natural ligands (VEGF-A, VEGF-C, and VEGF-D).
Regorafenib (Stivarga®)	Exhibits binary-targeted inhibitory activity against the tyrosine kinases VEGFR2 and TIE2.
Sorafenib (Nexavar®)	A protein kinase inhibitor that demonstrates activity against VEGFR, PDGFR, and RAF kinases. Among the RAF kinases, sorafenib exhibits greater selectivity for C-Raf compared to B-Raf.
Sunitinib (Sutent®)	A multitargeted tyrosine kinase inhibitor capable of binding to PDGF receptors (PDGF-Rs), VEGF receptors (VEGFRs), CD117 (c-KIT), RET, CD114, and CD135.
Vandetanib (Caprelsa®)	Inhibits the tyrosine kinase activity of two concurrent pathways by targeting VEGFR-2 and the epidermal growth factor receptor (EGFR).
Aflibercept (EYLEA™)	A soluble fusion protein that binds all isoforms of VEGF-A, as well as VEGF-B and placental growth factor, thereby inhibiting their receptor activation.
Zivafibercept (Zaltrap®)	A soluble decoy protein for the VEGF receptors, VEGFR-1 and VEGFR-2.
Brolucizumab (Beovu®)	A 26 kDa single-chain monoclonal antibody fragment capable of inhibiting the activation of VEGF receptors.
Ranibizumab (Lucentis®)	A recombinant humanized monoclonal antibody fragment targeting VEGF-A.

are presented in Table 1. The success rates of these agents vary due to several factors. Table 2 and Table 3 summarize some reported clinical outcomes, revealing two key limitations of these agents: Limited success rates and hypertensive side effects.

Hypothesis (Aim of work)

The limitations reported in the experimental and clinical studies can be attributed to several potential factors, as illustrated in Figure 1:

Table 2. Reported clinical success rates of some anti-angiogenic agents

Drug	Reported findings
Axitinib	In patients with cytokine-refractory metastatic renal cell carcinoma, Axitinib has the potential to yield an estimated 5-year survival rate of 20.6% [4].
Bevacizumab	A total of 167 patients with recurrent glioblastoma were enrolled in a multicenter, phase II, randomized, noncomparative trial. Patients who experienced a first or second relapse with progression while on temozolomide were randomized to receive either bevacizumab (10 mg/kg) alone or in combination with irinotecan, administered in 2-week cycles. The objective response rates observed were 28% in the single-agent group and 38% in the combination group. Six-month progression-free survival rates were 43% for the bevacizumab monotherapy group and 50% for the combination group. The median overall survival was 9.2 months for the bevacizumab-only arm and 8.7 months for the combination arm. The most common side effects included hypertension, seizures, neutropenia, and fatigue [5].
Cabozantinib	The phase 3 CheckMate 9ER trial randomly assigned patients with renal cell carcinoma to receive either cabozantinib in combination with nivolumab or sunitinib. The study reported an objective response rate (ORR) of 55.7% for the cabozantinib/nivolumab combination, with a complete response (CR) rate of 12.4%. In contrast, sunitinib demonstrated an ORR of 28.4% and a CR rate of 5.2%. The median duration of response (DOR) was 23.1 months for the cabozantinib nivolumab regimen, compared to 15.1 months for sunitinib [6].
Lenvatinib	According to GlobalData, the success rate of the transition phase in the phase III trial evaluating lenvatinib mesylate in patients with colorectal cancer was 43% [7, 8].
Pazopanib	In the SPIRE study, 211 patients with advanced soft tissue sarcomas were treated with pazopanib as a second-line or subsequent therapy. The median treatment duration was 3.1 months. The median progression-free survival was 3 months, while the median overall survival was 11.1 months. The overall clinical benefit rate across most histological subtypes was 46% [9, 10].
Ramucirumab	The administration of Ramucirumab in 355 patients with gastro-esophageal cancer demonstrated a response rate of 4%. However, it also showed a disease stability rate of 45%, compared to 21% in the placebo group, yielding an overall disease control rate of 45% versus 23% in the placebo group [11].

Table 2. Cont.

Drug	Reported findings
Regorafenib	Patients with metastatic colorectal cancer treated with Regorafenib had a progression-free survival of 2.9 months (interquartile range: 2.2 to 4.4 months), an overall response rate of 4% (n=2), and a disease control rate of 40% (n=19) [12].
Sorafenib	The use of Sorafenib in advanced-stage hepatocellular carcinoma demonstrated the following outcomes: a median overall survival of 26.1 months, 6- and 12-month survival rates of 92.1% and 85%, respectively, a median time to radiological progression of 8 months, and a progression-free survival rate of 64.3% [13].
Sunitinib	Objective response rates of 47% for Sunitinib and 12% for IFN- α (p<0.001) were observed in patients with metastatic renal cell carcinoma. The primary Sunitinib-related adverse effects included hypertension (12%), fatigue (11%), diarrhea (9%), and hand-foot syndrome (9%) [14].
Vandetanib	The use of Vandetanib in patients with locally advanced or metastatic medullary thyroid carcinoma yielded a pooled complete response rate of 0.7% and a disease stabilization rate of 47%, as determined by the RECIST criteria [15].
Zivafibercept	Patients with colorectal cancer treated with Zivafibercept demonstrated a median overall survival of 13.5 months and a median progression-free survival of 6.9 months, in contrast to 12.06 months and 4.67 months, respectively, for those receiving a placebo. Similarly, the response rate for the Zivafibercept plus FOLFIRI combination was 19.8%, compared to 11.1% for the FOLFIRI-only group [16].
Dovitinib	In a mutation-specific, single-arm, phase 2 study involving 80 cancer patients with colorectal, gastrointestinal stromal, or ovarian cancers, Dovitinib demonstrated a clinical benefit rate of 13.8% [17]. In an open-label, randomized phase 3 clinical trial evaluating dovitinib as a third-line targeted treatment for patients with metastatic renal cell carcinoma, the drug resulted in an increase of 3.7 months in progression-free survival and 11.1 months in overall survival [18].

- Hypoxia and relative ischemia are commonly observed alongside the rapid growth of solid tumors [19].
- Hypoxic conditions result in a significant decrease in NOSTRIN (Nitric-Oxide Synthase Trafficking Inducer) levels [20].
- Under hypoxic conditions, hypoxia-inducible factor 1- α (HIF-1 α) forms a dimeric complex with HIF-1 β through nuclear translocation. This complex binds to the hypoxia response element (HRE), interacting with the coactivator p300, which subsequently enhances the expression of VEGF-A, matrix metalloproteinases (MMPs), angiopoietin, and platelet-derived growth factor (PDGF) [21].
- Low NOSTRIN levels are associated with increased activity of endothelial nitric oxide synthase III (eNOS) [22], which, in turn, elevates VEGF-A [23], thereby inducing the release of soluble VEGF receptor 1 (sVEGFR-1 or sflt-1) [24].
- The presence of soluble VEGF receptor 1 (sVEGFR-1) has been reported to enhance the vasoconstrictive activity of endothelin-1 (ET-1) [25].

Clinically, low NOSTRIN has been linked to tumor progression, prognosis, and metastasis [26, 27]. Elevated levels of eNOS and VEGFs have been similarly associated with tumor progression, prognosis, recurrence, and metastasis [28]. Furthermore, high endothelin-1 (ET-1) levels have been clinically correlated with tumor progression and metastasis [29].

Therefore, the inhibition of the proangiogenic and vasodilatory effects of the eNOS/VEGF axis by anti-VEGF agents may lead to inadequate control of angiogenesis, potentially resulting in hypertension. This could be attributed to the continued proangiogenic activity and the unopposed vasoconstrictive effects of the endothelin-1 axis. The objective of this work is to validate the aforementioned principles.

Role of eNOS/VEGF axis in angiogenesis

During the conversion of L-arginine to L-citrulline, endothelial nitric oxide synthase (eNOS) acts as a catalyst, leading to the production of nitric oxide (NO). NO plays a critical role in mediating the angiogenic activity of various factors, including vascular endothelial growth factor (VEGF). The activation of eNOS is partially regulated by the upstream Akt/protein kinase B signaling pathway [29]. The VEGF family consists of seven known members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF), non-human genome encoded VEGF-E, and snake venom VEGF (svVEGF) [15]. VEGF-A is vital for supporting the vascular endothelium and serves as a key regulator of angiogenesis, contributing to tumor growth, proliferation, invasion, metastasis, angiogenesis, and drug resistance [30]. VEGF-B is involved in promoting neuronal survival and cardiovascular development through angiogenesis in specific organs. The roles of VEGF-C and VEGF-D are particularly significant in tumor growth and metastasis, as they are implicated in VEGFR-3-mediated lymphangiogenesis and lymphatic metastasis [30].

Role of endothelin -1 axis in angiogenesis

Endothelin-1 (ET-1) exerts a direct angiogenic effect on endothelial and peri-vascular cells [31]. It plays a crucial role in cell growth and proliferation, and its effects are mediated through the activation of the MAPK pathway [32]. Consequently, ET-1 is actively involved in tumor angiogenesis. Furthermore, ET-1 can enhance VEGF expression and promote angiogenesis via its endothelin A receptor (ETAR), integrin-linked kinase (ILK), Akt, and hypoxia-inducible factor-1 α (HIF-1 α) signaling pathways [33].

Materials and Methods

A systematic review of the literature was conducted in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The search

Table 3. Summary of clinical outcomes for anti-VEGF agents in solid tumors

Drug	Phase (trial)	Study population	Sample size (N)	ORR (%)	PFS (months)	OS (months)	Adverse events
Axitinib	Phase III	Metastatic RCC	Not specified	20.6 (5-yr survival)	Not specified	20.6 (5-yr)	Hypertension
Bevacizumab	Phase II	Recurrent glioblastoma	167	28–38	6-month PFS: 43–50%	9.2	Hypertension, seizures, fatigue
Cabozantinib	Phase III	Metastatic RCC	651	55.7	23.1	Not specified	Not specified
Lenvatinib	Phase III	Colorectal cancer	Not specified	43	Not specified	Not specified	Not specified
Pazopanib	Phase II	Soft tissue sarcoma	211	Not specified	3.0	11.1	Not specified
Ramucirumab	Phase III	Gastro-esophageal cancer	355	4	Not specified	Not specified	Hypertension
Regorafenib	Phase III	Metastatic colorectal cancer	Unclear	4	2.9	Not specified	Hypertension
Sorafenib	Phase III	Advanced HCC	Not specified	Not specified	8.0	26.1	Hypertension
Sunitinib	Phase III	Metastatic RCC	Unclear	47	Not specified	Not specified	Hypertension
Vandetanib	Phase III	Medullary thyroid carcinoma	Unclear	0.7	Not specified	Not specified	Not specified
Ziv-aflibercept	Phase III	Colorectal cancer	Not specified	19.8	6.9	13.5	Hypertension
Dovitinib	Phase II	Various solid tumors	80	13.8	3.7	11.1	Not specified

VEGFR: Vascular endothelial growth factor receptor; ORR: Objective response rate; PFS: Progression-free survival; OS: Overall survival; RCC: Renal cell carcinoma; HCC: Hepatocellular carcinoma.

strategy included queries in PubMed and Scopus using the following keywords and Boolean combinations: “Anti-VEGF therapy,” “angiogenesis inhibitors,” “VEGF antagonists,” “endothelin-1,” “cancer angiogenesis,” and “clinical trials.” The review was limited to English-language articles published between 2005 and 2024.

Inclusion criteria

- Peer-reviewed clinical trials or meta-analyses evaluating anti-angiogenic therapies.
- Studies reporting specific efficacy outcomes (e.g., ORR, PFS, OS) or adverse events, such as hypertension).
- Trials involving FDA- or EMA-approved anti-VEGF agents.

Exclusion criteria

- Non-clinical studies unless providing essential mechanistic insights.
- Conference abstracts without full datasets.
- Duplicate publications or interim analyses of the same trial.

After screening by title/abstract and applying inclusion/exclusion criteria, the selected studies were considered as sources for the informations included in this article.

In addition, HuH7 and HEK293T cells were sourced from affiliated research groups. The cells were harvested and washed with phosphate-buffered saline (PBS). Three independent biological replicates were performed, with 200,000 cells seeded into culture wells (6-well plates) and incubated overnight in 2 mL of supplemented medium at 37 °C in a humidified atmosphere with 5% CO₂ and ≥95% relative humidity. The medium used was DMEM, supplemented with 10% FBS (Gibco), 1X sodium pyruvate, 1X penicillin-streptomycin (Gibco), 1X Glutamax (Gibco), and 25 mM HEPES. The cells were subsequently treated with CoCl₂ (Sigma-Aldrich) according to the experimental protocol outlined below:

- Control non treated cells
- Cells treated with 200µM CoCl₂
- Cells treated with 300µM CoCl₂
- Cells treated with 400µM CoCl₂

Cells were incubated under the same conditions for an additional 72 hours before harvesting and subsequent processing. The impact of the treatments on HIF-1α and its target proteins was evaluated through immunoblotting, which was performed according to standard laboratory protocols. Equal amounts of total protein (50 µg per lane) were resolved on a 10% SDS-PAGE gel (Bio-Rad) and transferred to a nitrocellulose membrane via wet transfer. Membranes were blocked with 5% non-fat dry milk in TBST (Tris-buffered saline with 0.1% Tween-20) prior to antibody incubation. Primary antibodies specific to the target proteins were obtained from Proteintech, Germany. Band densities were analyzed using ImageJ software. An unpaired t-test was applied to compare the values of the experimental conditions to the control, with P-values less than 0.05 considered statistically significant.

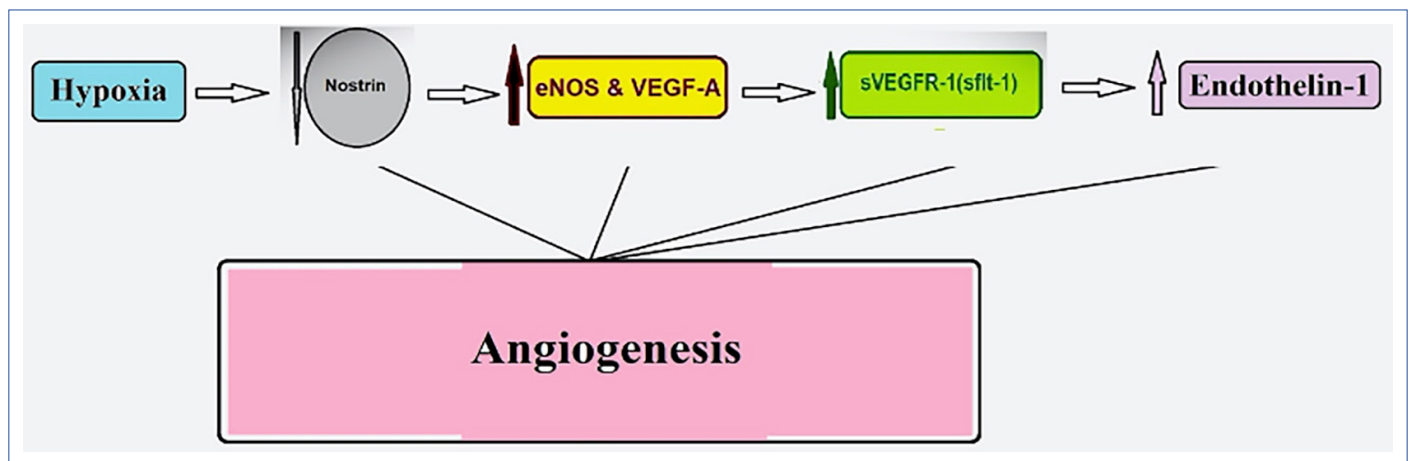


Figure 1. Diagrammatic representation of the article's hypothesis. Hypoxia is associated with decreased NOSTRIN and leads to increased eNOS, VEGF-A, sVEGFR-1 and ET-1.

eNOS: Nitric oxide synthase III; VEGF: Vascular endothelial growth factor; sVEGFR-1: Soluble VEGF receptor 1; ET-1: Endothelin-1; NOSTRIN: Nitric-Oxide Synthase Trafficking Inducer.

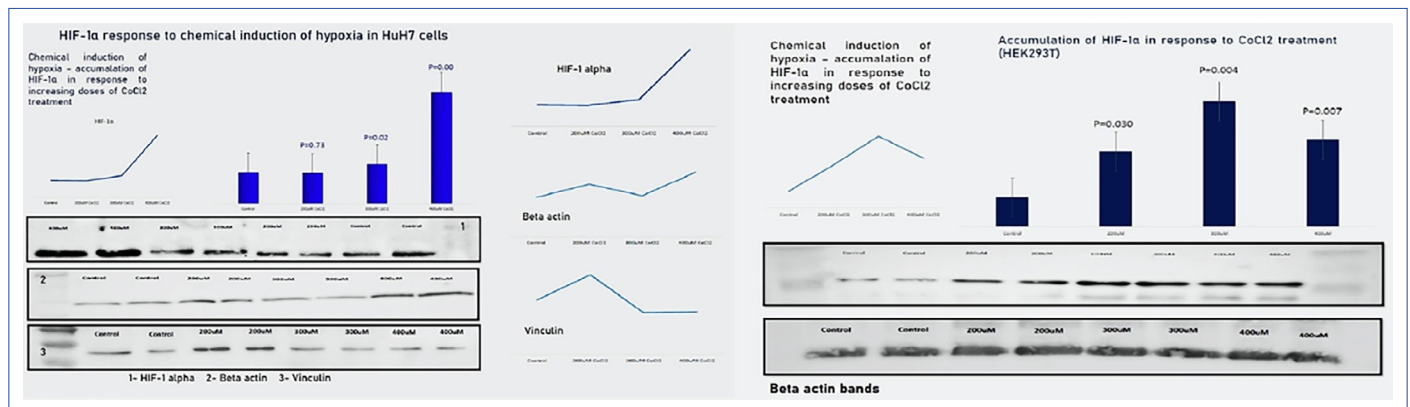


Figure 2. HIF-1α induction in HuH7 and HEK293T cells following treatment with various concentrations of CoCl₂. HIF-1α band intensities were normalized to their respective loading controls. Protein concentrations were estimated using the Bradford assay prior to gel loading. Equal amounts of total protein were loaded for each sample, and vinculin and β-actin were used as loading controls. Vinculin was favored over β-actin as a reference, given reports of β-actin's reactivity to hypoxia—an effect observed at the 400 μM treatment in HuH7 cells. CoCl₂ treatment induced dose-dependent changes in HIF-1α expression in HuH7 cells (p=0.73, 0.02, and 0.0001 for 200 μM, 300 μM, and 400 μM, respectively) and in HEK293T cells (p=0.03, 0.004, and 0.007 for 200 μM, 300 μM, and 400 μM, respectively).

HIF-1α: Hypoxia-inducible factor 1-alpha.

Results

The chemical induction of hypoxia was successfully achieved, as evidenced by the upregulation of HIF-1α (Fig. 2). In response to HIF-1α activation, the secretion of eNOS, VEGF-A, sVEGFR1, and Endothelin-1 (ET-1) was elevated in a dose-dependent manner (Fig. 3). Similar experiments conducted on human umbilical vein endothelial cells (HUVECs) also demonstrated an increase in ET-1 secretion (Fig. 4).

Discussion

The selected target proteins are well-established effectors that play crucial roles in endothelial physiology and vascular pathology. These proteins serve as indicators of tissue hypoxia and are involved in the process of angiogenesis [31]. To mitigate potential variations that may arise in studies uti-

lizing physical hypoxia, such as differences in the type (e.g., sustained or intermittent), duration (e.g., short-term or long-term), or extent of hypoxia, this study employed the previously validated chemical induction of hypoxia through the use of CoCl₂, which promotes the accumulation of HIF-1α and HIF-2α under normoxic conditions [34].

This study demonstrates that the secretion of ET-1 and VEGF-A increases concurrently in response to hypoxia, prior to the statistical significance peak of HIF-1α. This observation suggests that both effectors may exhibit heightened sensitivity to hypoxia and/or play simultaneous leading roles in the tissue's response to hypoxia, particularly at the paracrine and/or remote levels.

The roles of eNOS, NO, and VEGF-A in angiogenesis have been extensively investigated and thoroughly documented. The majority of clinically approved anti-angiogenic therapies target this specific pathway (Table 1). VEGF-A stim-

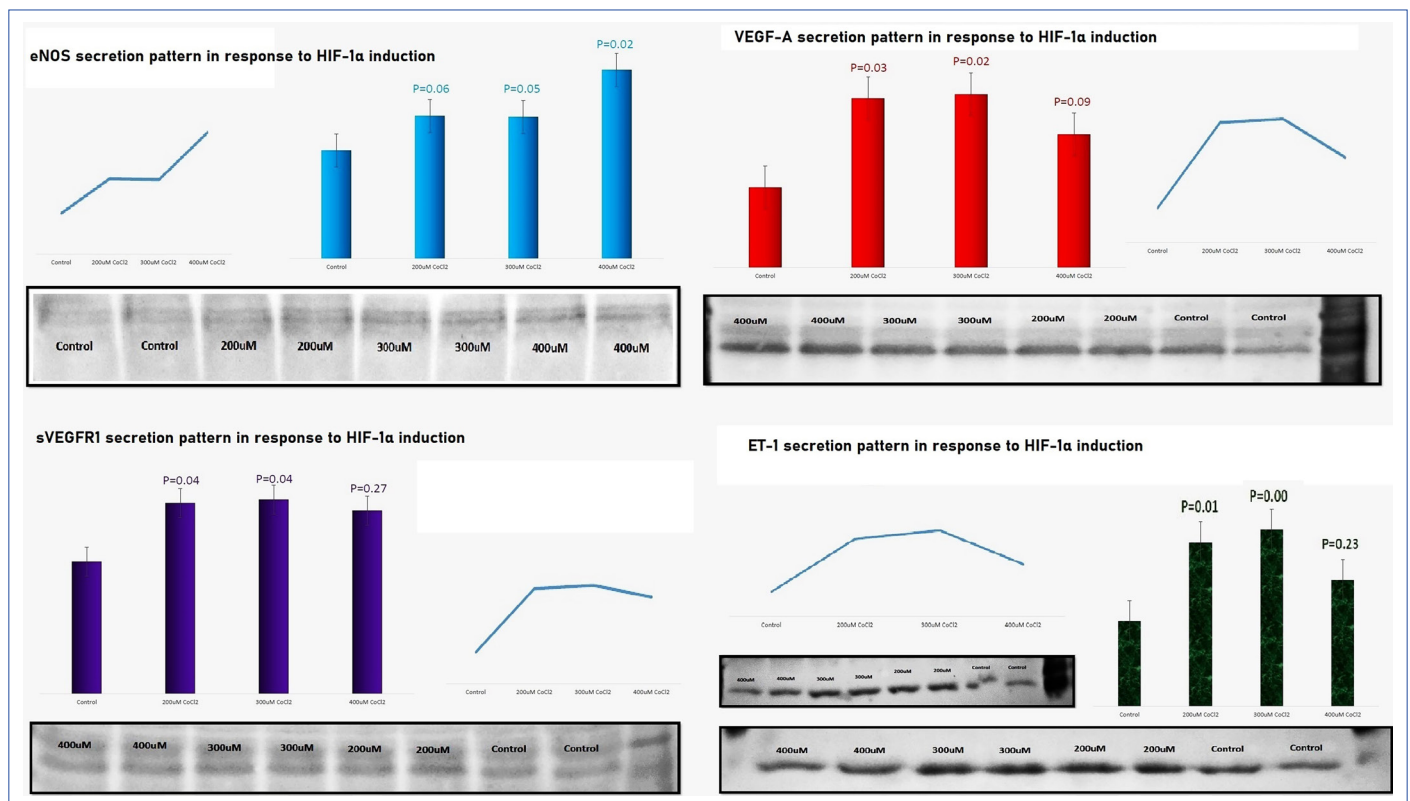


Figure 3. Secretion patterns of eNOS, VEGF-A, sVEGFR1, and ET-1 in response to chemically induced hypoxia. Band densities were quantified using ImageJ software. Each treatment group was compared independently to the control using an unpaired t-test. A P-value of less than 0.05 was considered statistically significant. No corrections for multiple comparisons were applied, as the values were analyzed independently. eNOS: Nitric oxide synthase III; VEGF: Vascular endothelial growth factor; sVEGFR-1: Soluble VEGF receptor 1; ET-1: Endothelin-1; NOSTRIN: Nitric-Oxide Synthase Trafficking Inducer.

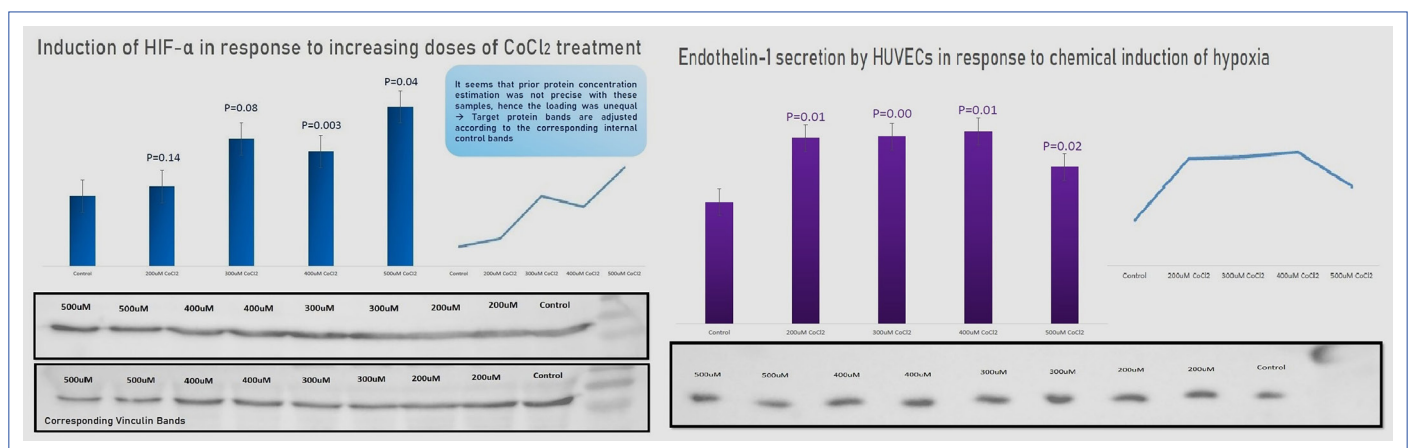


Figure 4. Effect of various doses of CoCl₂ treatment on HIF-1 α in HUVECs and the corresponding changes in ET-1 secretion. The induction of HIF-1 α was associated with corresponding increase in ET-1 secretion ($p < 0.05$).

HIF-1 α : Hypoxia-inducible factor 1-alpha; ET-1: Endothelin-1; HUVECs: Human umbilical vein endothelial cells.

ulates eNOS expression and enhances NO production by vascular endothelial cells. A reduction in NO production impairs angiogenesis and decreases the vascular permeability typically induced by VEGF-A [35].

Similarly, the elevated secretion of ET-1 by cultured cells in response to hypoxia has been previously reported, [36] along with other contradictory findings. *In-vivo* preclinical and clinical

studies have also reported similar outcomes, with intermittent hypoxia linked to ET-1 overexpression in animal models [37–39], and chronic intermittent hypoxia, as observed in patients with obstructive sleep apnea, associated with the accumulation of HIF-1 α and elevated circulating ET-1 levels [40–42]. Increased circulating ET-1 levels have been associated with vascular complications and endothelial dysfunction in humans [43].

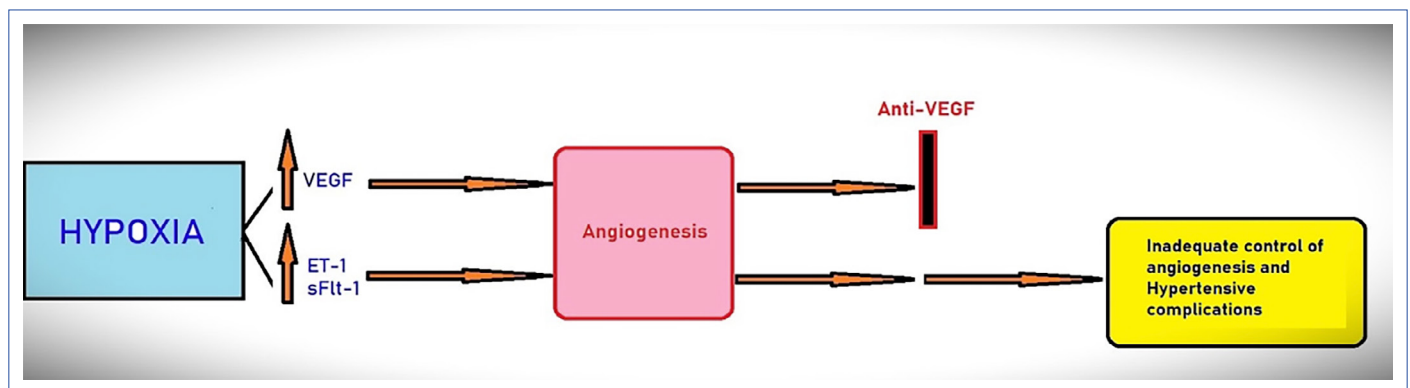


Figure 5. Diagrammatic representation of the main conclusion of the work: Anti-VEGF therapy may antagonize the effects of VEGF during hypoxia-induced angiogenesis; however, sVEGFR1 (sFlt-1) and ET-1 would remain elevated, which may explain the reported low success rates and hypertensive side effects.

VEGF: Vascular endothelial growth factor; sVEGFR-1: Soluble VEGF receptor 1; ET-1: Endothelin-1.

A recent study examined the impact of sustained and intermittent hypoxia (SH and IH, respectively) on HIF-1 α , VEGF, and ET-1 in HepG2 cells (hepatocellular carcinoma cell line). The study found an overexpression of HIF-1 α and VEGF in response to IH, but not to SH, whereas no such effect was observed for ET-1 [44]. While these findings may seem contradictory to those of the present study, several key considerations should be taken into account when interpreting these results; the hypoxia induction in the study was achieved physically through exposure to a low oxygen gas mixture. The cells employed were of cancerous origin, which may be associated with specific proangiogenic alterations that could make it challenging to detect additional induction of ET-1. In other words, cancerous cells may undergo a degree of hypoxia in culture, as indicated by their accelerated growth rates. As demonstrated in my experiments, ET-1 secretion increased with a 200 μ M CoCl₂ treatment but tended to decrease at the 400 μ M treatment, where HIF-1 α exhibited its peak expression (Fig. 3). In addition, the primary findings of this study focus on ET-1 secretion, which may not directly correspond to changes in mRNA or protein expression levels. In the context of cancer-related angiogenesis, where localized relative hypoxia is a constant feature of the tumor microenvironment, multiple studies have documented elevated circulating levels of ET-1 [45]. Therefore, the results of the present study appear to reflect a more realistic scenario.

Hypoxia-induced VEGF also stimulates the production of its truncated soluble form, VEGFR1, via the VEGFR-2-MEK-PKC signaling pathway, [46] which functions as a regulatory mechanism to prevent excessive activity. The ultimate consequence of hypoxia is the activation of angiogenesis, [47] a process characterized by a balance between proangiogenic and anti-angiogenic factors. Soluble VEGFR1 is part of the endogenous anti-angiogenic factors that help protect against uncontrolled angiogenesis, although it may also be actively involved in angiogenesis [48, 49]. These findings align with the results of the current study, which demonstrated an increase in sVEGFR1 following chemical induction of hypoxia (Fig. 3). However, a study reporting contradictory findings indicated that hypoxia led to a reduction in sVEGFR1

expression. This discrepancy may be attributed to the fact that their experiments were conducted on human microvascular endothelial cells isolated from neonatal dermis [50].

Soluble VEGFR-1 plays a significant role in angiogenesis, where perivascular cells interact with its isoforms via GM3 ganglioside. This interaction impacts actin cytoskeleton dynamics by destabilizing pericyte-endothelial cell interactions and altering adhesion contacts with the basement membrane, thereby contributing to vessel sprouting [51]. Moreover, the presence of sVEGFR-1 has been shown to shift $\alpha 5 \beta 1$ integrin signaling from a traditional adhesion pathway to a more dynamic one [52], while also enhancing its expression [53]. Consequently, the presence of sVEGFR-1 in the endothelial cell microenvironment during vessel sprouting is crucial [54]. These findings support the critical role of sVEGFR-1 in vessel sprouting and angiogenesis through mechanisms beyond VEGF binding [55], which aligns with the conclusions of the present study.

The intervention in the present study involved CoCl₂ treatment (chemical induction of HIF-1 α), and thus, the observed changes can be attributed to the activities of HIF-1 α . While the dependence of certain effectors on the upregulation or downregulation of others may be somewhat less considered based on the variations in response to different treatments, though it cannot be completely excluded yet.

The aim of this article was to confirm the dual activation of the eNOS/VEGF and ET-1 axes in response to hypoxia, which has been experimentally demonstrated, as well as to investigate the persistence of ET-1 activation despite anti-VEGF therapy (Fig. 5). As previously mentioned, the concurrent or parallel pattern of changes in VEGF-A and ET-1 secretion in response to hypoxia may suggest independent responses of both effectors. However, the appropriate approach to fully address this issue would have involved the introduction of various anti-VEGF agents followed by a reassessment of the levels of both effectors. Due to significant resource limitations, this investigation has not yet been conducted; thus, this issue will be further discussed based on existing published literature and experimental findings.

The secretion of VEGF-A in response to hypoxia persists for as long as the hypoxic stimulus is present [56]. Hypoxia induces angiogenesis, which is the process of new vessel formation [57]. Although the precise sequence of events remains unclear, this process involves both vascular endothelial and smooth muscle cells. VEGF-A acts as a specific mitogen for vascular endothelial cells, promoting their proliferation, while ET-1 stimulates the proliferation of vascular smooth muscle cells [58]. Therefore, both effectors are expected to increase concurrently, as observed in my experiments. Furthermore, it is anticipated that each effector can influence the expression of the other [58, 59]. From a biological perspective, the reduction in ET-1 observed following anti-VEGF therapy, despite the persistence of hypoxia and/or the initial stimulus, should be limited to the inhibition of the additional induction caused by VEGF overexpression, as its actions are suppressed by the therapy [60]. However, the response to the initial stimulus may remain unaffected. Consequently, reports of decreased ET-1 levels after anti-VEGF therapy may reflect scenarios where the stimulus for abnormal angiogenesis is simultaneously eliminated during the therapy [61]. In contrast, when the pathology persists, increased ET-1 levels have been observed post-therapy [61]. Therefore, in response to hypoxia and tumor-associated angiogenesis, driven by relative hypoxia within the tumor microenvironment, a reduction in ET-1 due to anti-VEGF therapy cannot be anticipated. For instance, VEGF-A, which normally reduces ET-1 production by 29%, loses this capability when its VEGFR2 receptor is blocked by SU5416, resulting in a 16% increase in ET-1 production under therapy [62].

Nevertheless, the current study demonstrated a notable increase in the levels of sVEGFR1 in the culture medium following hypoxia induction (Fig. 3). Soluble VEGFR1 is an endogenous antagonist of VEGF, and pharmacological anti-VEGF monoclonal antibodies exhibit structural and functional similarities to it [63]. Despite the elevation of sVEGFR1 in the culture medium, a significant increase in ET-1 levels was also observed (Fig. 3).

Conclusion

In summary, the limited success rates of anti-VEGF agents as adjuvant therapies in cancer treatment may be attributed to the principles discussed above. Additionally, any hypertensive side effects associated with these agents may be linked to the unopposed increase in ET-1 (Fig. 5). To achieve effective angiogenesis control without inducing hypertension, a dual antagonism of VEGF and ET-1 may be considered. Preclinical and clinical studies are necessary to evaluate the efficacy of such a dual therapy. Furthermore, since the secretion of the four effectors (eNOS, VEGF-A, sVEGFR1, and ET-1) significantly increases in response to hypoxia, which is a hallmark of angiogenesis, their levels may serve as biomarkers for monitoring the efficacy of therapy in angiogenesis-related pathologies, both before and after treatment. Thus, the SHEHATA MARKER OF ANGIOGENESIS has been introduced as a biomarker panel and is planned for further clinical validation [64].

Informed Consent: Not applicable. This study did not involve human participants, and no new patient data were collected or used. All data are derived from previously published sources or the author's own experiments on commercial cell lines.

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Research Article

Urokinase-type plasminogen activator and related microRNAs in hepatocellular carcinoma; a bioinformatic based study

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Abstract

Objectives: Hepatocellular carcinoma (HCC) is one of the most prevalent cancers worldwide. Urokinase-type plasminogen activator (uPA), which is encoded by the PLAUGene, is a serine protease involved in the degradation of the extracellular matrix. Increasing evidence indicates that PLAUGene is overexpressed in various cancers and is associated with poor prognosis, making it a potential biomarker for cancer. However, its potential role in HCC remains unclear. Therefore, this study aimed to investigate the role of PLAUGene and related microRNAs in HCC using multiple bioinformatics tools.

Methods: PLAUGene expression was evaluated using the TNMplot and GEPIA2 databases. Promoter methylation levels were assessed through UALCAN. Survival analysis (overall survival (OS) and recurrence-free survival (RFS) rates), was conducted using the Kaplan-Meier Plotter. Protein-protein interaction networks were examined with STRING. Target miRNAs were identified using TargetScan 8.0. Differential expression, survival analysis, and co-expression of miRNAs were investigated using ENCORI.

Results: PLAUGene expression was significantly upregulated in liver hepatocellular carcinoma (LIHC) compared to normal tissues ($p < 0.05$). Promoter methylation level of PLAUGene was significantly increased (hypermethylation) in LIHC tissues ($p = 5.43 \times 10^{-12}$). Elevated PLAUGene expression was not associated with OS ($p = 0.16$) and RFS ($p = 0.28$) rates. hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-181c-5p, and hsa-miR-181d-5p were positively correlated with PLAUGene in LIHC tissue ($p < 0.05$). The hsa-miR-181a-5p and hsa-miR-181b-5p were up-regulated in LIHC ($p < 0.05$).

Conclusion: In conclusion, our study highlights the potential role of PLAUGene and its related miRNAs (hsa-miR-181a-5p and hsa-miR-181b-5p) in HCC. However, elevated PLAUGene expression did not correlate with survival rates, indicating its involvement in tumor development but no prognostic significance. Further applicable studies are needed on this subject.

Keywords: Bioinformatic analysis, hepatocellular carcinoma, DNA methylation, microRNA, PLAUGene, prognosis, urokinase-type plasminogen activator

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Hepatocellular carcinoma (HCC), which constitutes approximately 90% of primary liver cancers, is one of the most prevalent cancers globally, ranking sixth in incidence and third in cancer-related mortality [1, 2]. HCC remains a significant global health concern, with rising incidence rates observed in both developed and developing countries [3, 4]. The

pathogenesis of HCC involves a complex array of molecular alterations, such as cell cycle dysregulation, immune modulation, DNA methylation changes, epithelial-mesenchymal transition (EMT), and microRNA (miRNA) dysregulation [5]. HCC is characterized by poor overall survival and a high recurrence rate [4]. Despite notable advances in surgical interventions,

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targeted therapies, and imaging techniques, the overall survival rates remain low. Early-stage cases may benefit from surgical approaches such as hepatic resection, liver transplantation, and local/regional therapies, while options for advanced stages remain limited, with median survival of 6 to 8 months. Therefore, understanding the mechanisms underlying HCC and identifying new biomarkers is crucial for improving early diagnosis, prognosis, and treatment [4–6].

Urokinase-type plasminogen activator (uPA), encoded by the PLA_U gene, is a serine protease that converts inactive plasminogen into active plasmin. This process plays a crucial role in the degradation of the extracellular matrix (ECM) and the basement membrane [7]. Such degradation facilitates cancer cell invasion and serves as a critical initial step in tumor progression. Numerous studies have demonstrated that uPA is integral to various stages of tumor progression, including tumor cell proliferation, migration, angiogenesis, and EMT [7–9]. Furthermore, research has shown that PLA_U expression, and consequently uPA levels, are significantly elevated in tumor cells, with higher PLA_U expression strongly correlating with poor prognosis [7, 8, 10–12]. Additionally, PLA_U levels have been found to be markedly increased in HCC; however, the number of studies on this topic remains limited [13–16].

MiRNAs are small non-coding RNA molecules that regulate target gene expression by binding to specific mRNAs, serving as key modulators of post-transcriptional gene silencing. They play a crucial role in the initiation and progression of cancer and are considered potential biomarkers for cancer diagnosis and treatment [17, 18]. The role of PLA_U in HCC, particularly its interaction with miRNAs, remains poorly understood. To date, no research has explored the relationship between PLA_U and its associated miRNAs in HCC. Therefore, this study aimed to investigate the role of PLA_U and related miRNAs in HCC using various bioinformatics tools.

Materials and Methods

Statement of ethics

Data for this study were retrieved from various publicly available databases; therefore, ethical approval was not necessary.

The analysis of differential gene expression of PLA_U using the tnmplot database

The TNMplot database (<http://www.tnmplot.com/>, accessed on December 12, 2024) is an online tool designed for analyzing differential gene expression in tumor, normal, and metastatic tissues. This resource comprises 56,938 unique samples collected from the Gene Expression Omnibus (GEO), the Genotypic-Tissue Expression (GTEx), the Cancer Genome Atlas (TCGA), and the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) databases [19]. We utilized the TNMplot database to investigate the differential gene expression of PLA_U in various tumor tissues and normal tissues derived from the TCGA (adjacent normal) and GTEx (healthy normal) datasets.

The analysis of PLA_U gene expression in LIHC using the GEPIA2 database

Gene Expression Profiling Interactive Analysis, version 2 (GEPIA2, <http://gepia2.cancer-pku.cn>, accessed on December 12, 2024), is a comprehensive bioinformatics tool designed for analyzing gene expression data derived from the TCGA and GTEx databases [20]. We used the GEPIA2 platform to analyze the expression levels of PLA_U in liver hepatocellular carcinoma (LIHC) tumor tissues compared to adjacent normal tissues from TCGA and health normal tissues from the GTEx database, using the “Match TCGA normal and GTEx data” option. Additionally, PLA_U expression was examined across different LIHC subtypes.

The analysis of gene expression and promoter methylation of PLA_U using the UALCAN database

The University of Alabama at Birmingham CANcer (UALCAN, <http://ualcan.path.uab.edu>, accessed on December 12, 2024) is an interactive and comprehensive web-based resource for the analysis of cancer OMICS data, including gene expression and promoter methylation profiles derived from TCGA datasets [21]. In this study, the UALCAN platform was utilized to evaluate PLA_U gene expression and promoter methylation levels in LIHC tissues compared to adjacent normal tissues. Gene expression analysis was performed across various clinical characteristics, including race, gender, age, weight, and nodal metastasis status.

The survival analysis of PLA_U in LIHC using the kaplan-meier plotter database

Kaplan-Meier plotter (KM plotter, <http://kmplot.com/analysis>, accessed on December 12, 2024) is a web-based database designed to explore the relationship between gene expression and prognosis across 21 different types of cancer using clinical data [22]. We utilized this database to investigate the overall survival (OS) and relapse-free survival (RFS) rates of PLA_U in LIHC tissue.

The analysis of the protein-protein interaction network and biological process (Gene Ontology) enrichment analysis of PLA_U using the STRING database

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, <https://string-db.org/>, accessed on December 12, 2024) is a widely used online database for exploring and predicting protein-protein interaction (PPI). Its objective is to establish a comprehensive and objective global network that encompasses both physical and functional interactions between two or more proteins [23]. We utilized the STRING database to examine the PPI networks and the biological process (Gene Ontology) enrichment of PLA_U.

The analysis of target miRNAs using the TargetScan 8.0 database

TargetScan 8.0 (https://www.targetscan.org/vert_80/, accessed on December 12, 2024) is a web resource utilized for predicting the target genes of miRNAs [24]. TargetScan

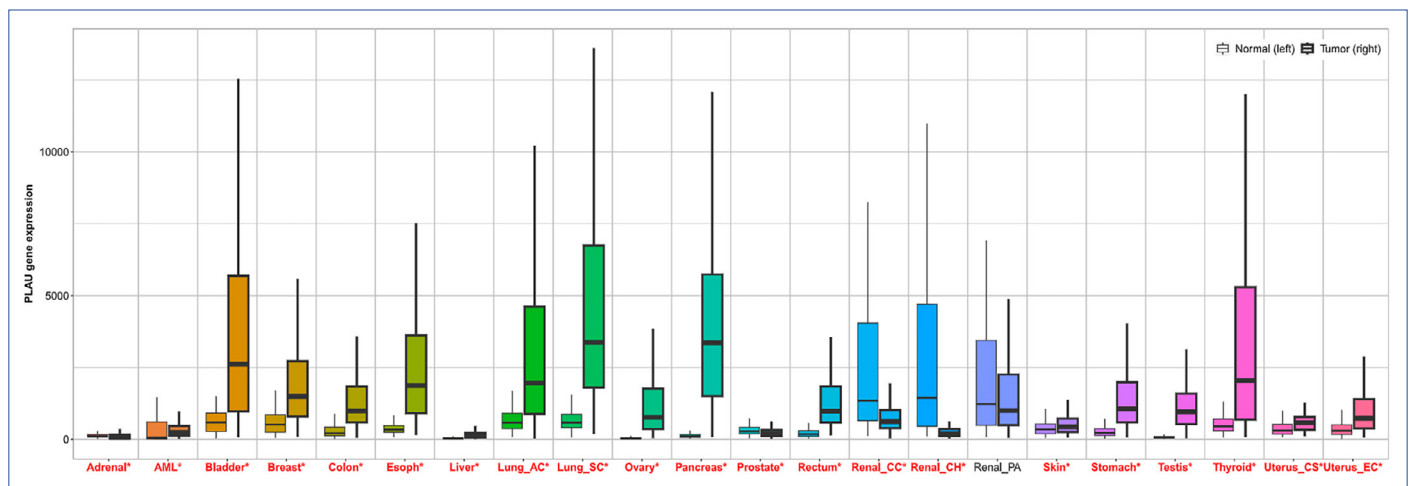


Figure 1. Box plots illustrating the differential PLAU expression analysis in normal (left) and tumor (right) tissues in TNMplot database.

Significant differences are indicated in red, with * $p < 0.05$.

predicts the biological targets of miRNAs by identifying conserved 8mer, 7mer, and 6mer sites that align with the seed region of each miRNA [25]. Furthermore, it provides predictions that encompass poorly conserved sites and nonconserved miRNAs. The tool also identifies sites with seed region mismatches that are compensated by conserved 3' pairing [26]. In mammals, predictions are prioritized based on their estimated targeting efficacy, which is determined using a biochemical model of miRNA-mediated repression. This model has been extended to all miRNA sequences through the application of a convolutional neural network [24]. We used this database to identify the target miRNAs of PLAU.

The analysis of differential expression, survival analysis, and co-expression of miRNAs using the ENCORI database

The Encyclopedia of RNA Interactomes (ENCORI, <https://rnasysu.com/encori/panCancer.php>, accessed on December 12, 2024) Pan-Cancer analysis platform is a comprehensive tool developed to decode Pan-Cancer Networks of long non-coding RNAs (lncRNAs), miRNAs, pseudogenes, small nucleolar RNAs (snRNAs), RNA-binding proteins (RBPs), and all protein-coding genes by analysing their expression profiles across 32 different cancer types [27]. We performed the this database to analyze differential expression, survival analysis, and co-expression of miRNAs in LIHC and normal tissues.

Statistical analysis

All statistical analyses were conducted using the default or recommended settings of each database. In the TNMplot database, the Mann–Whitney U test was used, and $p < 0.05$ was considered statistically significant. In the GEPIA2 database, differential expression analysis was performed using $|\log_2 \text{ fold change}| > 1$ and $p\text{-value} < 0.01$ as cut-off criteria. The data were $\log_2(\text{TPM}+1)$ transformed, and a one-way ANOVA was applied for comparisons. Additionally, pathological stage analysis was conducted using the same platform. In the UALCAN database, a Student's t-test was employed with statistical signifi-

cance defined as $p < 0.05$. In the Kaplan–Meier Plotter, survival analysis was performed using log-rank p-values to compare high and low expression groups in LIHC, with auto-selected best cutoff, and significance was set at $p < 0.05$. In the STRING database, $p < 0.05$ was considered statistically significant for PPI network analysis, and $\text{FDR} < 0.05$ was used for biological process enrichment analysis. In the ENCORI database, expression levels were presented as $\log_2(\text{RPM}+0.01)$.

Results

The differential gene expression of the PLAU in various tumor tissues

We conducted a pan-cancer analysis of the TNMplot database to evaluate the expression of PLAU across 22 different tumor types. The results demonstrated that PLAU was significantly expressed in 21 out of the 22 tumor tissues analyzed. As shown in Figure 1, PLAU expression was significantly upregulated in the adrenal, acute myeloid leukemia (AML), bladder, breast, colon, esophagus, liver, lung adenocarcinoma (lung-AC), lung squamous cell carcinoma (lung-SC), ovary, pancreas, rectum, skin, stomach, testis, thyroid, and both subtypes of uterine carcinoma (uterus-CS and uterus-EC) when compared to normal tissues. In contrast, PLAU expression was downregulated in prostate, renal clear cell carcinoma (renal-CC), and renal chromophobe carcinoma (renal-CH) tumor tissues ($p < 0.05$). There was no statistically significant difference in PLAU expression in renal papillary adenocarcinoma (renal-PA) ($p > 0.05$).

The gene expression level of PLAU in LIHC

We examined the expression levels of PLAU in LIHC tissues ($n=369$) compared to normal tissues ($n=160$) using GEPIA2. The results indicated that PLAU expression was significantly upregulated in LIHC compared to normal tissues ($p < 0.01$) (Fig. 2a). Box plot of the subtypes revealed that PLAU levels were upregulated in LIHC tissues compared to normal tissues in both iCluster_1 and iCluster_2 ($p < 0.01$). However, no signifi-

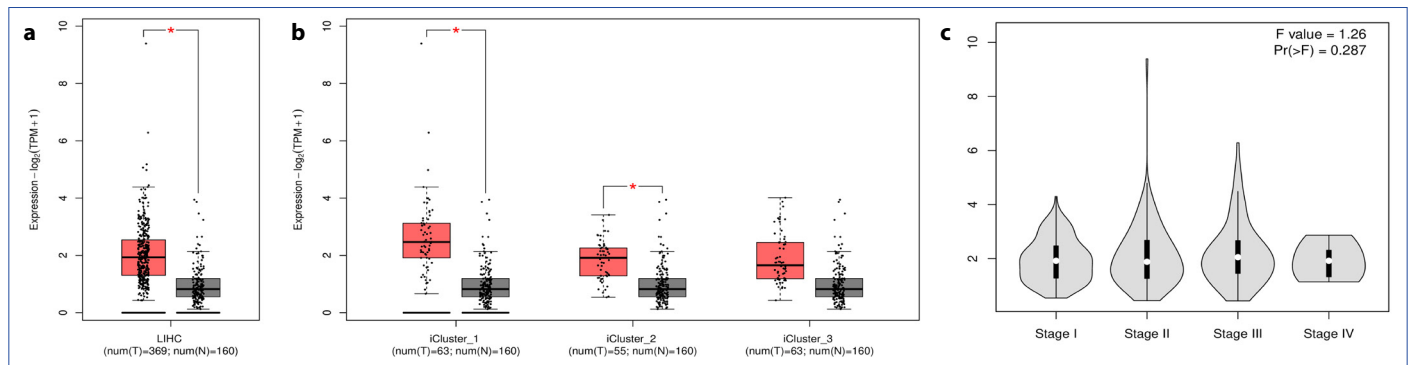


Figure 2. PLAU expression in LIHC tissue. (a) The box plot illustrating PLAU expression levels in LIHC (red) compared to normal tissues (gray) in GEPIA2 database. (b) The box plot showing PLAU expression levels at different iCluster groups in GEPIA2 database (c) The violin plot depicting PLAU expression levels at different stages of LIHC in GEPIA2 database.

Significant differences are indicated in red, with * $p < 0.01$. LIHC: Liver hepatocellular carcinoma.

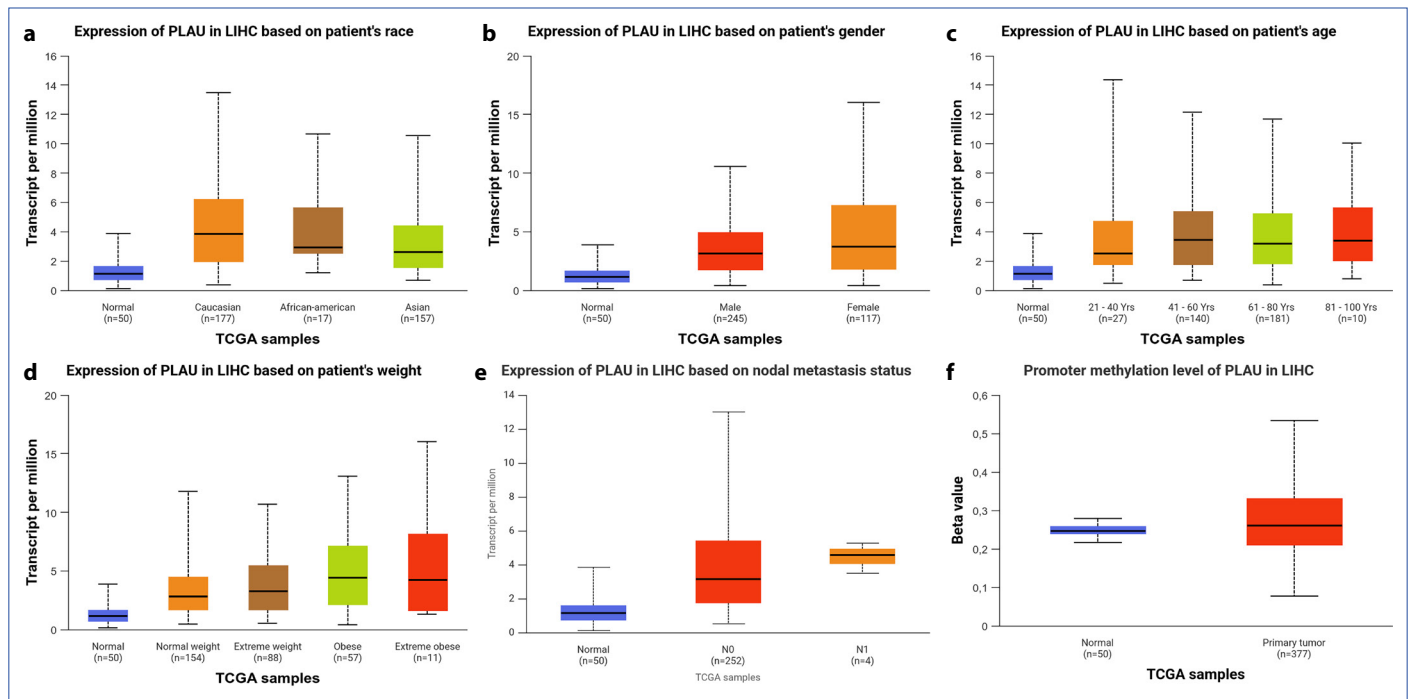


Figure 3. Box plots illustrating the gene expression and promoter methylation levels of PLAU in LIHC tissues compared to adjacent normal tissues using the UALCAN database. PLAU expression levels are shown based on (a) race, (b) gender, (d) age, (d) weight, and (e) nodal metastasis status. (f) Promoter methylation levels of PLAU.

TCGA: The cancer genome atlas.

cant difference was observed in iCluster_3 (Fig. 2b). Additionally, violin plot of the pathological stages showed no statistically significant differences among stages I, II, III, and IV of LIHC ($F = 1.26$; $Pr(>F) = 0.287$) (Fig. 2c).

The gene expression and promoter methylation level of PLAU in LIHC

The expression of PLAU in LIHC was analyzed based on patients' race, gender, age, weight and nodal metastasis status using TCGA data via the UALCAN platform. The results indicated that PLAU expression was significantly upregulated in tumor tissues compared to adjacent normal tissues in Cau-

casian ($p = 4.1 \times 10^{-15}$) and African-American ($p = 0.016$) patients, while no statistically significant difference was observed in Asian patients ($p = 0.089$). Additionally, there were no significant differences in PLAU expression among racial groups (Caucasian vs. African-American: $p = 0.991$; Caucasian vs. Asian: $p = 0.341$; African-American vs. Asian: $p = 0.355$) (Fig. 3a). PLAU expression was significantly upregulated in both male ($p = 0.0362$) and female ($p = 7.05 \times 10^{-11}$) patients compared to adjacent normal tissues. However, there was no statistically significant difference in PLAU expression between male and female patients ($p = 0.342$) (Fig. 3b). Age-stratified analysis showed significant upregulation of PLAU expression in the 21–40

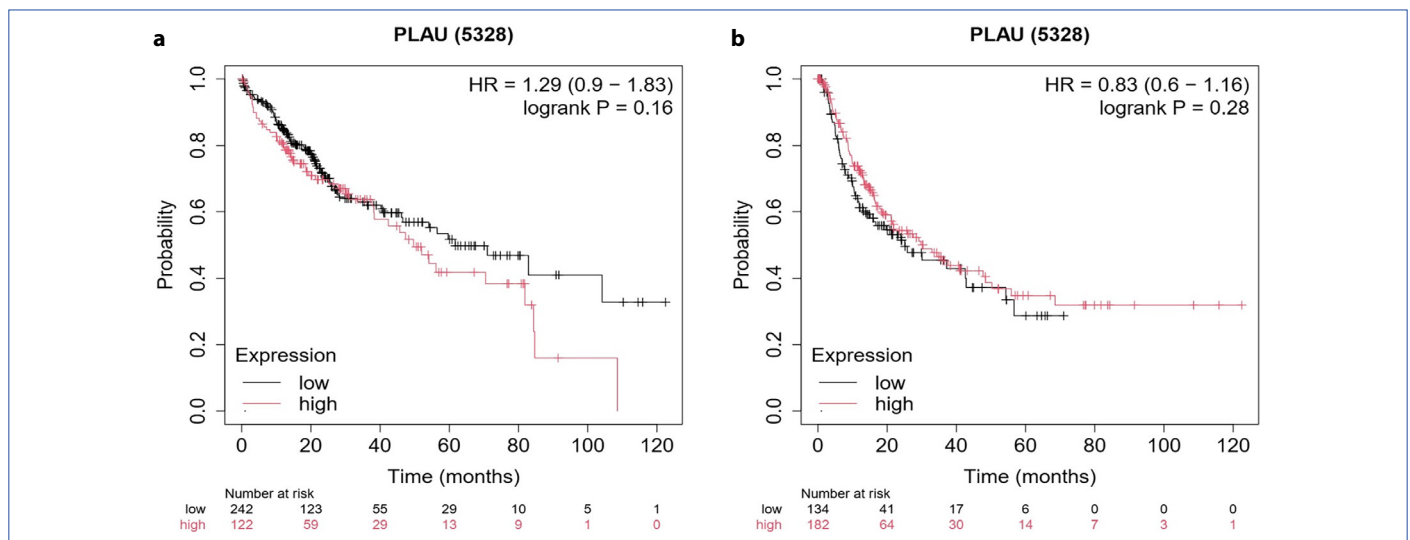


Figure 4. The survival analysis of PLAU in LIHC in the Kaplan-Meier plotter database. (a) Overall survival rates. (b) Relapse-free survival rates. HR: Hazard ratio; LIHC: Liver hepatocellular carcinoma.

($p=1.48 \times 10^{-3}$), 61–80 ($p=1.20 \times 10^{-12}$), and 81–100 ($p=0.040$) year groups. No significant upregulation was observed in the 41–60 group ($p=0.087$). Additionally, no significant differences were found among age groups (all $p>0.05$) (Fig. 3c). Regarding weight groups, PLAU expression was significantly upregulated in tumors from patients classified as extreme weight ($p=6.38 \times 10^{-8}$), obese ($p=2.38 \times 10^{-6}$), and extreme obese ($p=0.027$), whereas no significant upregulation was observed in the normal weight group ($p=0.079$). Additionally, no significant differences were found among the tumor weight groups (all $p>0.05$) (Fig. 3d). PLAU expression was significantly upregulated in patients without regional lymph node metastasis (N0) compared to adjacent normal tissues ($p=0.032$). However, no significant differences were observed in patients with limited lymph node involvement (N1, defined as metastasis in 1 to 3 axially lymph nodes) compared to adjacent normal tissues ($p>0.05$), nor between the N0 and N1 groups ($p>0.05$) (Fig. 3e). Additionally, promoter methylation levels of PLAU were investigated in LIHC using the UALCAN database. The results demonstrated that promoter methylation was significantly increased (hypermethylation) in LIHC tissues ($n=377$) compared to adjacent normal tissues ($n=50$), with median beta values of 0.26 and 0.247, respectively ($p=5.43 \times 10^{-12}$) (Fig. 3f).

The survival analysis of PLAU in LIHC

We examined the association between PLAU expression and the OS and RFS rates in LIHC. The analysis revealed that PLAU expression was not significantly associated with OS in LIHC patients (HR=1.29, 95% CI: 0.90–1.83, $p=0.16$) (Fig. 4a). The median survival rates for cohorts with low and high PLAU expression were 61.7 months and 49.7 months, respectively. PLAU expression was not significantly associated with RFS in LIHC patients (HR=0.83, 95% CI: 0.60–1.16, $p=0.28$) (Fig. 4b). The median survival rates for cohorts with low and high PLAU expression were 25.14 months and 30.4 months, respectively.

The analysis of protein-protein interactions and biological process enrichment of PLAU

The protein-protein interactions and biological process enrichment of PLAU were analyzed using the STRING database. The resulting PPI network comprises 11 nodes and 41 edges, with an average node degree of 7.45, an average local clustering coefficient of 0.856, and an expected number of edges of 12. The PPI enrichment p-value was 9.26×10^{-11} . The results demonstrated that PLAU interacts with serine protease inhibitor 1 (SERPIN1), serine protease inhibitor 2 (SERPIN2), serine protease inhibitor A5 (SERPIN5), serine protease inhibitor EB2 (SERPINEB2), plasminogen activator urokinase receptor (PLAUR), plasminogen (PLG), matrix metalloproteinase-9 (MMP9), vitronectin (VTN), cathepsin B (CTSB), insulin like growth factor 2 receptor (IGF2R) (Fig. 5a). Furthermore, the biological process enrichment analysis revealed that the interactions are associated with several biological processes, including the regulation of blood coagulation, fibrinolysis, plasminogen activation, and proteolysis (Fig. 5b).

The analysis of target miRNA

The miRNAs associated with PLAU were analyzed using the TargetScan 8.0 database. We identified five conserved miRNAs: hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-181c-5p, hsa-miR-181d-5p, and hsa-miR-4262.

The analysis of differential expression, survival, and co-expression of miRNAs

ENCORI analysis was conducted to compare the differential expression, survival analysis, and co-expression of hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-181c-5p, hsa-miR-181d-5p, and hsa-miR-4262 between LIHC ($n=370$) and normal tissues ($n=50$). The results indicated that hsa-miR-181a-5p and hsa-miR-181b-5p were significantly upregulated in LIHC

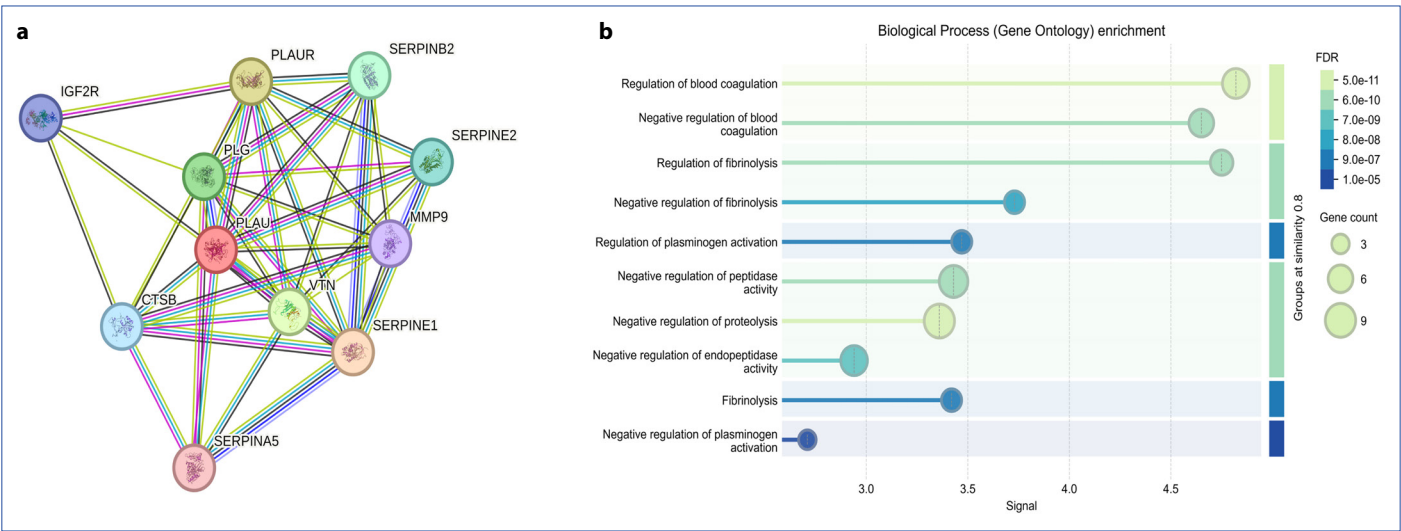


Figure 5. Interaction network of PLA in the STRING database. (a) Protein-protein interactions of PLA. (b) Biological process (Gene Ontology) enrichment analysis for PLA.
FDR: False discovery rate.

tissues compared to normal tissues ($p<0.05$) (Table 1; Fig. 6a). None of the miRNAs showed a statistically significant association with OS in LIHC tissues (Fig. 6b). According to the co-expression analysis, hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-181c-5p, and hsa-miR-181d-5p were positively correlated with PLA in LIHC tissues ($p=3.60\times10^{-14}$, 1.64×10^{-17} , $p=1.47\times10^{-23}$, and $p=3.79\times10^{-21}$, respectively) (Fig. 6c).

Discussion

The role of PLA in HCC, particularly its relationship with miRNAs, remains unclear. This study is the first to investigate PLA's involvement in HCC and its interaction with miRNAs through bioinformatic analysis. The urokinase-type plasminogen activator is an extracellular proteolytic enzyme that plays a pivotal role in remodeling tumor microenvironment and the progression of cancer [8]. Recently, uPA has garnered significant attention due to its involvement in tumor growth, metastasis, and angiogenesis, as well as its overexpression in various cancers. Elevated levels of uPA have been linked to poor prognosis, highlighting its potential as a valuable diagnostic, prognostic, and therapeutic biomarker [7, 8, 10]. Numerous strategies have been developed to target the uPA system by modulating its expression and activity in cancer

[7, 10, 28]. However, research on the role of PLA in HCC remains limited [13–16]. In the present study, we first assessed the differential expression of PLA across 22 different tumor types using the TNMplot database. Our findings demonstrated that PLA was significantly expressed in the majority of tumor types (21 out of 22), with expression levels varying according to the specific cancer type. Consistent with previous research, our analysis confirmed that PLA is consistently overexpressed in multiple cancers [12, 13, 15]. Subsequently, we analyzed PLA expression using the GEPIA2 database to investigate its levels in LIHC tissue. The results revealed that PLA expression was significantly upregulated in LIHC tissues compared to normal tissues, consistent with previous studies [13, 15]. Additionally, we examined PLA expression across different iCluster groups using the GEPIA2 database. Significant differences were observed in iCluster_1 (proliferative/stem cell-like) and iCluster_2 (intermediate/immune-active), while no significant difference was found in iCluster_3 (non-proliferative/metabolic). This may suggest a potential subtype-specific role of PLA in the tumor biology of LIHC. Furthermore, we assessed the expression of the PLA gene across different stages of cancer using the same database. The results indicated that PLA expression did not show a

Table 1. Differential expression table of pan-cancer analysis for miRNAs in LIHC			
miRNAs	Fold change	p	False discovery rate
hsa-miR-181a-5p	1.37	0.044	0.14
hsa-miR-181b-5p	1.76	0.00091	0.0055
hsa-miR-181c-5p	1.19	0.94	0.96
hsa-miR-181d-5p	1.52	0.39	0.72
hsa-miR-4262	1.0	0.71	0.78

miRNAs: microRNAs; LIHC: Liver hepatocellular carcinoma

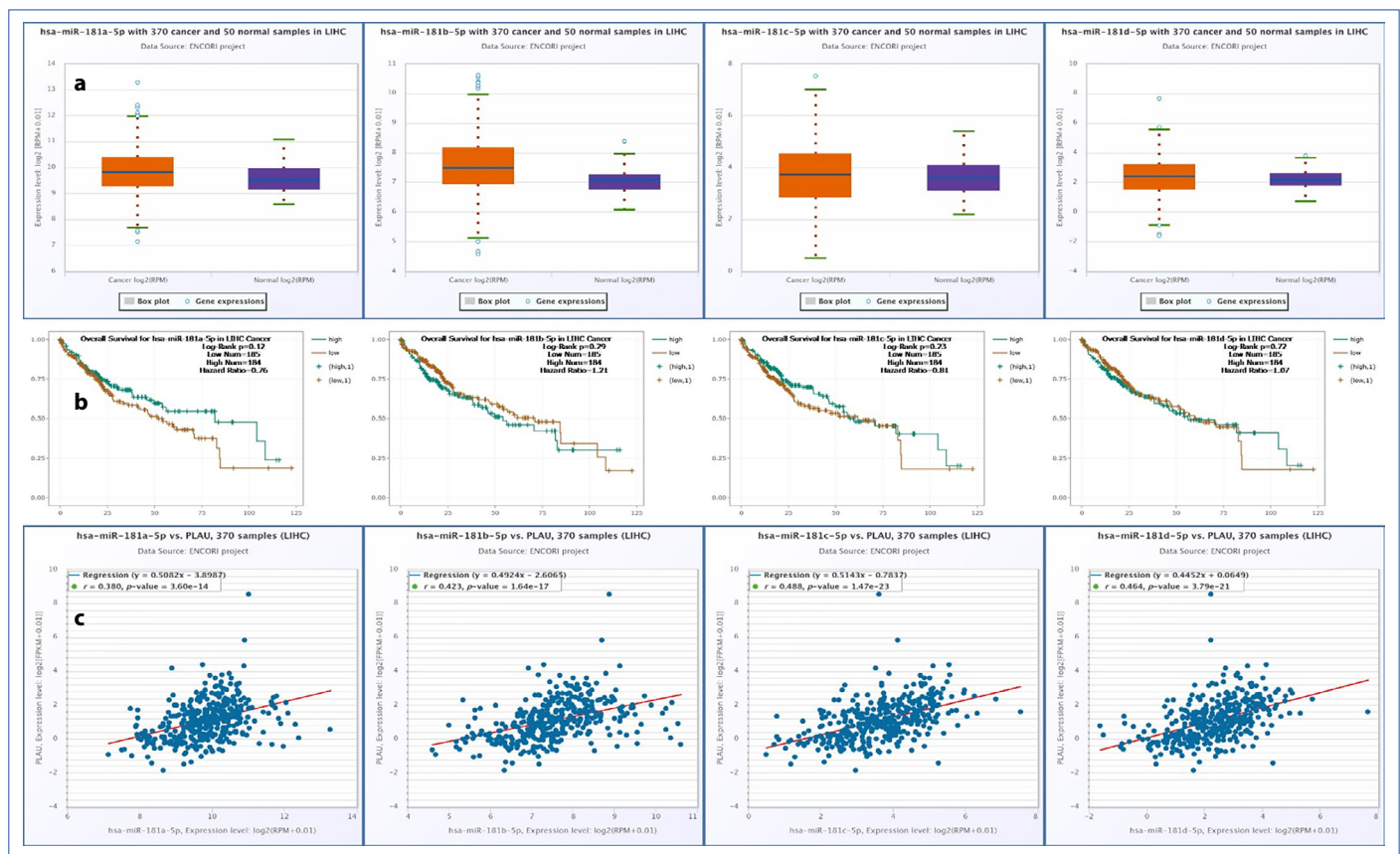


Figure 6. (a) The differential expression of hsa-miR-181a-5p, hsa-miR-181b-5p hsa-miR-181c-5p, and hsa-miR-181d-5p in LIHC in the ENCORI database. (b) Overall survival rates of hsa-miR-181a-5p, hsa-miR-181b-5p hsa-miR-181c-5p, and hsa-miR-181d-5p in LIHC in the ENCORI database. (c) Correlation between hsa-miR-181a-5p, hsa-miR-181b-5p hsa-miR-181c-5p, and hsa-miR-181d-5p expressions and PLAU expression in LIHC in the ENCORI database.

LIHC: Liver hepatocellular carcinoma.

statistically significant difference among stages I, II, III, and IV of LIHC. This suggests that PLAU expression remains relatively stable throughout disease progression, implying that its expression may not be stage-dependent. These findings underscore the need for further research into the functional role of PLAU in HCC subtypes. To our knowledge, this is the first study to explore this specific subject.

To further explore the clinical relevance of PLAU in LIHC, its expression was evaluated across various demographic and clinical subgroups. PLAU expression was significantly upregulated in Caucasian and African-American patients with LIHC, while no significant increase was observed in Asian patients. Moreover, no significant differences were found among the racial groups. These findings may suggest that PLAU plays a role in LIHC tumorigenesis in certain racial populations. PLAU expression was also significantly upregulated in both male and female patients with LIHC. However, no significant difference was observed between the sexes, suggesting that PLAU overexpression occurs independently of sex. Age-stratified analysis showed significant upregulation in the 21–40, 61–80, and 81–100 age groups, but not in the 41–60 group. Nonetheless, the lack of intergroup differences suggests that PLAU overex-

pression is not strongly age-dependent. Similarly, elevated PLAU expression in patients with extreme weight, obesity, and extreme obesity, but not in those with normal weight, was observed. However, the absence of significant variation among weight groups indicates a limited association with body weight. Notably, PLAU overexpression in patients without nodal metastasis (N0) suggests a potential role in early tumorigenesis. However, the absence of significant expression differences in N1 patients or between N0 and N1 groups, suggesting PLAU may not contribute to lymphatic spread in LIHC. Numerous studies suggest that uPA may serve as a prognostic marker, with elevated PLAU expression associated with poor prognosis in HCC [13–15]. Wu et al. [15] reported that high PLAU expression was associated with poorer OS. Tsai et al. [14] found that elevated serum uPA levels were linked to poorer OS in HCC patients after resection. Furthermore, Niu et al. [13] demonstrated that high uPA expression correlated with poor prognosis, indicating its potential role as a prognostic biomarker in HCC. Despite these findings, our analysis using the KM Plotter database did not reveal a statistically significant correlation between PLAU expression and OS or RFS in LIHC patients. The discrepancies between our findings and

those of previous studies may be attributed to differences in sample sizes, methodologies, or the specific databases utilized for analysis. Additionally, the heterogeneity of PLAU expression across different tumor stages, etiologies, and molecular subtypes of HCC may influence prognostic outcomes. Further validation studies are necessary to clarify the prognostic value of PLAU expression in LIHC.

HCC is commonly associated with genetic and epigenetic aberrations [29]. DNA methylation, an important epigenetic modification, plays a critical role in regulating gene expression. Aberrant DNA methylation is a hallmark of cancer, closely linked to the onset, development, and progression of cancer, and it holds potential as a biomarker for diagnosis and prognosis [30, 31]. Specifically, the epigenetic modification of the PLAU gene through DNA methylation has been implicated in cancer development [7]. Numerous studies have demonstrated that the promoter region of PLAU undergoes hypomethylation, which is linked to increased PLAU expression and contributes to its oncogenic effects [28, 30, 32, 33]. Pakneshan et al. [32] found that DNA hypomethylation at the PLAU promoter correlates with elevated expression in aggressive breast cancer, suggesting its potential as an early biomarker. Similarly, Wu et al. [30] reported an inverse relationship between PLAU promoter methylation and gene expression in differentiated thyroid cancer. Additionally, Huo et al. [28] identified a link between PLAU overexpression and DNA hypomethylation in head and neck squamous cell carcinoma, highlighting its role as an independent diagnostic and prognostic biomarker. In the present study, we examined the methylation of the PLAU promoter using the UALCAN database to investigate its role in LIHC. Contrary to existing literature, we found that the PLAU promoter was hypermethylated in LIHC tissues. While hypermethylation is typically linked to gene silencing, our results indicated increased PLAU expression, contradicting the conventional view that DNA methylation always suppresses gene expression [8, 31, 34]. Recent studies have highlighted instances where promoter hypermethylation correlates with increased expression, suggesting a more complex role for DNA methylation [31, 35–37]. Spainhour et al. [35] analyzed data from the TCGA and found that promoter methylation exhibited a positive correlation with gene expression, contrary to the expected negative correlation. This growing evidence suggests a potential link between hypermethylation and increased transcriptional activity. Several hypotheses have been proposed to clarify the molecular mechanisms underlying gene activation from hypermethylated promoters. These mechanisms include the binding of repressive transcription factors, interactions with distal elements, and expression from alternative promoters [31]. Our findings also offer new insights into the intricate relationship between methylation and transcriptional regulation. Further research is necessary to clarify the molecular mechanisms involved in gene activation in hypermethylated promoters and to understand the functional consequences of this epigenetic modification.

The uPA is a key protease that converts plasminogen into plasmin, playing a crucial role in fibrinolysis and coagulation [38]. Our PPI networks and enrichment analysis revealed that PLAU interacts with several proteins, including SERPIN1, SERPIN2, SERPIN5, SERPINEB2, PLAUR, PLG, MMP9, VTN, CTSB, and IGF2R. These interactions are involved in blood coagulation, fibrinolysis, plasminogen activation, and proteolysis. These findings highlight PLAU as a central regulator of the plasminogen system, contributing to tumor progression through proteolytic activity and ECM degradation. The interaction between PLAU and coagulation-related proteins suggests a dynamic crosstalk between fibrinolysis and tumor microenvironment remodeling, supporting the hypothesis that dysregulated hemostasis contributes to cancer progression [39]. While uPA is not a direct coagulation factor, it plays an essential role in the fibrinolytic system. Dysregulation of coagulation and proteolysis has been strongly linked to cancer progression, with proteases promoting tumor invasion and metastasis [38]. Further research is needed to elucidate the precise roles of PLAU-related proteins and to explore whether targeting PLAU or its associated pathways could offer novel therapeutic strategies for cancer treatment.

The miRNAs regulate key cellular processes such as proliferation, differentiation, and apoptosis. Their dysregulation is linked to various diseases, including cancer, where they play a complex role in tumor development and progression [17, 40]. To further investigate the mechanisms underlying PLAU upregulation in LIHC tissue, we conducted a bioinformatics analysis to predict miRNAs targeting PLAU. Our analysis identified hsa-miR-181a-5p and hsa-miR-181b-3p as upregulated in LIHC, showing a significant positive correlation with PLAU expression. Notably, no prior studies have explored the relationship between these miRNAs and PLAU in HCC. Recently, there has been growing interest in the roles of the miR-181 family in cancer. Research suggests that the miR-181 family members can act as either oncogenes or tumor suppressors, depending on the cellular context, and influence major pathways by targeting multiple genes [40–44]. Hsa-miR-181a-5p, a highly conserved microRNA, regulates crucial tumor-related processes, including proliferation, apoptosis, autophagy, angiogenesis, EMT, and migration [40]. Extensive studies have reported both upregulated and downregulated expression levels of hsa-miR-181a-5p across various tumor types [43–47]. These conflicting findings highlight the complexity of miRNAs, as their functions can vary significantly across tumor types. Hsa-miR-181a-5p has also been studied in HCC. Korhan et al. [44] demonstrated that hsa-miR-181a-5p is downregulated in HCC and directly targets c-Met, thereby inhibiting cell motility, invasion, and branching morphogenesis. Similarly, Bi et al. [45] reported that hsa-miR-181a-5p is downregulated in HCC and inversely correlated with Early Growth Response Factor 1 (Egr1) expression. Notably, overexpression of hsa-miR-181a-5p suppressed Egr1, inhibiting the TGF- β 1/Smad pathway and reducing proliferation. Conversely, Chang et al. [48] found that lncRNA-XIST enhances the expression of the tumor suppressor gene PTEN by inhibiting hsa-miR-181a-5p. Restoration of hsa-miR-181a-5p expression

was shown to promote HCC cell proliferation and invasion. Yadav et al. [49] demonstrated that free fatty acid-induced hsa-miR-181a-5p promotes apoptosis in hepatic cells by targeting and downregulating X-linked inhibitor of apoptosis protein and B-cell lymphoma 2, both of which are anti-apoptotic proteins. Numerous studies have shown that hsa-miR-181b-5p is overexpressed in various cancers, including HCC, where it promotes tumor progression through multiple signaling pathways [41, 42, 50]. Wang et al. [42] demonstrated that TGF- β signaling upregulates hsa-miR-181b in NASH-associated hepatocarcinogenesis by targeting tissue inhibitor of metalloproteinases 3 (TIMP3), leading to ECM degradation and tumor growth. These findings highlight the significance of the TGF- β /miR-181b/TIMP3 axis in hepatocarcinogenesis and its potential as a therapeutic target. Similarly, Yu et al. [50] found that cSMARCA5 suppresses HCC progression by sponging miR-181b-5p, thereby restoring TIMP3 expression. Our findings align with these studies. In conclusion, hsa-miR-181a-5p and hsa-miR-181b-5p play important roles in HCC progression by acting through multiple signaling pathways. Our study demonstrated that the upregulation of these miRNAs and their positive correlation with PLAU may be a shared mechanism promoting tumor progression. This highlights the potential of targeting PLAU and hsa-miR-181a/hsa-miR-181b as a therapeutic strategy in HCC. Further research is required to clarify the roles and regulatory mechanisms of hsa-miR-181a-5p and hsa-miR-181b-5p in HCC.

Conclusion

To our knowledge, our study is the first to examine the relationship between PLAU and miRNAs in HCC using several bioinformatic databases. This study indicates that PLAU may play a significant role in HCC development through epigenetic modification and miRNA interactions. The positive correlation with hsa-miR-181a-5p and hsa-miR-181b-5p suggest a complex regulatory network influencing tumor development. However, the lack of association with OS and RFS suggests that while PLAU may contribute to tumor development, its prognostic significance in HCC remains uncertain. Further investigation into their functional interplay and regulatory mechanisms is essential to understand their role in HCC pathogenesis better.

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Research Article

Tau protein expression and phosphorylation in a glucose-repressed yeast model: Insights into the cancer-alzheimer's disease link

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Abstract

Objectives: The microtubule-associated protein tau, responsible for stabilizing microtubules, plays a role in the pathology of neurodegenerative diseases called tauopathies, including Alzheimer's disease. In Alzheimer's disease, neurofibrillary tangle formation is observed as a result of tau hyperphosphorylation. Although it is known that tau protein plays a role in many cellular processes, all of its functions have not yet been elucidated. The inverse relationship between Alzheimer's disease and cancer has been a topic of research that has attracted attention in recent years. In addition, the role of tau protein in cancer has also gained importance with the determination of its direct relationship with DNA. In particular, the negative correlation between Alzheimer's disease and cancer points to two extremes of a common mechanism. Discovering a common molecule or pathway will allow understanding the cause of both diseases and developing treatments.

Methods: In this study, we obtained a cell model that mimics cancer metabolism by creating aerobic glycolysis-like conditions with glucose repression in *S. pombe* cells heterologously expressing human tau protein. We examined tau protein expression and phosphorylation (S262, S396 and S404) and various cellular processes (glucose metabolism, stress response, ER stress, autophagy, 20S proteasome activity, intracellular oxidation) at the molecular level in model cells.

Results: Under aerobic glycolysis-like conditions, we observed an approximately 2-fold increase in tau protein expression. In addition to this increase, we determined that the amount of phosphorylation at S396 residue of tau protein was decreased, while phosphorylation at S262 and S404 residues was increased.

Conclusion: These findings suggest a potential divergence in tau regulation under altered metabolic conditions, warranting further investigation.

Keywords: Cancer, glucose repression, heterologous expression, tauopathy, tau protein

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Microtubules play a role in morphogenesis, cell division, intracellular transport of macromolecules and organelles, and motility [1]. These processes occur through dynamic restructuring of microtubules mediated by microtubule-associated proteins such as tau. Tau protein is responsible for the stabilization of microtubules [2]. There is great interest in tau protein because it plays a major role in neurodegenerative diseases referred to as tauopathies, including Alzheimer's disease

(AD) [3]. There is increasing evidence that tau protein, in addition to its microtubule stabilizing function, is implicated in many significant signaling pathways, including proliferation, morphogenesis, and cell differentiation [4]. A lesser-known property of tau is its ability to bind to cancer-associated protein kinases. This suggests that tau has a potential role in regulating microtubule-independent cellular pathways [3]. It has also been determined that tau protein can bind to nu-

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cleic acids [5]. Tau has been suggested to be a DNA protector, specifically by preventing stress-induced DNA breaks [6, 7].

Over 55 million individuals globally suffer from dementia, with AD constituting 60–70% of these instances. The global prevalence of Alzheimer's disease is projected to surpass 138 million by 2050 [8]. Another leading disease with a high mortality rate is cancer. Approximately 10 million deaths attributed to cancer were reported globally in 2020, along with 19.3 million newly diagnosed cases of cancer. [9, 10]. In AD, alterations in behavior and cognitive impairments linked to neurodegeneration typically manifest around a decade following the beginning of proteinopathy. Its two characteristic features are A β plaques, and neurofibrillary tangles caused by tau hyperphosphorylation [11]. Cancer is a disease in which some cells uncontrolled divide by genetic mutations and they spread to other parts of the body. Brown and colleagues define cancer as a disease characterized by the uncontrolled multiplication of altered cells that undergo evolution through natural selection [12]. Multiple epidemiological studies indicate a negative correlation between cancer and AD [13]. This intriguing correlation is limited to certain types of cancer and neurodegenerative diseases. However, the underlying mechanisms of the two diseases are very different. While cancers evade cell death, neurodegeneration leads to cell death. As a result, it is possible that people with a neurodegenerative disease might have a lower likelihood of developing specific kinds of cancer, and the opposite might also be true [14]. Even though the underlying physiological processes of cancer and AD have been extensively researched, they are not yet distinctly understood [10].

Dysregulation of cellular energy metabolism is one of the hallmarks of cancer [15]. The Warburg effect refers to the transition from aerobic respiration to aerobic glycolysis, resulting in the production of lactate from glucose as the end-product. Warburg reported that even when oxygen is sufficient in an environment, cancer cells generally use glucose via glycolysis rather than oxidative phosphorylation [16]. Cancer cells typically exhibit increased glycolysis and diminished oxidative phosphorylation. While this long-term metabolic reprogramming is known as the Warburg effect, the short-term, reversible alteration of this metabolic process is known as the Crabtree effect [17]. The Warburg effect provides an advantage for cancer cells to survive and progress. In order to achieve high aerobic glycolysis rates, some types of cancer cells are induced to possess specific isoforms of glycolytic enzymes [18]. This metabolic adaptation helps cancer cells to proliferate and become more invasive. The relationship between high glucose and cancer development is still being investigated [19].

Glucose is the preferred carbon source for most living cells, and glucose metabolism provides the energy necessary for cell survival [20]. In yeast, glucose can induce the expression of various genes encoding some glucose transporters, ribosomal proteins, and glycolytic enzymes, by a process known as glucose induction. At the same time, the expression of numerous genes involved in respiration, gluconeogenesis, and the

utilization of alternative carbon sources can be suppressed, in the process named glucose repression. Yeasts are easy to use as models in studies examining glucose perception and signal transduction because of their similarity to complex multicellular eukaryotes in terms of glucose metabolism. Although yeast cells can use a wide variety of carbon sources, the presence of abundant glucose suppresses the utilization of alternative carbon sources, cellular respiration, and gluconeogenesis [21]. *Schizosaccharomyces pombe*, the fission yeast, is a single-celled model organism that can be used in studies on glucose sensing, intracellular signaling, and signal response processes. It shows high similarity with multicellular eukaryotic organisms in terms of molecular processes [22, 23].

Genes involved in various processes in human have orthologs in *S. pombe*, and *S. pombe* has 1514 orthologous transcripts (proteins and ncRNAs) associated with human diseases [24, 25]. Phosphorylation of tau protein, which plays a role in various diseases, is carried out by glycogen synthase kinase 3 beta (GSK-3 β), cyclin-dependent kinase 5 (CDK-5), and cAMP-dependent protein kinase A (PKA) and the orthologous of the human GSK3 β gene, which is the major kinase in the phosphorylation of tau protein, exists in the *S. pombe* genome [26–28]. Moreover, in our previous studies, we showed that human tau protein is phosphorylated in the *S. pombe* model, and we detailed several cellular processes [29, 30].

In this study, based on the inverse correlation seen in cancer and neurodegenerative diseases, we investigated the relationship between tau protein and the aerobic fermentation process observed in cancer, which we mimicked by glucose repression metabolism in yeast. We hypothesize that one of the factors affecting the dysregulation of glucose metabolism in neurodegenerative diseases and cancer may be the tau protein, which stands out with its role in microtubule stabilization and has a potential effect on glucose metabolism. In the present study, the conditions of glucose repression in *S. pombe* were referred to as "aerobic glycolysis-like state". For this purpose, we cultured *S. pombe* cells heterologously producing human tau protein under high glucose conditions (5%) to create glucose-repression and mimicked aerobic fermentation in cancer cells. We studied various processes at the molecular level in model cells with aerobic glycolysis-like metabolism in the presence of tau protein. In our model cells, a decrease in stress response was seen, as in cancer cells. We determined that under aerobic glycolysis-like conditions, the expression of tau protein in cells increased approximately 2-fold. Additionally, we observed a decrease in the phosphorylation level at the S396 site of tau protein and an increase in the phosphorylation levels at the S404 and S262 regions. Contrary to expectations under aerobic glycolysis-like conditions, the increase in tau level and the decrease in hexokinase, which plays a role in glycolysis, suggested that tau protein has a greater role in glucose metabolism than is known. The findings suggest that the inverse relationship between neurodegenerative diseases and cancer may be due to differences in the phosphorylation level of tau protein, which plays a role in

Table 1. Primer sequences used in real-time PCR

Gene	Forward Primer	Reverse Primer	Tm (°C)
<i>gpdh</i>	5' ggtgacaaccactcctccat 3'	5' tcaacaacacggtgggagta 3'	55
<i>mapt</i>	5' caagtgtggctcaaggaataat 3'	5' ggtttatgatggatgttcctaa 3'	55
<i>hvk2</i>	5' caacaaggactttgccaat 3'	5' aagggtgcgtctcctttga 3'	55
<i>fbp1</i>	5' gtatggtgcttcggctcatt 3'	5' ttcattgttcgatgggtaa 3'	55
<i>sod1</i>	5' attggccgtaccattgtcat 3'	5' gacaccacaagcggtacgtg 3'	55
<i>ctt1</i>	5' atcctcaatccgaccacttg 3'	5' aacgtcggtaattcgtcca 3'	55
<i>ire1</i>	5' attctcgacattctcggt 3'	5' aactgtgaatccgctcgtg 3'	55
<i>atg14</i>	5' tcaccctagttacttcaaca 3'	5' cggcaaatgtccataaaaactc 3'	55
<i>gsk3</i>	5' gatgcttctcctcgtcatt 3'	5' catcaagttcacgggtaaag 3'	55
<i>pp2a</i>	5' tattgtatcgtgtgtaatc 3'	5' ggtgtccttcgagctatt 3'	55

the molecular mechanisms of both diseases, or to an as-yet-unknown function of tau protein in glucose metabolism.

Materials and Methods

Yeast strain and culture conditions

In the present study, we used *S. pombe* cells (pMS-mapt) that produce human tau protein, which we obtained in our previous study [29]. pMS-mapt cells are auxotrophic for guanine and carry the human *MAPT* gene in the pSGP572 plasmid. pSGP572 contains the *ura4* marker gene, and the *GFP* gene at the 3'-terminal in the cloning site. So, the tau protein produced is a fusion protein with GFP. The *MAPT* and *GFP* genes are under the control of the *nmt* promoter, which is an inducible promoter and is repressed by thiamine [31].

In this study, we investigated the relationship between aerobic fermentation conditions and tau protein. Firstly, tau protein production was induced in pMS-mapt cells [24], then these cells were grown under different glucose conditions. For this purpose, cells were grown in standard EMM (1% glucose) medium containing thiamine (15 µM) and guanine (50 mg/L) at 30°C for 24 hours. Then, the cells were washed with PBS and cultured in standard thiamine-free EMM medium with guanine for 20 hours. After incubation, cells were washed with PBS and cultured in EMM containing different concentrations of glucose (3% and 5%) at 30°C for 4 hours.

Cell densities of pMS-mapt cells were measured spectrophotometrically at 600nm wavelength for 32 hours, and their growth under different conditions were compared. Cells grown under different glucose concentration conditions were examined under the microscope.

Gene expression analysis

After cells collected, total RNA isolation was performed by using "Thermo Scientific GeneJET RNA Purification Kit", according to the manufacturer's instructions with a minor modification. Cells in PBS were mechanically homogenized by using glass beads. After RNA isolation, cDNA was synthesized from 2 µg of total RNA by using a "Roche Transcriptor High Fidelity cDNA Synthesis Kit", according to the manufacturer's instructions.

We examined the expression level of genes related to various cellular mechanisms, including glucose metabolism (*hvk2*, *fbp1*), stress response (*sod1*, *ctt1*), ER stress (*ire1*), autophagy (*atg14*), and regulators of tau phosphorylation (*gsk3*, *pp2a*). Additionally, *mapt* gene expression was also examined.

Real-Time PCR was performed using "ThermoScientific Applied Biosystems PowerUp SYBR™ Green PCR Master Mix" kit and the primers, which we used in our previous study, listed in Table 1 [24]. Pairs of primers were designed using the "IDT PrimerQuest Tool". *S. pombe gapdh* gene was used as the reference gene. We applied the Pfaffl equation to analyse the relative expression levels of genes and we used *gapdh* gene expression levels for normalization [32]. All experiments were performed in three biological and technical replicates.

Immunoblotting analysis

Protein extraction from cells grown under different glucose concentration conditions was performed according to the method of Forsburg and Rhind (2006) with minor modifications [31]. The cells were mechanically homogenized by vigorous shaking using glass beads and lysis buffer (150 mM NaCl, 1mM PMSF, 0.5% (w/v) Nonidet-P40, 5 mM EDTA 50 mM Tris).

SMART™ bicinchoninic acid (BCA) protein assay kit (iNtRON Biotechnology) was used to quantify total protein. Equal amounts of protein (30 µg/well) was loaded on 10% SDS-PAGE, and transferred to PVDF membrane (Thermo Fisher Scientific). After blocking, the membrane blots were incubated overnight with the following primary antibodies: Anti-tau rabbit IgG (1/1000 dilution, Tau Rabbit mAb, Abclonal) and anti-phospho-tau (Ser396) rabbit IgG (E178) (1/1000 dilution, ab32057, Abcam), phospho-tau S404 Rabbit mAb (1/1000 dilution, AP1378 Abclonal) and phospho-tau S262 Rabbit pAb (1/1000 dilution, AP0397 Abclonal). Anti-GAPDH mouse IgG (1/2500 dilution, MA5-15738 Invitrogen) was used as internal control. The washed membranes were incubated with HRP-linked secondary antibody (1/5000 dilution anti-rabbit HRP Goat Anti-Rabbit IgG, Abclonal). Following washing, the blots were visualized using SuperSignal West Pico PLUS ECL reagent (Thermo Fisher Scientific). Immunoblots (heterologous MAPT protein levels) were imaged

using the ChemiDoc XRS system (BioRad). We performed quantification by using ImageLab 6.0.1 software (Bio-Rad).

Measurement of 20S proteasome activity

We determined 20S proteasome activity as suggested by Reinheckel et al. [33]. After protein extraction from grown cells under stated conditions, we diluted protein concentration to 50 µg/mL with assay buffer (50 mM Tris, 20 mM KCl, 0.5M DTT, 5 mM CH₃(COO)₂). To measure peptidase activity, we added 99 µL assay buffer and 1 µL Suc-Leu-Leu-Val-Tyr-AMC (Sigma) (stock 40 mM) to 100 µL of diluted protein sample. After 1 h incubation at 37°C, the reaction was stopped by adding an equal volume of cold ethanol and 1.6 mL of 125 mM sodium borate buffer (Na₂B₄O₇·10H₂O, pH 9.0). Then, we measured 20S proteasome activity by a spectrofluorometer plate reader (ex: 390nm, em: 470 nm).

Determination of intracellular oxidation level

We determined the intracellular oxidation level as recommended by Inoue et al. [34]. In this method, intracellular oxidation level is measured by conversion of DCFH-DA (2',7'-dichlorofluorescein diacetate) to the fluorescent compound 2',7'-dichlorofluorescein. pMS-mapt cells grown in EMM media containing guanine and different glucose concentrations (3%, 5%) were centrifuged for 5 minutes at 3000 g, and cells were dissolved in 1 mL EMM media with guanine. 40 µM DHCF-DA (Sigma) solution dissolved in ethanol was added and incubated at 30°C for 1 h, and the samples were washed with PBS. We kinetically measured intracellular oxidation level with a spectrofluorometer plate reader (Bio-tek FLx800) with excitation and emission wavelengths of 490 and 524 nm.

Imaging by microscopy

S. pombe cells grown under the stated conditions were fixed using 10% formaldehyde, and the cells were washed with PBS. Then, cells were examined under the microscopy (Olympus BX53).

Statistical data

We expressed all of our data as mean±SD. We used GraphPad Prism 9 software for statistical comparisons based on Student's t-test. The value of p<0.05 was considered statistically significant.

Results

Microscopic examination of the cells

We examined pMS-mapt cells grown under different glucose concentrations under the microscope, and observed morphological differences in cells grown under aerobic glycolysis-like conditions for 4 hours (Fig. 1a). Additionally, the absorbance graph of cell cultures measured every two hours at 600 nm wavelength for 32 hours is given in Figure 1b.

Aerobic glycolysis-like conditions

When pMS-mapt cells grown under standard glucose concentration conditions were compared with cells under aerobic

glycolysis-like conditions, a 3.58-fold decrease in the expression of the *hxx2* gene, which encodes the hexokinase 2 enzyme that plays a role in glycolysis in glucose metabolism, was observed under aerobic glycolysis-like conditions. A 4.52-fold decrease was observed in the expression of the *fbp1* gene, which encodes the fructose-1,6-bisphosphatase 1 enzyme that plays a role in gluconeogenesis under aerobic glycolysis-like conditions (Fig. 2).

Tau protein and its phosphorylation

When the expression of tau protein in cells grown under standard and aerobic glycolysis-like conditions was examined by western blot analysis, an increase in total tau was observed under repression conditions, consistent with gene expression analysis. However, it was determined that the phosphorylation level in the S396 region of the tau protein was lower in cells grown under aerobic glycolysis-like conditions, while the phosphorylation level in the S262 and S404 regions of the tau protein was higher in cells grown under aerobic glycolysis-like conditions (Fig. 3a).

Under aerobic glycolysis-like conditions, *MAPT* gene expression increased 2.68-fold. However, the expression of the *gsk3* gene, which is responsible for the phosphorylation of tau protein and encodes the glycogen synthase kinase 3 enzyme, decreased 2.67-fold. In addition, the expression of the *pp2a* gene, which encodes the protein phosphatase 2a enzyme that removes phosphate groups from the tau protein, decreased 2.78-fold (Fig. 3b).

Cellular stress response

When the intracellular oxidation level was examined, a significant 1.86-fold increase was observed under aerobic glycolysis-like conditions (Fig. 4a). The expression of *sod1* and *ctt1* genes, which encode superoxide dismutase and catalase enzymes that play a role in the stress response in the cell, decreased 1.68 and 3.39 times, respectively (Fig. 4b).

Under aerobic glycolysis-like conditions, 20S proteasome activity, one of the pathways responsible for the degradation of proteins in the cell, was found to be 10% higher than standard conditions (Fig. 5a). When the expression of the *atg14* gene, which is related to autophagy, another degradation pathway, was examined, no significant change was observed under aerobic glycolysis-like conditions. The expression of the *ire1* gene, a marker of endoplasmic reticulum stress, decreased 2.14-fold under glucose suppression conditions. (Fig. 5b).

Discussion

In recent years, interest in the relationship between Alzheimer's disease and cancer has increased. There are studies showing that the risk of developing cancer decreases in Alzheimer's patients and, conversely, the risk of developing AD decreases in cancer patients [10, 13, 35, 36]. In their study, Musicco and colleagues reported that the risk of cancer was

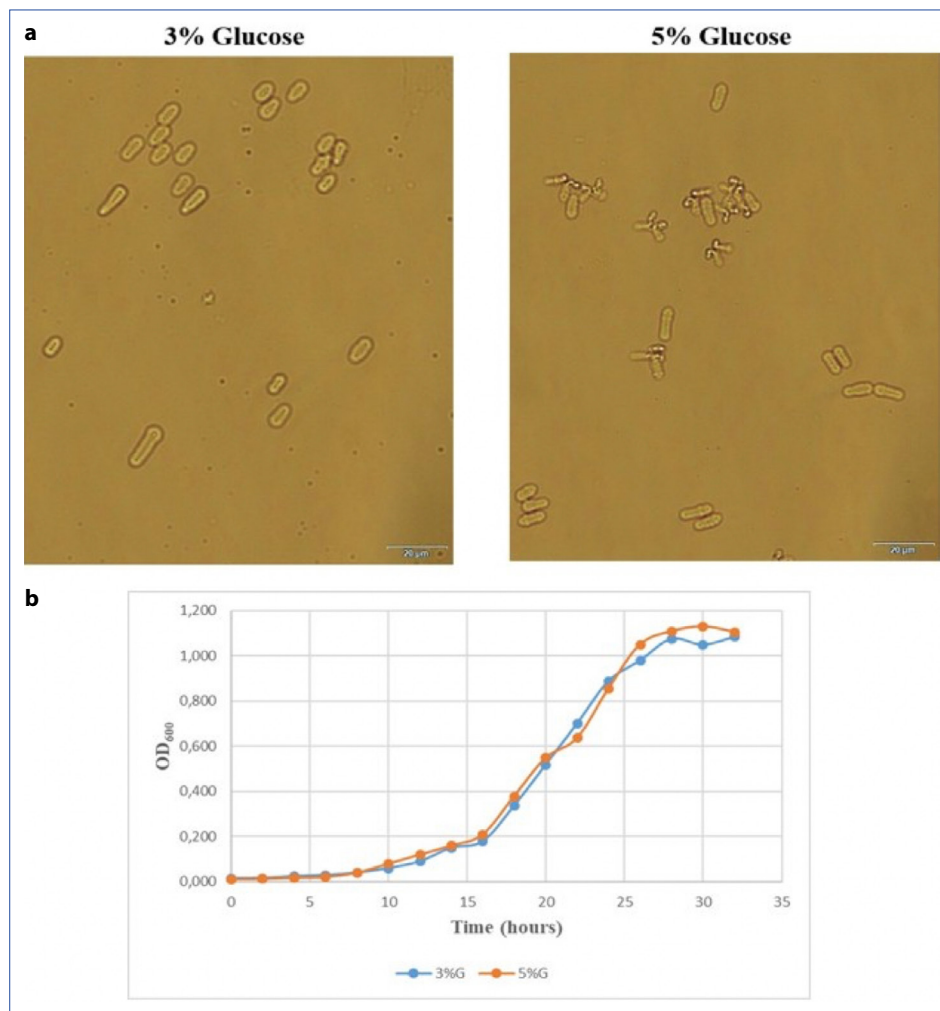


Figure 1. Growth and imaging of pMS-mapt cells under different conditions. (a) Microscope images of pMS-mapt cells grown under different glucose concentrations (40X magnification and bar represents 20 μm). (b) Growth curves of pMS-mapt cells grown under different glucose concentrations.

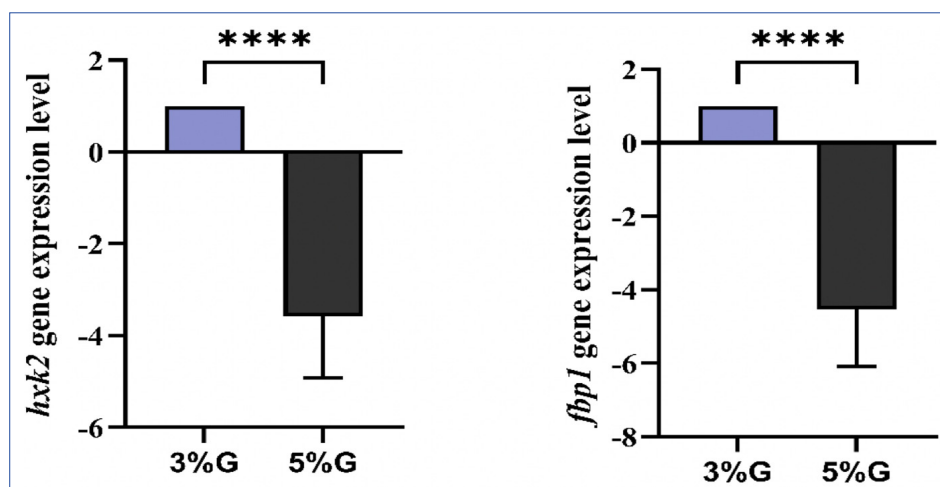


Figure 2. Expression levels of *hxx2* and *fbp1* genes related to glucose metabolism in pMS-mapt cells grown at different glucose (G) concentrations (3%G, 5%G).

Data (mean±SD) was derived from three independent experiments and analyzed using unpaired Student's t-test (****p<0.0001).

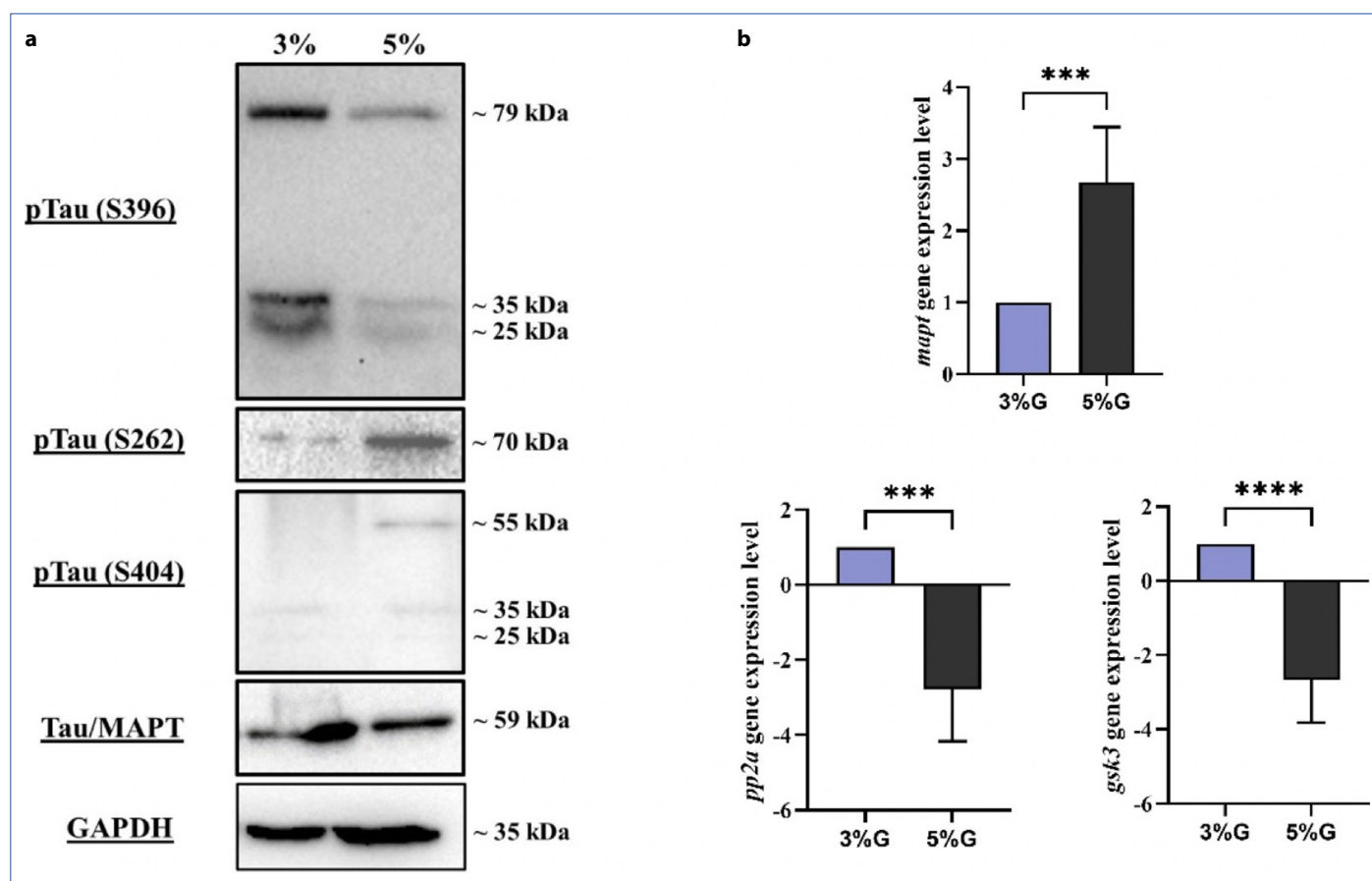


Figure 3. Indication of tau protein expression and phosphorylation in pMS-mapt cells grown under different conditions. (a) Membrane images as a result of western blot of total tau and phosphorylated tau protein (S262, S396 and S404) in pMS-mapt cells. (b) Expression levels of the *MAPT* gene encoding tau protein and the *gsk3* and *pp2a* genes encoding glycogen synthase kinase 3 and protein phosphatase 2a, which are responsible for the phosphorylation and dephosphorylation of tau protein.

Data (mean±SD) was derived from three independent experiments and analyzed using unpaired Student's t-test (**p<0.001; ****p<0.0001). SD: Standard deviation.

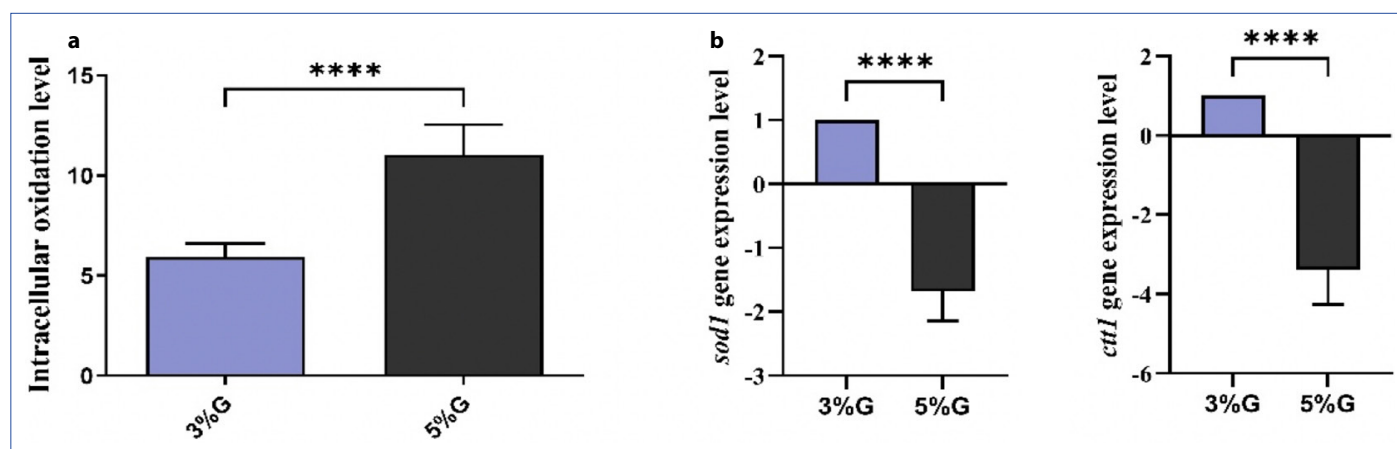


Figure 4. Intracellular oxidative stress response in pMS-mapt cells grown under different conditions. (a) Intracellular oxidation level in cells (b) Expression levels of stress response genes *sod1* and *ctt1*.

Data (mean±SD) was derived from three independent experiments and analyzed using unpaired Student's t-test (****p<0.0001).

halved in AD patients and the risk of AD in cancer patients was reduced by 35% [37]. Although the pathophysiological mechanisms of both cancer and AD have been widely stud-

ied, they have not been elucidated. However, an inverse relationship between them is noticed. It has been determined that patients with AD have a 61% lower risk of cancer [10].

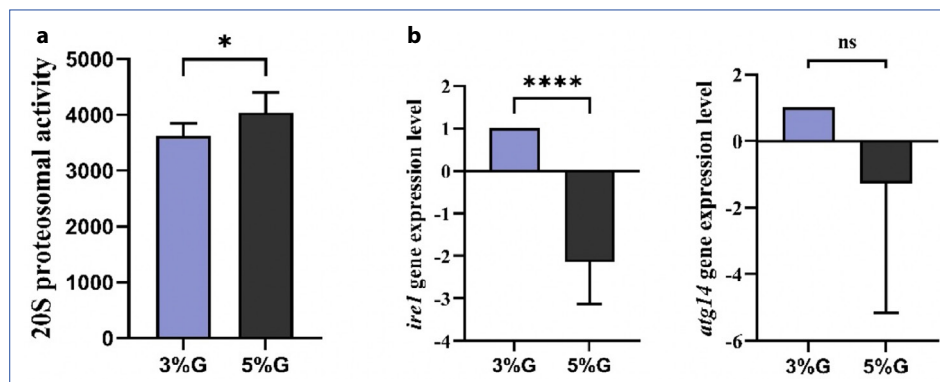


Figure 5. 20S proteasome activity in pMS-mapt cells grown under different conditions. (a) 20S proteasome activity in cells (b) Expression levels of *ire1* genes, which play a role in ER stress response, and *atg14* genes, which play a role in autophagy.

Data (mean±SD) was derived from three independent experiments and analyzed using unpaired Student's t-test (* $p < 0.05$; **** $p < 0.0001$). ns: Non-significant.

Tau protein is recognized for its involvement in neurodegenerative disorders. Research indicates that Tau may contribute to the advancement of several tumors and the resistance to cancer therapies [3]. Numerous studies have documented abnormal levels of tau in cancer cells of the brain, breast, stomach, and prostate [38]. Moreover, the level of tau expression has been associated with resistance to anti-microtubule drugs in cancer [39, 40]. Gargini and colleagues showed that higher levels of MAPT were inversely correlated with glioma aggressiveness [41].

Aerobic glycolysis (Warburg effect) is observed in cancer cells under high glucose conditions. In some types of cancer, glycolytic enzymes are stimulated to achieve high rates of aerobic glycolysis [18]. Glycolysis is promoted, and this metabolic adjustment enables cancer cells to reproduce and invade more rapidly. This strengthens the competition between cancer cells with normal cells [19].

Yeasts also prefer fermentation despite the presence of oxygen, similar to cancer cells, when there is plenty of glucose in the environment. Additionally, the expression of numerous genes that play a role in gluconeogenesis and respiration, the use of alternative carbon sources, and stress response pathways are suppressed in cells [21, 41].

There are both similarities and differences between aerobic glycolysis, where cancer cells turn to glycolysis even in the presence of oxygen (the Warburg effect), and processes where yeast cells prefer fermentation over cellular respiration in the presence of high glucose and oxygen (the Crabtree effect). In both cancer cells and yeast, this fermentative metabolism is associated with rapid growth and proliferation [17]. In both processes, fermentation occurs in an oxygenated environment; however, the end products are lactate (resulting from aerobic glycolysis) or ethanol (resulting from fermentation). Additionally, aerobic glycolysis and fermentation, glycolysis is rapid, and mitochondrial respiration is repressed. Besides their similarities, one of the most important differences is that the Warburg effect is permanent in cancer cells, but in yeast, when glucose is depleted in the environment, aerobic respiration be-

gins [42]. Santos and Hartman (2019) mimicked the Warburg effect by repressing respiration in the presence of glucose in *S. cerevisiae*. They examined the effects of doxorubicin used in chemotherapy in a yeast model and reported that glucose-repressed yeasts could be a suitable model for cancer research [43]. Although yeast, a single-celled organism, cannot fully provide the Warburg effect, it has the potential to elucidate cancer mechanisms due to the similarities between the processes.

In *S. pombe*, glucose is sensed by G protein-coupled receptor (GPCR), and signal transduction occurs via cAMP-dependent protein kinase A (PKA) [22, 44]. Glucose repression signaling represses *fbp1* gene expression via activation of the cAMP-dependent PKA pathway [45]. In our study, under aerobic glycolysis-like conditions, the expression of the *fbp1* gene encoding the fructose-1,6-bisphosphatase-1 enzyme that plays a role in gluconeogenesis was repressed.

Hexokinase-2 enzyme is encoded by the *hxx2* gene and is a rate-limiting enzyme that functions in the first step of the glycolytic cascade [19]. Hxx2 is an important enzyme in glucose repression. Under high glucose conditions, PKA causes hyperphosphorylation of Rgt1, followed by expression of the *hxx2* gene [46, 47]. In our study, transcription of the *hxx2* gene was unexpectedly decreased under aerobic glycolysis-like conditions in pMS-mapt cells. This may be due to the increase in the expression of the MAPT gene.

When total tau and the phosphorylation status of tau in the S262, S396, and S404 regions were examined by immunoblot analysis, aerobic glycolysis-like caused an increase in the expression of tau protein, while a decrease in phosphorylation was observed at S396 residue. Tau phosphorylation at S396 and S404 residue is one of the earliest events in AD [48, 49]. We observed that under aerobic glycolysis-like conditions, the increase in MAPT gene expression in cells is consistent with the increase in tau protein. However, under aerobic glycolysis-like conditions, higher phosphorylation occurred at residues S262 and S404 of tau protein. Tau phosphorylation at sites such as Ser262 in the proline-rich region and Ser396/404 at the edges of the microtubule binding

site induces conformational change of tau protein and weakens the binding of tau protein to microtubules [50]. Full-length tau has 85 potential phosphorylation sites [51]. Since only three phosphorylation sites (S262, S396, and S404) were analyzed in the present study, our interpretation remains limited. Future studies should include additional AD-relevant phosphorylation sites to better assess tau modification patterns.

Glycogen synthase kinase-3 (GSK3) is a protein kinase composed of GSK3 α and GSK3 β subunits that phosphorylates a large number of substrates. Increased GSK3 expression has been seen in the brains of AD patients and models. GSK3 directly promotes tau phosphorylation, modulates amyloid precursor protein (APP) breakage, results in A β production, and either directly or indirectly incites neuroinflammation and oxidative injury [52]. In our study, under aerobic glycolysis-like conditions, the expression of the *gsk3* gene, which encodes GSK3, which phosphorylates tau protein, was reduced in cells, and the decrease in the phosphorylation level of tau protein was associated with this decrease in the expression of the *gsk3* gene. PP2A, a serine/threonine protein phosphatase, is a tumor suppressor [53] and regulates the cell cycle by interacting with more than 300 cell cycle-related substrates [54]. Expression of protein phosphatase 2A (PP2A) is reduced in both cancer and neurodegenerative diseases. A decrease in PP2A-A α subunit expression to approximately 50% of the normal level induces tumor formation, while a decrease in PP2A-A α expression by more than 63% results in apoptosis [14]. Similar to cancer conditions, we observed that decreased expression of the *pp2a* gene in pMS-mapt cells under aerobic glycolysis-like conditions.

It was seen that the cells were both smaller and had different cellular shapes compared to cells in normal conditions. However, these differently shaped cells are not dead cells, because the cells were observed to reproduce for 32 hours.

Under glucose repression conditions, the stress response in the cell is repressed [22, 55, 56]. We also observed a decrease in the gene expression of stress response genes *sod1* and *ctt1*, as expected. Suppression of stress response genes in pMS-mapt cells resulted in an increase in the level of intracellular oxidation. ER stress and unfolded protein response (UPR) are molecular events in the development of AD. Failure of these mechanisms results in the formation of pathological structures such as neurofibrillary tangles formed by hyperphosphorylated tau. ER stress is an imbalance between protein synthesis and ER protein folding capacity. This results in the accumulation of misfolded proteins in the ER. As a result of ER stress, unfolded proteins are proteasomally degraded [57–59]. In the present study, we observed that the expression of the *ire1* gene, which is involved in the ER stress response, was reduced under aerobic glycolysis-like conditions. A decrease in autophagy is an expected result due to decreased ER stress in pMS-mapt cells, and this may explain the decrease in *atg14* gene expression. No significant change in 20S proteasome activity was observed under aerobic glycolysis-like conditions in pMS-mapt cells. Decreases in ER stress response and autophagy are the expected results under repression conditions.

Several studies on diabetes and tau protein suggest a role of tau protein, particularly in regulating glucose homeostasis. In some studies, Alzheimer's disease has been referred to as type III diabetes. However, the relationship between impaired glucose signaling and tau has not yet been elucidated [60]. One of the limitations of this study is that the effect of insulin, one of the most important components of glucose metabolism, could not be demonstrated in model cells. Another limitation is that the DNA protection function of tau protein has not been examined in our model. Emerging evidence for tau's functions in P53 regulation and DNA repair suggests that it is associated with cancer. Studies have shown that the MAPT gene is a potential factor in many types of cancer. However, the role of tau in cancer is still unclear. Whether tau is positively or negatively associated with cancer type may be because it plays a role in different cellular processes [7].

In the present study, aerobic fermentation metabolism in cancer was mimicked by creating glucose repression conditions in the tau protein-producing *S. pombe* model organism. Under aerobic glycolysis-like conditions, we observed an increase in tau protein expression and a decrease in its phosphorylation. In contrast, it is the increase in tau phosphorylation that causes pathology in tauopathies. Thus, under cancer-mimicking conditions, a situation in tau phosphorylation that was opposite to that in neurodegeneration was observed. The fact that a different response than expected occurred also in glucose metabolism in these tau-producing cells suggests that tau protein may be a common component that plays a greater role in the mechanisms underlying the diseases than thought. In our study, we examined only one phosphorylation site, and although this is not sufficient for a definitive conclusion, it provides data on the change of phosphorylation status. In future studies, target phosphorylation sites in Alzheimer's disease should be examined in more detail in cancer. Based on previous studies and our findings, tau protein may play a more important and multifunctional role in cellular mechanisms than thought. Therefore, the cellular mechanisms in which tau protein is involved and the molecular components it interacts with should be investigated in more detail.

Conclusion

More detailed studies are needed to understand the molecular and cellular mechanisms related to the different or common features between cancer and neurodegeneration. Both diseases are common and have high mortality rates. Most studies investigating the relationship between cancer and neurodegenerative diseases demonstrate the potential for one disease to protect against the other. At this point, it is of great importance to determine the mechanism that brings together two opposing processes, such as uncontrolled cell proliferation and degeneration, and the intermediary molecules involved in this mechanism. Understanding the underlying mechanisms linking AD and cancer will enable the development of prevention strategies and treatment for both diseases.

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Research Article

Adaptive mitochondrial modules: Going with the flow of cancer-specific metabolic rewiring

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Abstract

Objectives: Mitochondrial gene networks constitute a fundamental subsystem of cellular homeostasis, integrating bioenergetic, metabolic, and signaling functions. In cancer, the rewiring of these networks represents a critical mechanism of metabolic adaptation, enabling tumor cells to sustain growth and survival under diverse microenvironmental constraints. To systematically characterize these alterations, we analyzed transcriptomic data from The Cancer Genome Atlas (TCGA) with a specific focus on mitochondrial genes, aiming to uncover cancer-type-specific patterns of differential expression and their potential biological implications.

Methods: Transcriptomic data from The Cancer Genome Atlas (TCGA) were analysed to identify differential expression patterns in mitochondrial genes. Weighted Gene Co-expression Network Analysis (WGCNA) was applied to detect co-expressed gene modules. The biological relevance of these modules was assessed through functional enrichment analysis and survival modelling using Cox regression and Kaplan–Meier estimations. Dimensionality reduction techniques including PCA and UMAP were used to evaluate module-driven clustering patterns across cancer types.

Results: Seven mitochondrial gene modules were identified, six of which demonstrated significant associations with specific cancer types. Modules ME2, ME4, ME5, ME6, and ME7 were associated with improved overall survival, while ME3 correlated with poorer prognosis. Functional enrichment analyses revealed distinct mitochondrial processes including oxidative phosphorylation, apoptosis, fatty acid β -oxidation, and ketone body metabolism. Dimensionality reduction analyses supported the presence of module-specific expression patterns with cancer-type-dependent clustering.

Conclusion: The observed cancer-type-specific expression and prognostic associations of mitochondrial gene networks reflect their central involvement in the metabolic flexibility of tumors. By underscoring the clinical and biological significance of mitochondrial subsystems, these findings suggest that they may serve not only as prognostic markers but also as promising targets for therapeutic modulation.

Keywords: Cancer metabolism, co-expression modules, gene expression profiling, gene co-expression networks, mitochondrial genes, systems biology

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Biological systems are intrinsically complex, dynamic, and deeply interconnected. To maintain cellular homeostasis, they rely on multilayered regulatory networks that combine structural redundancy with exceptional adaptive flexibility [1, 2]. This adaptability may allow cancer cells to emerge as reorganized—yet still coordinated—deviations from the original regulatory architecture. Even in the disease state, internal logic

and systemic coordination may persist through altered but non-random arrangements of regulatory configurations [3].

Understanding these transformations is particularly challenging due to the high-dimensional, non-linear, and interdependent nature of molecular interactions. Numerous molecular components operate simultaneously and influence one another in non-linear ways, making it difficult to isolate individual

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effects or predict system-wide behavior. This complexity poses significant challenges for both computational modeling and biological interpretation, especially when attempting to capture the emergent properties of the system as a whole [4–6].

One rational strategy to navigate this complexity is to focus on key functional groups of genes or proteins (regulatory nodes) that coordinate specific biochemical pathways or molecular processes. These groups are critical for cellular survival and proliferation and may be maintained or repurposed by cancer cells to sustain viability, differentiation, and growth [7, 8]. Among these, mitochondria are pivotal due to their roles in metabolic reprogramming, redox signaling, and apoptotic regulation [9]. Beyond these functions, while mitochondrial functions are modulated by nuclear-encoded proteins (1,138 genes), their compact genome (37 genes), defined metabolic pathways, and membrane-bound localization render them a relatively self-contained and tractable subsystem for dissecting cancer's regulatory rewiring [10–14].

Given these considerations, we hypothesize that differential mitochondrial gene expression patterns can reveal cancer-type-specific prognostic modules. Their regulatory roles are not fixed but dynamically adapted to meet the context-specific demands of diverse tumor types. This plasticity may underlie resistance to single-agent therapies, as tumors exploit the flexibility of these mitochondrial subsystems—groups of interacting genes or proteins performing coordinated functions—to sustain survival under therapeutic pressure [15–20]. In this study, we adopt a systems biology approach to investigate mitochondrial gene networks as a model regulatory subsystem—a group of interacting genes or proteins that jointly perform a functional role. Our aim is to identify adaptive mitochondrial modules that contribute to cancer-type-specific regulatory reorganization, with a particular focus on their prognostic significance and functional diversity across tumors.

Materials and Methods

Ethical considerations

This study was conducted exclusively using publicly available data from The Cancer Genome Atlas (TCGA) project (<https://www.cancer.gov/tcga>). All data were fully deidentified and used in accordance with the TCGA publication guidelines and data access policies. No new human or animal data were collected or generated by the authors. Therefore, this research is exempt from institutional review board (IRB) approval under current regulations [21]. All procedures performed in this study complied with the ethical standards of the TCGA consortium and with the 1964 Helsinki Declaration and its later amendments. The study complies with the U.S. Department of Health and Human Services policy for the protection of human research subjects (45 CFR 46). The TCGA provides an invaluable and ethically curated resource for studying cancer biology at the molecular level, enabling reproducible and large-scale *in silico* analyses [22, 23].

Study design and overview

To investigate mitochondrial gene regulatory networks across diverse cancer types, we used a systems biology framework that combines co-expression network analysis, module–phenotype association, and mechanistic enrichment. Our middle-out strategy—anchored at the module level where eigengenes represent the dominant expression pattern of co-expressed genes—links gene-level perturbations to higher-order phenotypes. This design enables the detection of biologically meaningful modules first and their subsequent association with phenotypes, balancing molecular detail with system-level interpretation.

This integrative analysis was conducted in three key phases; first, we analysed RNA-seq data from TCGA to identify tumor-specific co-expression modules (Fig. 1, steps 1 to 3). Second, we correlated these modules with clinical outcomes including survival and molecular subtypes (Fig. 1, steps 4 to 6). Third, we performed pathway enrichment analysis using pathway enrichment results obtained via Enrichr-KG which integrates GO, KEGG and Reactome databases to annotate mechanistic functions (Fig. 1, step 7) [24]. This integrative strategy enabled systematic mapping of mitochondrial regulatory programs in cancer while maintaining biological interpretability.

Data acquisition and preprocessing

RNA-seq data and clinical metadata were retrieved from TCGA using the GDCRNATools R package [25]. Raw HTSeq count data and corresponding clinical metadata were downloaded for 23 cancer types. Only cancer types with ≥ 2 matched Solid Tissue Normal samples were retained, excluding other tissue types and technical duplicates. This filtering yielded 17 cancer types: BLCA, BRCA, CESC, COAD, ESCA, GBM, HNSC, KICH, KIRC, KIRP, LIHC, LUAD, PRAD, READ, STAD, THCA, UCEC. Normalization was performed using the Trimmed Mean of M-values (TMM) method, followed by voom transformation, as implemented in the GDCRNATools package. Only genes annotated as mitochondrial in the MitoCarta3.0 [26] human gene set ($n=1,138$) were included, resulting in expression profiles for 7,874 samples (7,202 Primary Tumor, 672 Solid Tissue Normal). Sample counts per cancer type ranged from 91 (KICH) to 1,208 (BRCA).

Delta expression matrix calculation

To quantify tumor-specific transcriptional alterations, a delta expression matrix was constructed by subtracting the mean expression of each gene in Solid Tissue Normal samples from Primary Tumor expression values, separately within each cancer type. Sample types were assigned using clinical metadata. The final matrix contained 1,138 mitochondrial genes (rows) and 7,202 tumor samples (columns). All identifiers were checked for dimensional consistency prior to further analysis. A limitation of this approach is the small number of normal samples in a few cancer types, which may reduce the robustness of the differential expression scores.

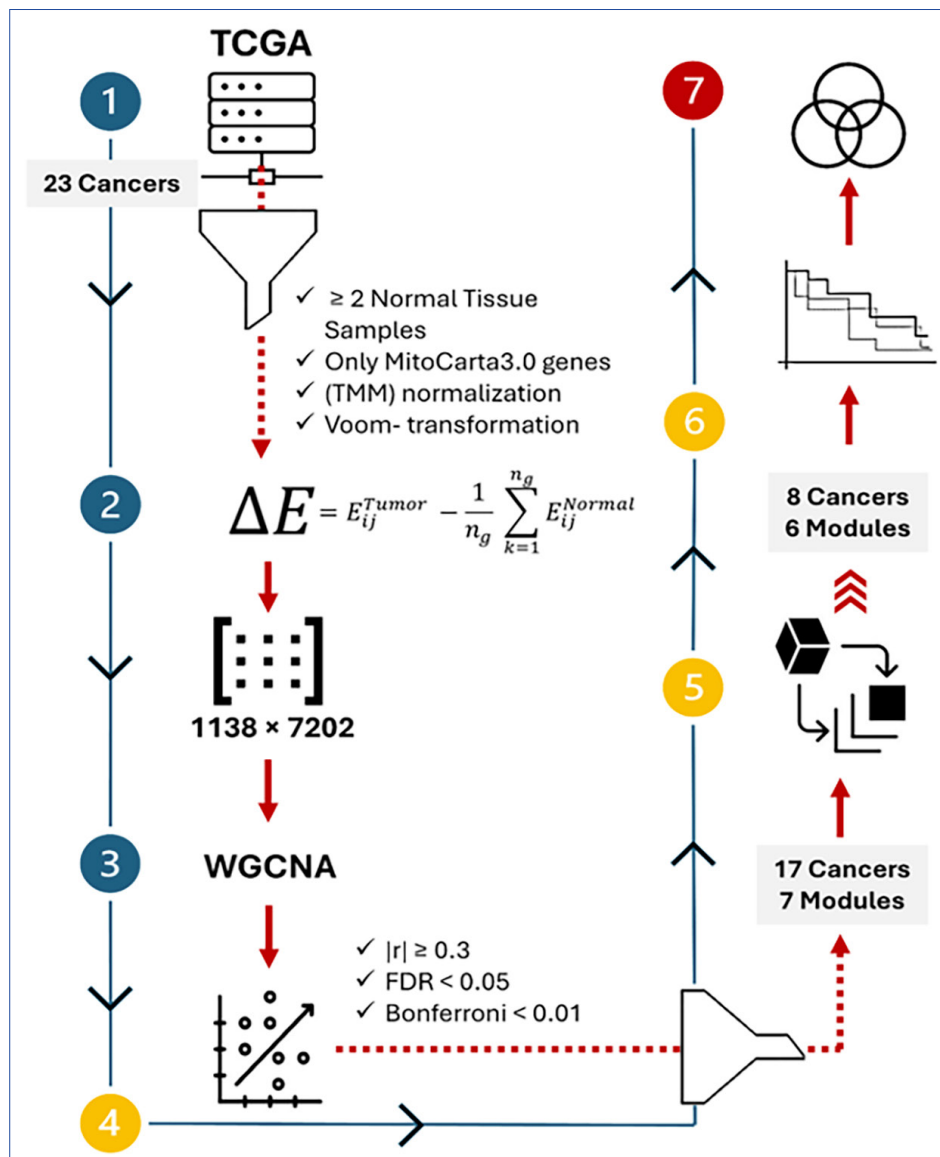


Figure 1. Schematic overview of the module-based cancer analysis pipeline. The workflow consists of seven main steps: (1) acquisition and preprocessing of gene expression and phenotype data, (2) calculation of delta expression, (3) construction of a gene co-expression network and module detection via WGCNA tool, (4) correlation analysis between modules and cancer types, (5) dimensionality reduction and visualization of module-trait relationships, (6) survival analysis based on module expression, and (7) functional enrichment analysis to infer biological relevance. Funnel icons represent filtering steps, with specific exclusion criteria indicated adjacent to each filter.

WGCNA: Weighted Gene Co-expression Network Analysis.

Co-expression network construction and module detection

Weighted Gene Co-expression Network Analysis (WGCNA) was performed on the delta expression matrix to identify modules of co-expressed mitochondrial genes [27]. A soft-thresholding power of $\beta=4$ was selected based on scale-free topology and mean connectivity criteria ($R^2=0.89$), as illustrated in Appendix 1a-b. The resulting adjacency matrix was used to compute the topological overlap matrix (TOM), followed by hierarchical clustering and dynamic tree cutting, which identified seven distinct co-expression modules (Appendix 1c).

To evaluate module stability, the dataset was randomly split into reference (70%) and test (30%) subsets, and module preservation was assessed across 20 permutations using both Z-summary and median rank statistics, with higher Z-summary and lower median rank values indicating stronger and more biologically coherent preservation; median rank values supported the hierarchy suggested by Z-summary, hub genes were identified based on intra-module connectivity (Appendix 2). The Topological Overlap Matrix (TOM) was used to compute k_{Within} values, and genes with $k_{Within} > 1$ SD above the

module mean were designated as hub genes. These genes were retained for downstream functional analyses.

Module-cancer type correlation analysis

Cancer type metadata was one-hot encoded to match the sample order in the module eigengene (ME) matrix. Pearson correlations between MEs and binary cancer-type variables were computed to identify module–cancer associations, with significance assessed via Student’s t-distribution. Multiple testing correction was performed using both False Discovery Rate (FDR) and Bonferroni methods. Correlations with $|r| \geq 0.3$ and adjusted p-values <0.05 and $FDR<0.01$ (Bonferroni) were considered significant (Appendix 2).

Dimensionality reduction and visualization of module-trait relationships

To explore module–cancer associations, dimensionality reduction was applied to delta expression data restricted to genes within significant modules. Principal Component Analysis (PCA) was used to project samples into lower-dimensional space while preserving variance. Clustering patterns by cancer type were visualized along the first two principal components, and cluster quality was assessed using silhouette scores. To capture nonlinear structure, UMAP and t-SNE were also performed, both supporting PCA-derived groupings and revealing distinct cancer type separations based on module gene expression.

Functional enrichment analysis

Functional enrichment analysis was conducted for each module using the Enrichr-KG platform, which integrates curated databases such as WikiPathways, Reactome, KEGG, and Gene Ontology [24]. Enrichment was based on the statistical overrepresentation of module genes within known pathways, assessed via adjusted p-values. Only modules significantly correlated with cancer types were included to focus on biologically relevant gene networks. Significant terms were summarized and visualized to aid interpretation of predominant functional themes within each module.

Survival analysis

The prognostic relevance of mitochondrial gene co-expression modules was assessed using Kaplan–Meier survival curves and univariate Cox proportional hazards models based on module eigengene expression. Module scores were matched with clinical survival data (time-to-event and event status) from 7,202 tumor samples with complete metadata. Samples were dichotomized into "High" and "Low" groups by the median eigengene value per module. While median-based grouping is common practice, it may lead to some information loss, which should be considered when interpreting results. Survival differences were evaluated with log-rank tests, and hazard ratios (HR) with 95% confidence intervals were estimated via Cox models. P-values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) method (Appendix 2). This approach provided robust prognostic assessment across cancer types while avoiding assumptions related to continuous variable modeling.

Table 1. Mitochondrial gene co-expression modules and preservation statistics

Module	Gene count	Z-summary	Median rank
ME1	443	28.85	2
ME3	107	16.54	4
ME2	162	13.89	7
ME4	99	13.29	5
ME6	58	12.27	2
ME5	72	10.13	5
ME7	46	9.85	4

Preservation was assessed using Z-summary, a composite statistic reflecting module stability across datasets, and median rank metrics over 20 permutations. Higher Z-summary and lower median rank values indicate stronger and more biologically coherent module preservation across cancer types.

Software and tools

All analyses were performed using R version 4.4.1 (2024-06-14) on Windows 11 x64. Key R packages included GDCRNATools (v1.18.0), WGCNA (v1.73), survival (v3.8-3), survminer (v0.5.0), dynamicTreeCut (v1.63-1), fastcluster (v1.2.6), ggplot2 (v3.5.2), ggpubr (v0.6.0), tidyverse (v2.0.0), umap (v0.2.10.0), and Rtsne (v0.17).

Results

Module preservation and structural robustness

WGCNA identified seven mitochondrial gene co-expression modules (ME1–ME7), ranging from 46 to 443 genes in size (Table 1). Genes not assigned to any module (ME0) were grouped into the gray module and excluded from downstream analyses. Module preservation was evaluated using Z-summary and median rank statistics across 20 permutations. Four modules—ME1, ME3, ME2, and ME4—showed strong preservation. ME6 and ME5 also met the threshold for high preservation, while ME7 demonstrated moderate stability. Median rank values supported the Z-summary-based hierarchy of module robustness. Collectively, these results indicate that the identified modules represent reproducible and biologically coherent co-expression structures among mitochondrial genes across cancer types.

Module–cancer type associations

To evaluate the biological relevance of mitochondrial gene modules across cancer types, we assessed the correlations between module eigengenes and tumor labels. Six of the seven modules (ME2–ME7) showed statistically significant associations with at least one cancer type (Fig. 2). In total, twelve significant module–cancer type pairs were identified, involving eight distinct cancer types. Full correlation coefficients and adjusted p-values are presented in Table 2. The strongest positive associations were observed for ME6 with KIRC and ME5 with THCA, while ME7 exhibited the most pronounced negative correlation with LIHC.

These findings suggest that mitochondrial gene co-expression patterns vary systematically across cancer types, potentially reflecting tumor-specific mitochondrial reprogramming. Based on significance filtering, a refined dataset was

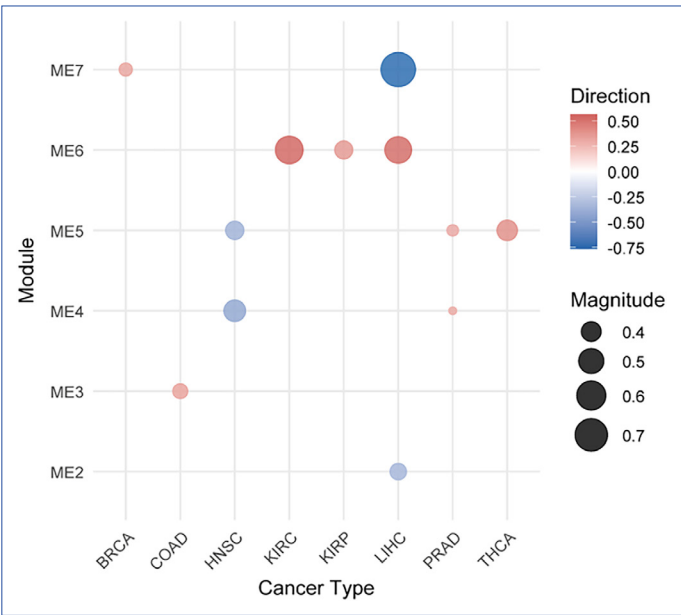


Figure 2. Module–cancer correlations. Bubble plot showing correlations between gene expression modules (ME2–ME7) and cancer types after significance filtering. Bubble size reflects the absolute correlation, while color indicates direction (red: positive, blue: negative).

generated comprising six modules (ME2 to ME7) and eight cancer types (BRCA, LIHC, COAD, KIRC, KIRP, HNSC, PRAD, and THCA). This subset included 544 genes and 4,269 tumor samples and was used for subsequent clustering, survival, and functional enrichment analyses. To provide a comprehensive overview, we included the full module–cancer correlation matrix, the module-level delta expression heatmap, and eigengene distributions across cancer types, shown in Appendix 3, 4, and 5, respectively.

Dimensionality reduction and clustering of module activity

To assess whether mitochondrial module activity could stratify tumors by type, we applied PCA, UMAP, and t-SNE to the expression profiles of 544 genes across 4,269 tumor samples. PCA accounted for a moderate portion of variance but yielded limited clustering performance for most cancer types (Fig. 3a). In contrast, both UMAP and t-SNE revealed clearer separation, with UMAP achieving the highest overall cluster quality and strongest within-type cohesion across several cancer types, notably PRAD, LIHC, and HNSC (Fig. 3b, c).

These findings indicate that non-linear dimensionality reduction techniques better capture the underlying mitochondrial expression patterns that differentiate tumor types.

Survival associations of mitochondrial modules

Univariate Cox proportional hazards analysis demonstrated significant associations between mitochondrial gene co-expression modules and overall survival across 7,202 tumor samples. Modules ME5, ME7, ME4, ME6, and ME2 were associated with improved prognosis, with hazard ratios ranging from approximately 0.40 to 0.79 (all adjusted $p < 0.001$). Converse-

Table 2. Prognostic mitochondrial gene modules and their cancer-type-specific associations		
Module	Cancer	Survival effect (HR)
ME5	HNSC (-), PRAD (+), THCA (+)	0.40 (protective)
ME7	BRCA (+), LIHC (-)	0.46 (protective)
ME4	HNSC (-), PRAD (+)	0.47 (protective)
ME6	KIRC (+), KIRP (+), LIHC (+)	0.72 (protective)
ME2	LIHC (-)	0.79 (protective)
ME3	COAD (+)	1.25 (risk increasing)

Cancer	Modules	Survival effect (HR)
BRCA	ME7 (+)	0.46 (protective)
COAD	ME3 (+)	1.25 (risk increasing)
HNSC	ME4 (-), ME5 (-)	0.47 , 0.40 (protective)
KIRC	ME6 (+)	0.72 (protective)
KIRP	ME6 (+)	0.72 (protective)
LIHC	ME7 (-), ME2 (-), ME6 (+)	0.46 ,0.79, 0.72 (protective)
PRAD	ME4 (+), ME5 (+)	0.47 ,0.40 (protective)
THCA	ME5 (+)	0.40 (protective)

Mitochondrial modules (ME2–ME7) showing significant associations with specific cancer types and corresponding hazard ratios (HR) from survival analysis are summarized. The top section lists each module and its correlated cancer types; the bottom section takes a cancer-centric view, indicating associated modules and their prognostic effects. Modules with HR < 1 indicate protective associations, while HR>1 suggests increased risk.

ly, ME3 showed a significant association with poorer survival (HR>1, adjusted $p < 0.001$). These findings were consistently supported by Kaplan–Meier survival analyses (Fig. 4) and further quantified by module-specific hazard ratios calculated from scaled eigengene expression.

Functional signatures of mitochondrial modules

Each identified module represents a coordinated gene program reflecting distinct aspects of mitochondrial biology. Functional enrichment analyses revealed that modules are associated with specific mitochondrial processes as follows.

ME2 (Aminoacyl-tRNA and mitochondrial protein synthesis)

ME2 is enriched in mitochondrial aminoacyl-tRNA synthetases and components involved in mitochondrial translation, with pathway enrichments in Aminoacyl-tRNA biosynthesis, Mitochondrial tRNA Aminoacylation, and Translation. It also includes genes related to the TCA cycle, suggesting a link between protein synthesis and central carbon metabolism. Functionally, ME2 likely regulates mitochondrial translational capacity critical for bioenergetic demands. Its expression is negatively correlated with tumor presence and positively associated with better overall survival in liver hepatocellular carcinoma (LIHC), indicating that preserved mitochondrial translation supports favorable prognosis in metabolically active tumors. Disease association analysis highlights links to mitochondrial disorders such as lactic acidosis, reflecting mitochondrial dysfunction that may underlie LIHC metabolic reprogramming.

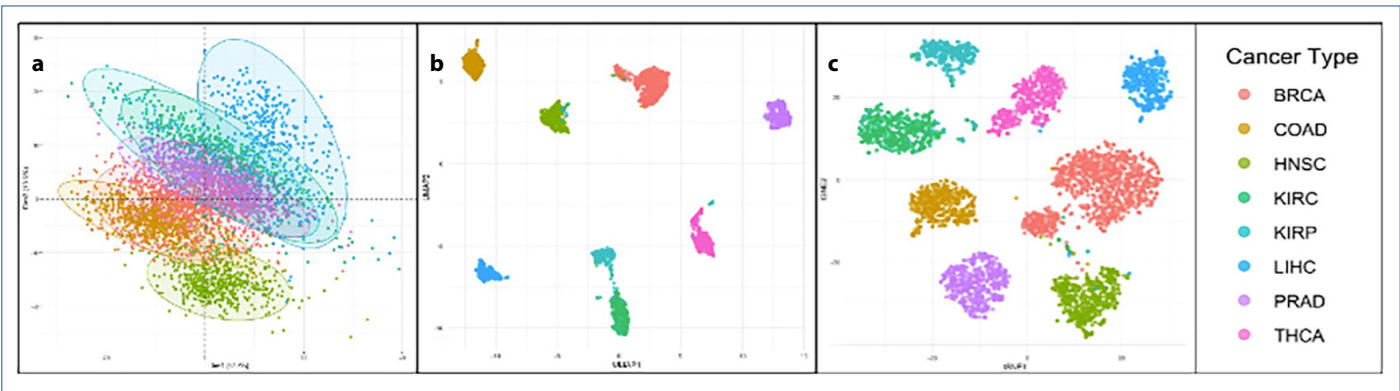


Figure 3. Dimensionality reduction analysis of module–trait relationships. (a) PCA, (b) UMAP, and (c) t-SNE plots illustrate the distribution of samples based on module eigengene expression profiles. Each point represents a sample, and colors correspond to different types of cancer as indicated in the legend. These visualizations highlight the clustering patterns and potential separability of cancer types based on module-level expression signatures.

PCA: Principal component analysis; UMAP: Uniform Manifold Approximation and Projection; t-SNE : t-Distributed Stochastic Neighbor Embedding.

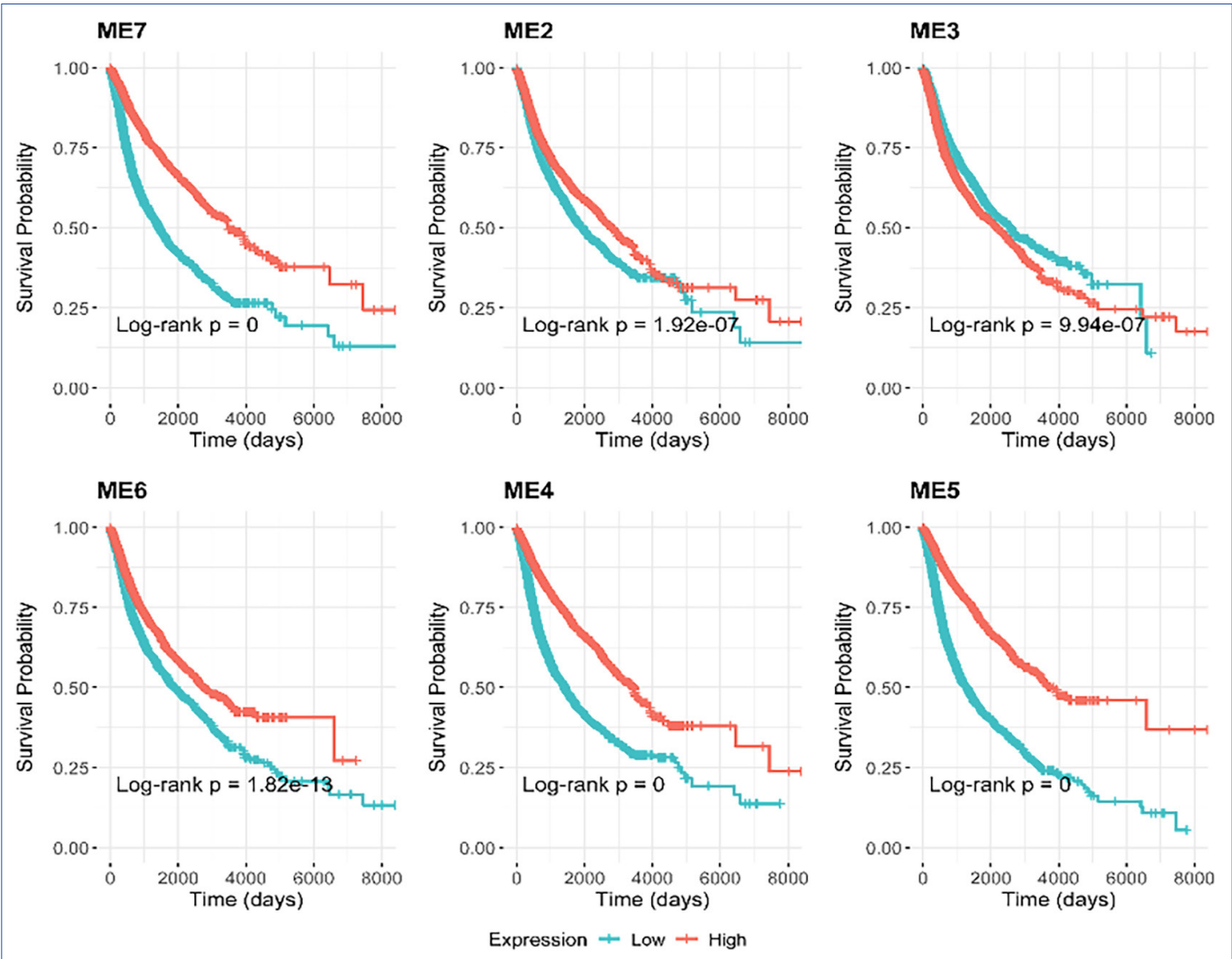


Figure 4. Kaplan-Meier survival curves for cancer correlated modules. Survival probabilities over time (days) are shown for patients stratified by high (red) versus low (blue) module expression. All differences between groups were statistically significant based on log-rank tests (adjusted $p < 0.001$).

ME3 (Oxidative phosphorylation and mitochondrial translation initiation)

ME3 is enriched in genes related to mitochondrial translation initiation, oxidative phosphorylation (OXPHOS), and respiratory complex assembly, reflecting a coordinated bioenergetic program essential for ATP production. Key enriched pathways include Oxidative Phosphorylation, Respiratory Electron Transport, and Mitochondrial Translation Initiation. Clinically, ME3 expression positively correlates with tumor presence and poorer overall survival in colon adenocarcinoma (COAD), suggesting that elevated mitochondrial energy metabolism is associated with tumor aggressiveness. This module likely represents a mitochondrial bioenergetic signature contributing to cancer progression in COAD.

ME4 (Fatty acid β -oxidation and branched-chain amino acid catabolism)

ME4 is enriched for genes involved in mitochondrial fatty acid β -oxidation and branched-chain amino acid (BCAA) catabolism, with pathway enrichments highlighting lipid degradation, acyl-CoA metabolism, and peroxisomal lipid processing. Key enzymes in valine, leucine, and isoleucine degradation underscore ME4's role in maintaining mitochondrial energy homeostasis through versatile substrate utilization, especially under metabolic stress or nutrient scarcity.

ME5 (Apoptosis, mitochondrial dynamics, and calcium homeostasis)

ME5 is enriched in genes regulating intrinsic apoptosis, mitochondrial dynamics, and calcium transport, with key pathways including Apoptosis, Neurodegeneration, and Mitochondrial Calcium Ion Transport. This module likely coordinates mitochondrial quality control and stress responses. Clinically, ME5 expression is reduced in head and neck squamous cell carcinoma (HNSC), correlating with tumor presence and poorer prognosis, whereas in prostate adenocarcinoma (PRAD) and thyroid carcinoma (THCA), higher ME5 levels associated with better survival despite positive tumor correlation. These findings suggest a protective role of ME5 across cancers, with context-dependent transcriptional regulation reflecting mitochondrial integrity and apoptosis pathways.

ME6 (Lipid biosynthesis, Acyl-CoA metabolism, and amino acid conjugation)

ME6 is enriched in genes regulating fatty acid biosynthesis, acyl-CoA metabolism, glycine conjugation, and pathways related to detoxification and amino acid catabolism. Key pathways include Fatty Acid Beta-Oxidation, Peroxisome function, and Amino Acid Metabolism, indicating a role in lipid catabolism and mitochondrial-peroxisomal crosstalk. Clinically, ME6 expression positively correlates with tumor presence in kidney cancers (KIRC, KIRP) and liver hepatocellular carcinoma (LIHC), and associates with improved overall survival, suggesting a protective metabolic program that may limit tumor progression. This contrasts with modules

like ME7, characterized by downregulation of mitochondrial translation and negative correlation with tumors such as LIHC. While ME6 reflects an active metabolic state supporting fatty acid oxidation and detoxification linked to better prognosis, ME7 indicates mitochondrial dysfunction or repression of mitochondrial protein synthesis associated with more aggressive tumor behavior. Together, these differences highlight the complex and diverse mitochondrial adaptations across cancer types that shape tumor biology and patient outcomes.

ME7 (Ketone body metabolism, urea cycle, and sulfur amino acid turnover)

ME7 is enriched in genes involved in ketone body metabolism, urea cycle, and sulfur amino acid metabolism. Pathway annotations highlight ketone metabolism, nitrogen metabolism, and sulfur relay systems, suggesting roles in metabolic reprogramming during fasting or nutrient fluctuations, integrating nitrogen detoxification, energy substrate switching, and redox buffering. Functionally, ME7 is composed mainly of mitochondrial ribosomal proteins and oxidative phosphorylation components, reflecting a core mitochondrial translational and bioenergetic program essential for maintaining a balanced proteome, apoptosis regulation, and ATP production. Clinically, ME7 expression correlates positively with overall survival (HR=0.46), indicating preserved mitochondrial function may suppress tumor progression. ME7 shows cancer-type specific expression patterns: Upregulated in breast cancer (BRCA) and downregulated in liver hepatocellular carcinoma (LIHC). These findings may reflect tissue-specific metabolic reprogramming. In BRCA tumors, the retention of mitochondrial translation and apoptotic signaling is associated with better prognosis, whereas LIHC exhibits metabolic dedifferentiation and hypoxic adaptation. The loss of ME7 module expression in LIHC further supports a shift toward aggressive tumor phenotypes. Downregulation of ME7 in LIHC mirrors disruption of mitochondrial metabolic pathways including amino acid and nitrogen metabolism, supporting aggressive tumor phenotypes. Conversely, ME7 upregulation in BRCA aligns with preserved mitochondrial function and metabolic flexibility, promoting controlled tumor growth and apoptosis. Overall, ME7 represents a mitochondria-centered tumor suppressive module whose context-dependent expression is prognostically informative, underscoring the interplay between mitochondrial translation, apoptosis, and metabolic adaptation in cancer biology.

Discussion

Our integrative analysis of mitochondrial-related gene expression modules across multiple cancer types reveals distinct module-cancer specificity patterns with significant prognostic implications as summarized in Table 2. Modules ME2, ME4, ME5, ME6, and ME7 generally demonstrate protective effects on overall survival, whereas ME3 shows a risk-increasing effect, highlighting the heterogeneous roles

of mitochondrial functions in cancer progression. Notably, ME2 exhibits a strong protective association uniquely in liver hepatocellular carcinoma (LIHC), consistent with its role in mitochondrial aminoacyl-tRNA synthetase function and bioenergetic regulation. ME3, conversely, correlates positively with tumor presence and worse prognosis specifically in colon adenocarcinoma (COAD), reflecting heightened oxidative phosphorylation activity potentially driving tumor aggressiveness. Modules ME4 and ME5 show complex, cancer-specific correlation directions, protective in some cancers (e.g., HNSC) but positively correlated in others (e.g., PRAD, THCA), indicating context-dependent mitochondrial pathway engagement. Modules ME6 and ME7 also display strong protective effects with positive correlations in kidney cancers (KIRC, KIRP) and breast cancer (BRCA), respectively, supporting the notion that mitochondrial functional states may influence survival in a tumor-type-specific manner.

Overall, our results emphasize the potential of mitochondrial functional modules as robust prognostic biomarkers and promising therapeutic targets across diverse cancer types. For instance, ME2's strong protective association specifically in liver hepatocellular carcinoma (LIHC) highlights how preserving mitochondrial translational capacity may suppress tumor progression in metabolically demanding tumors. Conversely, the risk-increasing profile of ME3 in (COAD) suggests that elevated mitochondrial oxidative phosphorylation activity contributes to tumor aggressiveness in this cancer type. These cancer-specific patterns suggest that mitochondrial dysfunction and metabolic rewiring may vary across tumors, reflecting distinct bioenergetic adaptations. This modular perspective may inform metabolic precision oncology, where therapeutic strategies can be tailored based on the dominant mitochondrial module dysregulated in a patient's tumor. Such an approach may enhance treatment efficacy by addressing cancer-specific metabolic dependencies, as exemplified by ME2-associated modules in LIHC potentially benefiting from therapies that restore mitochondrial translation and bioenergetics, while ME3-associated pathways in COAD might be targeted by inhibitors of oxidative phosphorylation. Therefore, integrating mitochondrial module profiling into clinical decision-making offers a promising avenue for developing more effective, personalized cancer treatments grounded in tumor metabolic phenotyping.

Furthermore, when stratifying tumors by their estimated metabolic phenotypes, we observed a striking pattern: Nearly all tumors classified as HGLO (High Glycolysis, Low OXPHOS (Oxidative Phosphorylation))—meaning they rely mainly on glycolysis and have suppressed mitochondrial respiration—belonged to the subset of cancers that showed no significant correlation with mitochondrial gene modules. In contrast, all tumors classified as HGHO (High Glycolysis, High OXPHOS)—which maintain both glycolytic and mitochondrial activity—were exclusively found among cancers with strong and consistent correlations with mitochondrial modules.

This distribution aligns with prior pan-cancer metabolic classifications [28], and suggests that mitochondrial module engagement may be shaped by the tumor's dominant metabolic strategy. Specifically, tumors with suppressed oxidative phosphorylation (HGLO) may show lower activity of mitochondrial gene modules, which can reduce ATP production and alter redox balance, explaining the lack of correlation. Conversely, tumors with active mitochondrial metabolism (HGHO) rely more on mitochondrial energy production and biosynthetic pathways, resulting in enhanced energy production and robust module engagement. These findings support the view that mitochondrial module expression may be both cancer-type specific and metabolically contextual and highlight the importance of integrating metabolic phenotyping into mitochondrial biomarker interpretation.

Our findings align with the evolving paradigm of mitochondria as dynamic cancer regulators. While early studies focused on the Warburg effect, we now recognize their pleiotropic roles in metabolic reprogramming, ROS signaling, and apoptosis [7, 13, 29, 30]. Notably, our results supports that mitochondrial adaptations are highly context-dependent across tumor types [13, 31]. These modules—particularly in translation and bioenergetics—may explain observed therapeutic resistance [7, 32], suggesting that targeting mitochondrial plasticity requires personalized approaches. Consequently, stratifying tumors by mitochondrial module expression profiles may thus provide a framework for metabolic subtyping and inform therapeutic strategies targeting mitochondrial vulnerabilities. Although key mitochondrial modules with prognostic and subtype-specific relevance were identified, functional validation is needed to clarify their causal roles. Integrating additional data such as mutations, epigenetics, and metabolomics could deepen mechanistic insights. Future studies should assess the potential of these modules as predictive biomarkers for patient stratification and therapies targeting metabolic vulnerabilities.

Interestingly, the cancer-type-specific behavior of mitochondrial modules may reflect a form of adaptive pleiotropy, a concept previously described in microbial systems [33–35]. In such contexts, early adaptive mutations often occur in global regulators, leading to broad transcriptomic shifts that influence multiple traits simultaneously [36]. Analogous regulatory dynamics may underlie the divergent prognostic roles of modules like ME2 and ME7 across tumor types. The lack of module association in HGLO tumors may further support this interpretation, consistent with stress-induced mitochondrial suppression. These observations suggest that mitochondrial modules may operate within regulatory architectures that favor coordinated multi-trait adaptation, reinforcing their role as context-sensitive hubs in cancer evolution [37–39]. In summary, our findings support the concept of adaptive mitochondrial modules that 'go with the flow' of cancer-specific metabolic rewiring, highlighting their potential as context-sensitive biomarkers and therapeutic targets.

Online Appendix Files: [https://jag.journalagent.com/ijmb/abs_files/IJMB-32656/IJMB-32656_\(3\)_IJMB-32656_Appendix.pdf](https://jag.journalagent.com/ijmb/abs_files/IJMB-32656/IJMB-32656_(3)_IJMB-32656_Appendix.pdf)

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Research Article

Can cinnamon reduce endoplasmic reticulum stress in diabetic nephropathy?: An experimental rat model

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Abstract

Objectives: The aim was to investigate the effects of cinnamon on the kidney tissue serum endoplasmic reticulum (ER) stress marker, reticulone (RTN)1A, receptor for advanced glycation end products (RAGE) and the lipid peroxidation indicator, malondialdehyde (MDA) in an experimental diabetes mellitus (DM) rat model.

Methods: Twenty-eight male Wistar Albino rats (six months old and weighing 350–400 g) were divided equally into four groups: 1) Control group - citrate buffer (0.2 M, pH 4.4; ip); 2) Cinnamon group - cinnamon (600 mg/kg/day, orogastric tube); 3) DM group - STZ (35 mg/kg, ip); and 4) DM + cinnamon group; Cinnamon and STZ were given at the same doses and route as in Groups 2 and 3, respectively. At the end of the 12-week experiment period, serum, urine and kidney tissue samples were taken from all groups. Serum RTN 1A, RAGE, MDA, urea, blood urea nitrogen (BUN), creatinine levels and kidney tissue RTN 1A, RAGE and MDA levels were measured.

Results: Our biochemical results showed that there was a statistically significant decrease in RAGE and MDA levels in the DM + cinnamon group compared to the DM group ($p < 0.05$). In addition, the decrease in serum urea, BUN, and creatinine levels in the DM + cinnamon group was also remarkable ($p < 0.05$). Although histologically no widespread necrosis was observed, cortical interstitial vascular dilatation was observed in DM+cinnamon group.

Conclusion: Cinnamon was effective in reducing markers of oxidative stress and ER stress including RAGE and MDA, in kidney tissue in an animal model of diabetic nephropathy.

Keywords: Cinnamon, diabetic nephropathy, endoplasmic reticulum stress, malondialdehyde, receptor for advanced glycation end products, reticulone 1A

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Diabetic nephropathy (DN) is a major cause of end-stage renal disease worldwide. Between 20 and 40% of diabetic patients develop DN [1]. DN is characterized by thickening of the glomerular and basement membranes, renal inflammation, tubular interstitial fibrosis, and progressive decrease in kidney function. Proteinuria and microalbuminuria are important markers for evaluating the progression of DN [2, 3].

Chronic hyperglycemia is the main cause of metabolic, biochemical and vascular abnormalities in DN. Oxidative stress (OS) caused by increased levels of reactive oxygen species

(ROS) in the cell, triggered by chronic hyperglycemia, due to mitochondrial dysfunctional cellular respiration and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity, can lead to DN [4–6]. As a result of OS, critical cellular components, especially protein, lipid, and DNA, are damaged and can lead to podocyte damage, endothelial cell dysfunction, mesangial cell damage, microalbuminuria, and apoptosis in the kidney [7]. Hyperglycemic states can also trigger an unfolded protein response (UPR) by inducing ER stress [5]. It has been suggested that hyperglycemia, protein-

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uria, and increased advanced glycation end products (AGEs) and free fatty acids in DN can trigger UPR in kidney cells, and chronic increased UPR response may result in cell death and increased kidney damage [6–8].

It is thought that the accumulation of AGEs has an important place in the pathophysiology of DN by contributing to the deterioration of ER homeostasis. AGEs show their abnormal effects by binding to their receptor, RAGE. Binding of AGEs with RAGE induces signal transduction and activates ROS production through dysregulation of NOX activity in endothelial cells. ER stress is induced not only in hyperglycemic states but also by hypoxia or oxidative stress, and UPR is triggered [9–11].

Reticulone 1 (RTN1)A is another molecule thought to contribute to ER stress-mediated kidney damage in DN [12]. Reticulones (RTNs) are proteins located in the ER membrane of the cell. There are three human RTN1 isoforms: RTN1A, RTN1B, RTN1C [13]. The human RTN1A protein consists of 776 amino acids, with a hydrophobic region in the ER membrane and hydrophilic regions extending out of the membrane (N-terminal and C-terminal) [14]. Increased expression of RTN1A has been reported to be associated with the progression of DN and the severity of kidney damage, but its mechanism is not yet clear [12, 15]. Increased expression of RTN1A in tubular epithelial cells induces apoptosis through the activation of ER stress [16]. Generally, agents that induce apoptosis at low doses induce necrosis at higher doses. Depending on the severity of exposure to the stimulus, apoptosis and cell necrosis may follow each other, both of which may copresent in most pathological conditions [17]. In this case, RTN1A cell content, indicated by increased renal expression, may be detected in the blood.

Cinnamon is widely consumed around the world as a spice and dietary supplement. The bark of cinnamon is peeled and dried from the body of a small tropical tree. Although there are about 250 types of cinnamon grown in the world, there are two types of cinnamon spices that humans consume: *Cinnamomum* (C.) *zeylanicum* and *C. cassia* [18]. Cinnamon has been shown to modulate glucose and lipid metabolism [19] and has shown pharmacological functions including inhibition of OS, and anti-inflammatory, antihypertensive, and antimicrobial effects [20, 21]. There are also studies suggesting that cinnamon is both preventive of the development of DN and protective against its progression by inhibiting the formation of AGEs [22, 23]. To date, no studies have investigated the impact of cinnamon on diabetic nephropathy (DN) in relation to RTN1A, an established marker of endoplasmic reticulum (ER) stress. In this study, we hypothesized that cinnamon would affect RTN1A, RAGE, and the lipid peroxidation indicator, MDA, in an animal model of DN.

Materials and Methods

Animals

Twenty-eight male Wistar Albino rats, six months old and weighing 350–400 g, were used. All rats were adapted for one week before the experimental procedure. During the adaptation and

treatment periods, all the animals were housed in cages at room temperature (22 ± 2 °C) and humidity ($55\pm 5\%$), and maintained under standard conditions with 12-hour light/dark cycles. They were fed a standard pellet diet and tap water ad libitum throughout the study. The study protocol was approved by the Institutional Animal Care and Ethical Committee of the University. (Approval Number: KOU HADYEK 2/1-2020). The study was designed in accordance with the Helsinki Declaration.

Experimental protocol

After the adaptation period, the rats were divided into four equally sized groups ($n=7/\text{group}$). These groups were: 1) the control group, which received the citrate buffer placebo (0.2 M, pH 4.4; Sigma Aldrich Co., St. Louis, MO., USA) intraperitoneally (i.p), in a single dose. 2) the cinnamon group which received cinnamon (stem barks of *C. Zeylanicum* (Ceylon cinnamon), 600 mg/kg/day) via orogastric tube suspended in distilled water, as previously described [24]; 3) the diabetes mellitus (DM) group, which received streptozotocin (STZ, Cat. No. S0130, Sigma Aldrich, St Louis, MO, USA) at a dose of 35 mg/kg dissolved in 0.2 M pH 4.4 citrate buffer i.p., was given as a single dose, as previously described [25]; And 4) the DM + cinnamon group: Diabetic rats were given cinnamon (600 mg/kg/day, orogastric tube) for 10 weeks.

Three days after STZ injection, rats with blood glucose levels of ≥ 300 mg/dL measured by glucometer (Accu-Chek, Roche, Basel, Switzerland) were considered diabetic and selected for the study [25]. This measurement was used solely as an inclusion criterion and was not part of the follow-up data. The experiment was continued for 12 weeks, including a two-week adaptation period [26]. Blood glucose levels were monitored at baseline and at the end of the first, third, and tenth weeks following STZ administration. Cinnamon was administered simultaneously with the STZ injection.

Collection of blood, urine and tissue samples

Spot urine samples of rats in all groups were collected into Eppendorf tubes and stored at -40 °C until tested. At the end of the experimental period, intracardiac blood was collected from rats in all groups under general anesthesia with 75 mg/kg ketamine + 15 mg/kg xylazine (90 + 12 mg/kg, i.p. single dose). Blood taken into a plain tube was centrifuged at 3500 g for 15 minutes and stored at -40 °C for biochemical analysis. Kidneys were perfused via abdominal aorta with 100 ml of phosphate buffered saline (PBS). Right kidney tissues were weighed, and 1/10 weight/volume PBS (0.1 M and pH 7.4) was added, and the tissues were homogenized. The homogenates were centrifuged at 3500 g for 15 minutes; the supernatants were separated, taken into Eppendorf tubes, and stored at -40 °C until analysis. Left kidney tissues were fixed with Bouin for 48 hours and prepared for routine light microscopy.

Biochemical measurements

In all groups, urea and creatinine levels of serum and urine were measured with an automated chemistry analyzer (AU

Table 1. Serum RTN1A, RAGE and MDA levels in all groups

Parameter	Control group (n=7)	Cinnamon group (n=7)	DM group (n=7)	DM+cinnamon group (n=7)
δ RTN1A (pg/mL)	307.34 $\pm$ 87.67	327.91 $\pm$ 74.40	428.28 $\pm$ 204.44	336.50 $\pm$ 23.68
ψ RAGE (ng/mL)	168.74 $\pm$ 4.27	179.98 $\pm$ 3.76*	186.19 $\pm$ 4.75*	181.26 $\pm$ 3.44*
δ MDA (nmol/mL)	0.76 $\pm$ 0.25	0.86 $\pm$ 0.13	1.48 $\pm$ 0.68**	0.96 $\pm$ 0.07

The values are expressed as mean $\pm$ standard deviation (SD). δ : p values are calculated with Mann-Whitney U test; ψ : p values are calculated with independent sample test; *: Compared with Control group; p<0.05, **: Compared with Cinnamon group; p<0.05. RTN1A: Reticulone 1 (RTN1)A; RAGE: Receptor for advanced glycation end products; MDA: Malondialdehyde; DM: Diabetes mellitus.

Table 2. Renal tissue RTN1A, RAGE and MDA levels in all groups

Parameters	Control group (n=7)	Cinnamon group (n=7)	DM group (n=7)	DM+cinnamon group (n=7)
ψ RTN1A (pg/mg protein)	31.50 $\pm$ 3.24	32.53 $\pm$ 3.17	48.92 $\pm$ 7.86**	36.45 $\pm$ 4.99
δ RAGE (ng/mg protein)	29.58 $\pm$ 1.84	30.69 $\pm$ 1.29	48.72 $\pm$ 8.48**	33.45 $\pm$ 3.06*+
δ MDA (nmol/mg protein)	0.14 $\pm$ 0.04	0.15 $\pm$ 0.01	0.33 $\pm$ 0.09**	0.18 $\pm$ 0.10*+

The values are expressed as mean $\pm$ standard deviation (SD). ψ : p values are calculated with independent sample test; δ : p values are calculated with Mann-Whitney U test; *: Compared with Control group, p<0.05; **: Compared with Cinnamon group, p<0.05; +: Compared with DM group, p<0.05.

5800, Beckman Coulter Inc., Brea, CA, USA). Rat RTN1A and rat RAGE concentrations in kidney tissue and serum were measured by rat-specific ELISA (Cat. No: 201-11-4817 and Cat. No: 201-11-4438, Sunred Biological Technology Co., Shanghai, China). Serum and kidney tissue malondialdehyde (MDA) concentrations were measured using the method of Buege and Aust [27]. Protein concentrations of tissues were determined by the method of Lowry et al. [28].

Light microscope procedures

Sections were trimmed into 20 μ m sections up to the beginning of the kidney tissue using a Leica SM2000 R microtome (Leica microsystems, Wetzlar, Germany). When the tissue was reached, serial sections of 4–6 μ m were taken. Five preparations were made by skipping ten sections from each kidney. The sections were taken from a hot water bath set at 450°C and mounted on standard glass slides. Then, the sections were stained with either hematoxylin and eosin (H&E) or periodic acid Schiff (PAS). The microscopic structure of kidney tissue was routinely evaluated with H&E. The development of diabetic nephropathy in rats was evaluated according to histopathological criteria. Histologically, necrotic changes in the renal cortex were quantified according to 5 parameters: 1. Interstitial Edema, 2. Epithelial Changes, 3. Tubular Degeneration, 4. Capillary Congestion, 5. Leukocyte Infiltration. PAS stains glomerular capillaries, mesangium, basement membranes of tubules, and Bowman capsules as positive (+). Examination using PAS thus allows for evaluation of the thickness of the basement membrane and possible defects in renal structures. Renal sections from all groups stained with H&E and PAS, were examined under an Olympus Light Microscope (Olympus CX41RF, Olympus Corporation, Tokyo, Japan) and photographed with an Olympus DP26 (Olympus Corporation, Tokyo, Japan) camera.

Statistical analysis

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 10 (GraphPad Software Inc.; San Diego, CA, USA). Normality of data distribution was assessed using the Shapiro–Wilk test. Descriptive statistics were expressed as mean $\pm$ standard deviation (SD) for normally distributed variables. Independent sample t test was employed for normally distributed data, for non-parametric variables, Mann-Whitney U test was performed.

In addition, two-way ANOVA followed by Tukey's post hoc multiple comparison test was conducted to evaluate changes in glucose levels across four time points. All tests were two-tailed, and a p value<0.05 was considered statistically significant.

Results

Effects of cinnamon on serum RTN1A, RAGE and MDA levels

The effects of cinnamon on serum RTN1A, RAGE and MDA levels in this diabetic rat model are shown in Table 1. In the DM group, serum RAGE and MDA levels significantly increased compared to the control group (p<0.05). No statistically significant difference was found between the DM + Cinnamon group and the DM group in terms of any parameter.

Effect of cinnamon on kidney tissue RTN1A, RAGE and MDA levels.

The effects of cinnamon on kidney tissue RTN1A, RAGE, and MDA levels in this diabetic rat model are shown in Table 2. In the DM group, RTN1A, RAGE, and MDA levels were significantly increased compared to the control group (p<0.05). In the DM + Cinnamon group compared to the DM group, a statistically significant decrease was found in RAGE and MDA levels (p<0.05).

Table 3. Effect of cinnamon on serum urea, BUN and creatinine, and urinary albumin and creatinine levels in all groups

Parameters		Control group (n=7)	Cinnamon group (n=7)	DM group (n=7)	DM+cinnamon group (n=7)
Serum	^o Urea (mg/dL)	38.83±2.07	36.65±5.32	57.88±8.55 ^{*&}	39.59±2.27 ⁺
	^o BUN (mg/dL)	18.42±1.13	16.43±2.99	29.58±7.99 ^{&}	17.43±2.99 ⁺
	^ψ Creatinine (mg/dL)	0.34±0.04	0.29±0.08	0.47±0.06 ^{*&}	0.38±0.06 ⁺
Urine	^o Albumin (mg/dL)	2.69±1.13	4.26±1.45	21.85±15.47 ^{*&}	22.33±19.48 ^{*+}
	^o Creatinine(mg/dL)	28.92±10.79	23.32±8.97 [*]	25.84±6.72 [*]	36.15±12.60 [*]

The values are expressed as mean±standard deviation (SD). ^o: p values are calculated with Mann-Whitney U test; ^ψ: p values are calculated with independent sample test;

^{*}: Compared with Control group; p<0.05; [&]: Compared with Cinnamon group, p<0.05; ⁺: Compared with DM group; p<0.05. BUN: Blood urea nitrogen; DM: Diabetes mellitus.

Table 4. Blood glucose concentrations (mean±SD, mg/dL) in control and experimental groups of rats measured at baseline and at 1, 3, and 10 weeks following STZ injection

Experimental groups	Baseline	1 st week after STZ injection	3 rd week after STZ injection	10 th week after STZ injection
Control group	86.6±12.8	81.6±14.2	87.2±13.4	85.4±14.7
Cinnamon group	86.7±9.4	79.6±9.6	88.6±7.2	85.4±8.7
DM group	85.4±6.9	403.6±31.6	410.5±26.2	420.6±21.6
DM+cinnamon group	87.6±8.7	352.8±28.7	342.8±24.6	345.6±26.1
Group comparison	Baseline	1 st week	3 rd week	10 th week
Control vs. cinnamon	NS	NS	NS	NS
Control vs. DM	NS	p<0.001	p<0.001	p<0.001
Control vs. DM+cinnamon	NS	p<0.001	p<0.001	p<0.001
Cinnamon vs. DM	NS	p<0.001	p<0.001	p<0.001
Cinnamon vs. DM+cinnamon	NS	p<0.001	p<0.001	p<0.001
DM vs. DM+cinnamon	NS	p<0.001	p<0.001	p<0.001

The values are expressed as mean±standard deviation (SD). Results of two-way ANOVA followed by Tukey's post hoc multiple comparison test. SD: Standard deviation; STZ: Streptozotocin; DM: Diabetes mellitus; NS: Non-significant.

Effect of cinnamon on serum and urine parameters

Serum urea, BUN, and creatinine, and urine albumin and creatinine levels in the four groups are shown in Table 3. DM group: Serum urea, BUN, creatinine and urinary albumin levels significantly increased compared to the control group, while urinary creatinine levels decreased (p<0.05). Serum urea, BUN, and creatinine levels were significantly decreased in the DM + Cinnamon group compared to the DM group (p<0.05).

Blood glucose levels were analyzed after the STZ injection, at the 1st, 3rd, and 10th weeks, and it was determined that diabetes developed in the rats. Two-way ANOVA with Tukey's post-hoc multiple comparison test demonstrated that baseline glucose values did not differ among groups. From week 1 onward, diabetic (DM) animals exhibited markedly elevated glucose levels compared with controls (p<0.001). Cinnamon supplementation in DM animals produced a significant, progressive reduction in glucose concentrations versus DM alone (week 1: -50.8; week 3: -67.7; week 10: -75.0 units; all p<0.001), while no differences were observed between Control and Cinnamon groups at any time point (Table 4).

Histological evaluation

Normal parenchymal morphology of glomeruli, Bowman's capsular spaces, and tubules were observed in the kidney sections of the Control group under light microscopy. Tubules and arterioles were observed in the areas between the glomeruli. Larger arteries and vessels were traced at the interface between cortex and medulla. Only tubules and blood vessels were observed in the medulla. PAS staining revealed that the basement membranes in the tubules the Bowman capsule surrounding the glomeruli were thin (Fig. 1a-d). Similar morphological findings were observed in the Cinnamon group (Fig. 1e-h).

Granulo-vacuolar epithelial cell degeneration areas, and desquamation in tubules, hyaline cast, and leukocytic infiltration were observed in the DM and DM + cinnamon groups (Fig. 1i-p). PAS staining enabled examination of basement membrane thickness of renal tubules and Bowman capsules. PAS (+) areas in both the DM and DM + cinnamon group kidney sections, and PAS (+) staining in glomerulo-basement membranes were increased in comparison to tissues obtained from control animals (Fig. 1d, h, l, p).

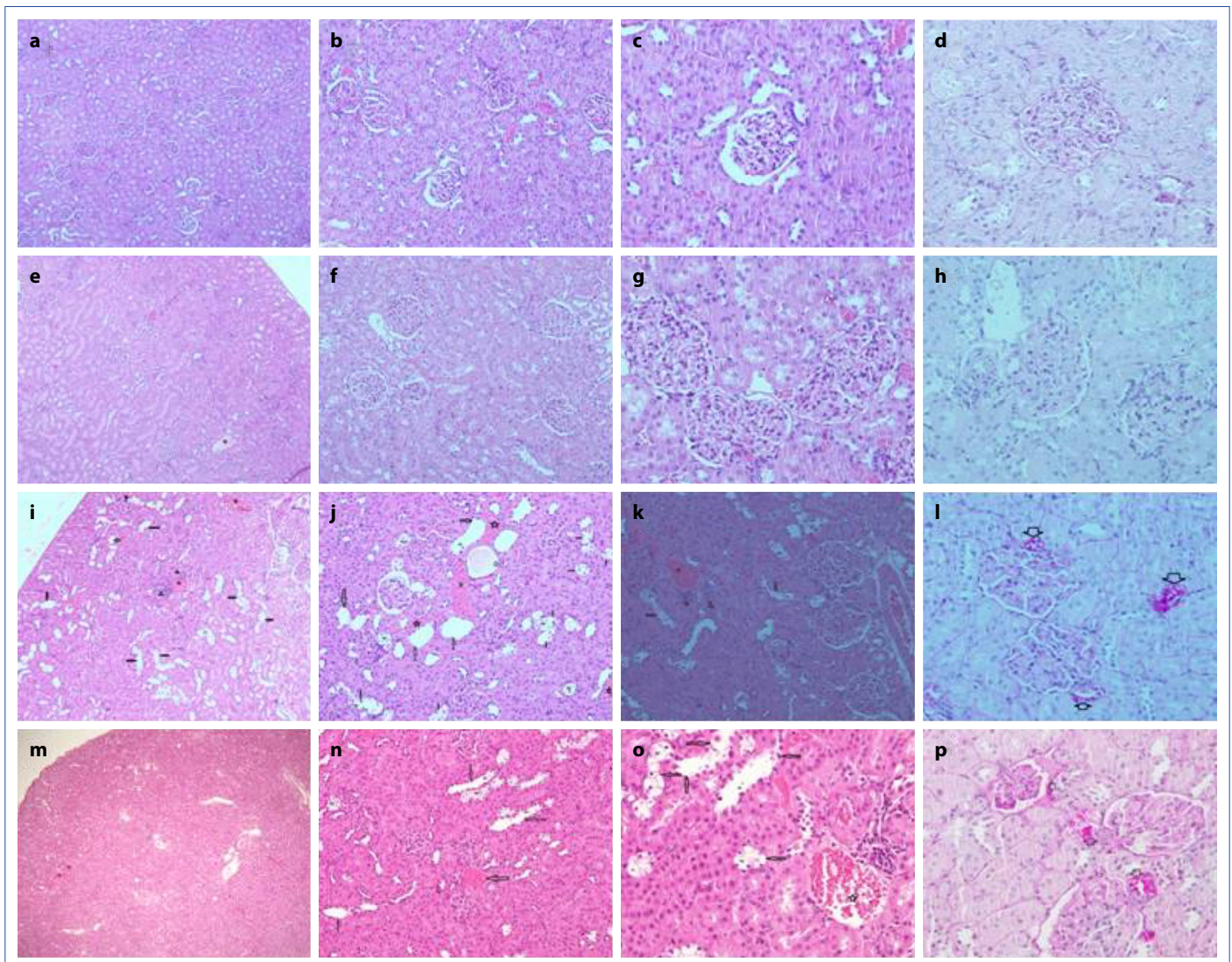


Figure 1. Photomicrographs of diabetic rats showing the effects of cinnamomum zeylanicum treatment on renal histology.

Histopathological changes were more prominent in the DM group (Fig. 1i-l) compared to the DM + cinnamon group (Fig. 1m-p). Although no widespread necrosis was observed, cortical interstitial vascular dilatation was observed in the DM+cinnamon group, (Fig. 1m-o).

These histological results suggest that cinnamon ingestion in the DM + cinnamon group did not completely eliminate microscopic renal damage. However, renal damage was not as common as in the DM group.

Discussion

This study demonstrates that cinnamon supplementation has significant effects on endoplasmic reticulum (ER) stress and oxidative stress (OS) in diabetic nephropathy (DN). In particular, RAGE and MDA levels in renal tissue were found to be significantly lower in the DM + cinnamon group compared with the DM group, suggesting an improvement in stress-related molecular pathways. Histologically, renal tissue damage such as tubu-

lar degeneration, leukocytic infiltration, vascular dilatation, and PAS(+) thickening of the glomerular basement membrane was observed in the diabetic groups; however, the severity of these changes was less pronounced in the DM + cinnamon group. Overall, these findings indicate that cinnamon intake reduced necrotic areas and alleviated renal damage in diabetic rats.

DN is one of the major microvascular complications of DM. The formation of AGEs, associated with hyperglycemia, is thought to play a central role in the pathophysiology of DN [29]. RAGE, a transmembrane receptor belonging to the immunoglobulin superfamily and found in almost all cell types in the kidney, plays key roles in innate immunity and inflammatory processes [30, 31]. It has been shown that increased AGEs due to DM stimulate RAGE expression in the kidneys [32]. It is thought that RAGE activation induces OS, ER stress, and UPR activation by stimulating NOX-mediated ROS production, causing inflammation, glomerular hypertrophy, podocyte damage and renal fibrosis. NOXs are sources of ROS, induced ER stress in kidney cells, and NOX

activity is induced by hyperglycemia, aggregation of AGEs, and activation of protein kinase C (PKC) [33]. UPR consists of three main signaling pathways initiated by the activation of three ER membrane receptors: Activating transcription factor 6 (ATF 6), enzyme 1 α that requires inositol (IRE1 α), and pancreatic ER eIF2 α kinase (PERK) [34]. These UPR-transducer proteins form the inflammatory signal cascade and regulate the expression of pro-inflammatory gene products through nuclear factor- κ B (NF- κ B) as well as other ER stress-inducible transcription factors that modulate ER functions [35]. Consistent with our study, Neto et al. [36] also concluded that cinnamaldehyde treatment reduced ER stress.

An interaction between ER stress and OS has been shown during the development and progression of DN [37]. OS can cause chronic inflammation in kidney tissue, tubule-interstitial fibrosis, and renal hypertrophy. It may also contribute to thickening of tubular and glomerular membranes, podocyte dysfunction, and development of apoptosis [38]. OS caused by chronic hyperglycemia can lead to metabolic and cellular disorders, including lipid peroxidation, protein oxidation, and DNA damage. Non-enzymatic glycosylation of endogenous antioxidants may also contribute to increased OS. Consequently, an imbalance between pro-oxidant and antioxidant processes in DN results in an increase in ROS [39]. The mechanisms of ROS formation in chronic hyperglycemia include oxidative phosphorylation of glucose, which may inhibit regeneration of reduced glutathione and increase superoxide production due to excessive consumption of NADPH in the polyol pathway, AGEs production, mitochondrial respiratory processes, and separation of NOX [40]. In a study investigating the effects of cinnamon on DN, it was reported that procyanidin-B2, one of the active metabolites of cinnamon, inhibited the accumulation of AGEs in diabetic rat kidney, caused a decrease in urinary albumin and creatinine levels, and that it had a curative effect on AGEs-mediated pathogenesis of DN. Recent reports have shown that accumulation of AGEs induced apoptosis through ER stress in various cell types, including glomerular mesangial cells [41, 42]. In agreement with these studies, our results suggest that cinnamon has a protective effect against ER stress by reducing the RAGE level.

Recent studies have suggested that RTN1A analysis plays a critical role in the development of renal tubular cell damage and renal fibrosis [16]. In our study, RTN1A levels in kidney tissue were significantly higher in the DM group compared to the control groups. However, there was no lowering effect of cinnamon on RTN1A levels. These results may be due to the dose, and duration of the cinnamon applied to the experimental groups. The increased expression of RTN1A was associated with progression of DN and the severity of kidney damage [15]. RTN1A has been shown to contribute to both glomerular and tubular cell damage in DN through regulation of ER stress. Fan et al. [6] showed that RTN1A interacts with PERK through its N-terminal and C-terminal domains, and mutation of these PERK domains prevents ER

stress [12]. PERK is known as an important UPR sensor in ER and is activated by phosphorylation under ER stress. Overexpression of RTN1A is thought to increase PERK phosphorylation in kidney cells, leading to the expression of the C/EBP homologous protein (CHOP), a transcription factor that is activated during ER stress [43].

Cinnamon is thought to modulate the production of antioxidant glutathione and phase II detoxifying enzymes, and can prevent the initiation and progression of DN by removing ROS through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [44, 45]. In agreement with these studies, in our study, the concentrations of the lipid peroxidation product, MDA, in the DM + cinnamon group were significantly lower than in the DM group. Similarly, Mishra et al. [46] showed that cinnamon reduces lipid peroxidation products and increases antioxidant capacity in a dose-dependent manner (5, 10, 20 mg/kg; i.p.) in the DN model.

In our study, cinnamon administration markedly improved serum markers of renal dysfunction, including serum urea, BUN, and creatinine, in diabetic rats. These findings are consistent with previous experimental studies demonstrating that cinnamon or its active components, such as cinnamaldehyde, reduce serum urea and creatinine levels and ameliorate renal histopathology in STZ-induced diabetic models [47–49]. This effect of cinnamon may be attributed to its antioxidant and anti-inflammatory properties, which alleviate oxidative stress and metabolic disturbances commonly associated with diabetic nephropathy [50].

However, despite the improvements in serum biochemical parameters, urinary albumin excretion remained elevated in the cinnamon-treated diabetic group compared with the DM group. This finding contradicts some studies reporting that cinnamon or its bioactive fractions reduced albuminuria in diabetic rats [23, 51], but it may indicate that the impact of cinnamon on glomerular permeability depends on the formulation, dose, and duration of treatment. Our results suggest that, at the dose and duration applied in our study, cinnamon ameliorated metabolic and oxidative stress-related damage but showed limited ability to reduce proteinuria.

We have shown both biochemical and histopathological changes in kidney tissues in diabetic animals supplemented with cinnamon. Our histological results suggested that cinnamon ingestion in the DM cinnamon group did not eliminate completely microscopic renal damage. However, it should be kept in mind that biochemical changes are likely to be detectable before histopathological changes are evident using basic light microscopy. Therefore, we believe that further studies are warranted using different cinnamon doses and different measures of ER stress and OS in DN models. In addition, performing histological evaluations by more sensitive methods, such as electron microscopy, may allow us to both observe the damage in more depth and show the possible protective effect of cinnamon in more detail.

Conclusion

These data suggest that cinnamon exerts a protective effect against DN in STZ-induced diabetic rats by reducing the ER stress response and OS. Cinnamon may have a role as an additional supportive agent in preventing the development of diabetic complications, especially those caused by the AGEs and the OS-mediated pathologies such as diabetic nephropathy. However, larger prospective animal studies followed by clinical trials would be necessary to confirm and expand upon these findings.

Ethics Committee Approval: The study was approved by the Kocaeli University Institutional Animal Care Local Ethics Committee (no: 2/1-2020, date: 27/02/2020).

Informed Consent: Informed consent was obtained from all participants.

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Research Article

Platelet-normalized biomarkers as diagnostic and prognostic indicators in crimean-congo hemorrhagic fever

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Abstract

Objectives: Crimean-congo hemorrhagic fever (CCHF) is a viral disease characterized by thrombocytopenia and systemic inflammation. In this study, we evaluated the role of platelet-normalizing biomarkers as diagnostic and prognostic indicators of CCHF.

Methods: This study included 60 patients with CCHF and 30 age-/sex-matched healthy controls. Biochemical parameters, including aspartate aminotransferase, alanine aminotransferase (ALT), gamma-glutamyl transferase, alkaline phosphatase, C-reactive protein and interleukin-6 (IL-6) levels were measured using photometric or electrochemiluminescence methods (Roche Cobas 8000, c702 and e801). Coagulation parameters' levels; activated partial thromboplastin time, international normalized ratio, fibrinogen, and D-dimer were determined using Roche Cobas t511. These parameters were expressed as ratios to platelet count (Plt). Comparisons were performed between the CCHF cohort and control group. Subgroup analyses evaluated associations with intensive care unit (ICU) admission and mortality risk.

Results: Statistically significant differences were observed between CCHF patients and healthy controls in all parameters ($p < 0.05$). Patients admitted to the ICU or those who did not survive exhibited a significant increase in all platelet-normalized ratios ($p < 0.05$), except ALT/Plt. ROC analysis revealed that IL-6/Plt (AUC=0.998, cut-off>0.018, sensitivity=98.3%, specificity=100%) and D-dimer/Plt (AUC=0.992, cut-off>0.002, sensitivity=95%, specificity=96.7%) had the highest diagnostic accuracy for CCHF. Furthermore, IL-6/Plt and D-dimer/Plt ratios also showed high predictive accuracy for predicting the need for ICU admission and mortality risk.

Conclusion: Platelet-normalized biomarkers, particularly IL-6/Plt and D-dimer/Plt, demonstrate strong diagnostic and prognostic potential for CCHF. Their inclusion in clinical protocols could improve early detection, risk assessment and treatment decisions for CCHF patients.

Keywords: Biomarkers, Crimean-congo hemorrhagic fever, inflammation, mortality prediction, platelet indices

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Crimean-congo hemorrhagic fever (CCHF) is a serious viral disease caused by Crimean-congo hemorrhagic fever virus, a member of the Nairoviridae family [1, 2]. CCHF is endemic in several regions, including Africa, the Middle East, Asia and Southeast Europe, and there has been a notable increase in its incidence over the past decade [3]. The disease is characterized by a range of symptoms, including high fever, muscle pain, vomiting and severe hemorrhagic manifestations, and can lead to a mortality rate ranging from 5% to 30%, depending on the outbreak and region [4].

One of the hallmarks of CCHF is thrombocytopenia, a critical indicator of disease severity and progression [5]. Thrombocytopenia in CCHF is often accompanied by life-threatening conditions such as petechiae, ecchymosis, and gastrointestinal bleeding. The pathogenesis of CCHF involves a complex interaction between the virus and the host. Infection triggers an inflammatory response that can develop into a cytokine storm, a hyper-inflammatory condition characterized by excessive release of pro-inflammatory cytokines. This cytokine storm is associated with severe tissue damage and can cause hemorrhag-

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ic symptoms observed in CCHF patients [6, 7]. Studies have shown that CCHF virus can target immune cells, leading to their activation and subsequent cytokine release, which can exacerbate the inflammatory response [6, 8]. The resulting cytokine storm can lead to multiple organ failure, which is a common cause of death in severe cases of CCHF. Liver damage in CCHF is often evaluated through biomarkers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are indicators of hepatocellular damage. High levels of these enzymes have been consistently reported in CCHF patients, reflecting the degree of liver involvement during infection [9].

Platelet indices are increasingly recognized as potential biomarkers for disease severity and prognosis in a variety of infectious and inflammatory conditions [10, 11]. However, the utility of platelet and liver enzymes, inflammatory markers, and coagulation parameters in assessing the inflammatory and coagulopathic response in CCHF has not been adequately studied. In this study, it aims to determine whether these ratios provide clinically meaningful information about disease progression and severity, potentially improving risk stratification and guiding therapeutic interventions. For this purpose, aspartate aminotransferase to platelet ratio (AST/Plt), alanine aminotransferase to platelet ratio (ALT/Plt), gamma-glutamyl transferase to platelet ratio (GGT/Plt), alkaline phosphatase to platelet ratio (ALP/Plt), C-reactive protein to platelet ratio (CRP/Plt), interleukin-6 to platelet ratio (IL-6/Plt), activated partial thromboplastin time to platelet ratio (APTT/Plt), international normalized ratio to platelet ratio (INR/Plt), fibrinogen to platelet ratio (Fibrinogen/Plt), and D-dimer to platelet ratio (D-dimer/Plt) were evaluated in CCHF patients and healthy controls.

Materials and Methods

Patients

The study included 60 patients aged over 18 years who were admitted to the Infectious Diseases Clinic between March and October 2022 with a preliminary diagnosis of Crimean-congo hemorrhagic fever (CCHF). The diagnosis of CCHF was subsequently confirmed using PCR or serological methods. Additionally, a control group consisting of 30 age-/sex-matched healthy people, with no history of chronic disease or drug use, was included for comparison.

The minimum total sample size required to detect a moderate effect size (Cohen's $d=0.65$) at a significance level (α) of 5%, with 80% statistical power ($1 - \beta$) and a group allocation ratio ($N1/N2$) of 2, was calculated to be 90. While enlarging the sample size may enhance the detection of statistically significant differences between groups, such differences risk being clinically irrelevant. To prioritize the identification of biologically and clinically meaningful effects, a moderate effect size was selected a priori, balancing statistical sensitivity and practical significance. Power analysis indicated that a total sample size ($n=90$) would minimize the possibility of overinterpreting insignificant differences and provide sufficient power for the study. All human research protocols were in compliance with

relevant national regulations, institutional policies, and the principles outlined in the Declaration of Helsinki. The study was approved by the Institutional Review Board (Ethical Committee approval No: 2025-01/62, Date: 16/01/2025). Informed consent was obtained from all participants involved in the study.

Laboratory analyses

Platelet count, AST, ALT, GGT, ALP, CRP, IL-6, INR, APTT, fibrinogen, and D-dimer levels were measured in both patient and control groups. Additionally, data regarding the need for intensive care unit (ICU) and the survival status of patients were recorded. Laboratory tests for AST, ALT, GGT, ALP, and CRP were conducted using photometric methods on a Roche Cobas c702 analyzer (Roche Diagnostics, Germany), while IL-6 levels were assessed using an electrochemiluminescence method on a Roche Cobas e801 analyzer. Complete blood count tests were performed using a Sysmex XN-1000 (Sysmex Corporation, Japan) analyzer, and coagulation tests were conducted on a Roche Cobas t511 analyzer.

Statistical analysis

New indices were derived by dividing the laboratory data by platelet count. These indices were compared between the patient and control groups and, within the patient group, based on the need for intensive care and survival status. The assumption of normality was assessed using the Shapiro-Wilk test. The non-parametric Mann-Whitney U test was used for comparisons between two groups. Furthermore, receiver operating characteristic (ROC) analyses were conducted to evaluate the performance of the indices in predicting CCHF diagnosis and disease prognosis, including the need for intensive care and survival status. The area under the curve (AUC), sensitivity, and specificity were calculated. Data were analyzed using SPSS software (IBM Corp., SPSS Statistics for Windows, Version 23.0, USA), and GraphPad Prism version 8.3.0 (GraphPad Software, www.graphpad.com, USA) was employed for data visualization. A significance level of $p<0.05$ was considered for all statistical tests.

Results

Statistically significant differences were observed between CCHF patients and healthy controls in all parameters studied. All indices were higher in patients than in healthy controls (Table 1, Fig. 1).

INR/Plt, APTT/Plt, D-dimer/Plt, fibrinogen/Plt AST/Plt, GGT/Plt and ALP/Plt, CRP/Plt and IL-6/Plt values of patients admitted to ICU were significantly increased in the ICU group, however, ALT/Plt ($p=0.068$) was not statistically significant between the groups (Table 2). Similar results were obtained for patients who did not survive.

IL-6/Plt (AUC=0.998), D-dimer/Plt (AUC=0.992), and AST/Plt (AUC=0.990) had the highest predictive values in the ROC analysis to predict the diagnosis of CCHF, with sensitivity and specificity reaching nearly 100% at cut-off values (>0.018 , >0.002 , and >0.162 , respectively). The highest probability

Table 1. Comparison of platelets indexes between CCHF patients and control groups

Parameters	Groups		p
	Control (n=30)	Patient (n=60)	
INR/Plt	0.004 (0.004–0.005)	0.012 (0.009–0.02)	<0.001
APTT/Plt	0.118 (0.099–0.146)	0.409 (0.268–0.628)	<0.001
D-dimer/Plt	0.001 (0.001–0.001)	0.033 (0.011–0.112)	<0.001
Fibrinogen/Plt	1.14 (0.923–1.373)	3.358 (2.287–4.821)	<0.001
AST/Plt	0.074 (0.06–0.093)	1.311 (0.38–4.991)	<0.001
ALT/Plt	0.077 (0.063–0.097)	0.568 (0.295–2.162)	<0.001
GGT/Plt	0.07 (0.047–0.121)	0.587 (0.25–1.137)	<0.001
ALP/Plt	0.304 (0.241–0.336)	0.981 (0.589–1.554)	<0.001
CRP/Plt	0.005 (0.002–0.009)	0.156 (0.046–0.477)	<0.001
IL-6/Plt	0.007 (0.006–0.009)	0.26 (0.15–1.142)	<0.001

Continuous variables are expressed as median and quartiles (Q1-Q3). Groups were compared using the Mann-Whitney U test. Significant p-values are shown in bold. CCHF: Crimean-congo hemorrhagic fever; INR: International normalized ratio; Plt: Platelet; APTT: Activated partial thromboplastin time; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; CRP: C-reactive protein; IL-6: Interleukin-6.

Table 2. Comparison of platelets indexes of CCHF patients according to intensive care unit requirements

Parameters	Intensive care unit requirements		p
	No (n=53)	Yes (n=7)	
INR/Plt	0.012 (0.009–0.016)	0.038 (0.029–0.048)	<0.001
APTT/Plt	0.368 (0.259–0.544)	1.364 (0.824–1.524)	<0.001
D-dimer/Plt	0.021 (0.01–0.06)	1.273 (0.824–1.524)	<0.001
Fibrinogen/Plt	3.2 (2.186–4.548)	6.048 (4.452–7.182)	0.005
AST/Plt	0.745 (0.348–3.365)	5.818 (2.843–6.071)	0.006
ALT/Plt	0.551 (0.288–1.895)	1.394 (0.832–3.129)	0.068
GGT/Plt	0.472 (0.244–0.947)	1.524 (0.686–13.581)	0.009
ALP/Plt	0.875 (0.584–1.275)	2.636 (1.69–3.452)	<0.001
CRP/Plt	0.132 (0.036–0.286)	2.806 (0.686–5.952)	<0.001
IL-6/Plt	0.24 (0.124–0.688)	7.182 (2.706–15.806)	<0.001

Continuous variables are expressed as median and quartiles (Q1-Q3). Groups were compared using the Mann-Whitney U test. Significant p-values are shown in bold.

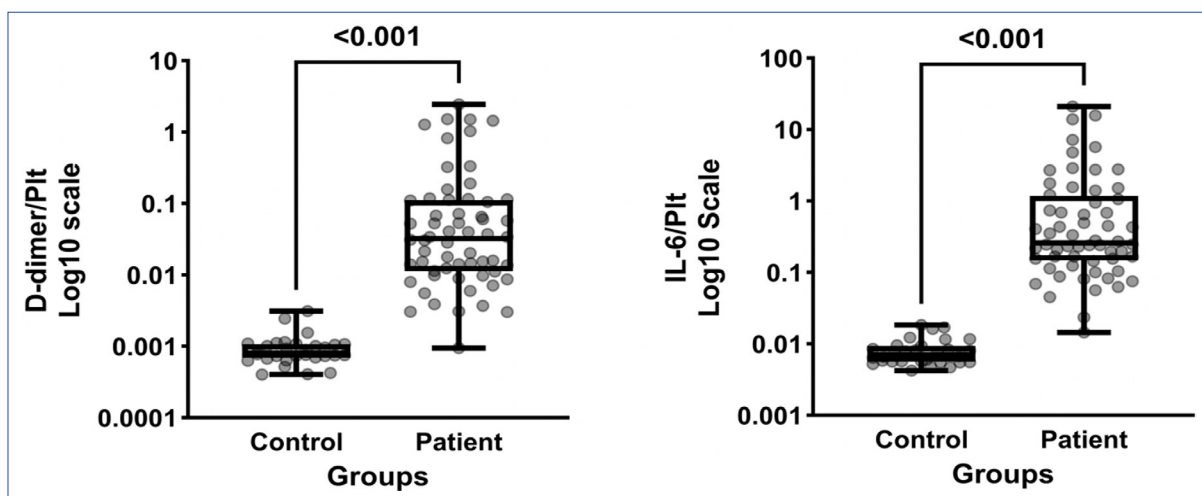


Figure 1. Box-plot comparison of D-dimer/Plt and IL-6/Plt levels between CCHF (Crimean-congo hemorrhagic fever) patients and healthy controls.

Table 3. ROC analysis results for predicting the CCHF diagnosis

Parameters	Cut-off value	AUC	Sensitivity (%)	Specificity (%)	LR (+)	LR (-)
INR/Plt	>0.007	0.973	90 (79.5–96.2)	96.7 (82.8–99.9)	27 (3.92–185)	0.1 (0.048–0.22)
APTT/Plt	>0.196	0.958	93.3 (83.8–98.2)	96.7 (82.8–99.9)	28 (4.07–192)	0.069 (0.027–0.18)
D-dimer/Plt	>0.002	0.992	98.3 (91.1–100)	96.7 (82.8–99.9)	29.5 (4.29–202)	0.017 (0.003–0.12)
Fibrinogen/Plt	>2.03	0.970	88.3 (77.4–95.2)	100 (88.4–100)		0.12 (0.058–0.23)
AST/Plt	>0.162	0.990	96.7 (88.5–99.6)	100 (88.4–100)		0.033 (0.009–0.13)
ALT/Plt	>0.162	0.957	90 (79.5–96.2)	96.7 (82.8–99.9)	27 (3.92–185)	0.1 (0.048–0.22)
GGT/Plt	>0.188	0.930	86.7 (75.4–94.1)	93.3 (77.9–99.2)	13 (3.40–49.8)	0.14 (0.074–0.27)
ALP/Plt	>0.434	0.976	95 (86.1–99.0)	93.3 (77.9–99.2)	14.3 (3.73–54.4)	0.054 (0.018–0.16)
CRP/Plt	>0.014	0.978	93.3 (83.8–98.2)	100 (88.4–100)		0.067 (0.026–0.17)
IL-6/Plt	>0.018	0.998	98.3 (91.1–100)	100 (88.4–100)		0.017 (0.002–0.12)

ROC: Receiver operating characteristic; CCHF: Crimean-congo hemorrhagic fever; AUC: Area under the curve; LR: Likelihood ratio; INR: International normalized ratio; Plt: Platelet; APTT: Activated partial thromboplastin time; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; CRP: C-reactive protein; IL-6: Interleukin-6.

Table 4. ROC analysis results for predicting the intensive care unit requirements of CCHF patients

Parameters	Cut-off value	AUC	Sensitivity (%)	Specificity (%)	LR (+)	LR (-)
INR/Plt	>0.025	0.895	85.7 (42.1–99.6)	94.3 (84.3–98.8)	15.1 (4.84–47.4)	0.15 (0.025–0.93)
APTT/Plt	>0.762	0.895	85.7 (42.1–99.6)	92.5 (81.8–97.9)	11.4 (4.22–30.6)	0.15 (0.025–0.95)
D-dimer/Plt	>0.118	0.978	100 (59.0–100)	92.5 (81.8–97.9)	13.3 (5.16–34.0)	0
Fibrinogen/Plt	>4.43	0.819	85.7 (42.1–99.6)	73.6 (59.7–84.7)	3.24 (1.89–5.58)	0.19 (0.031–1.20)
AST/Plt	>1.94	0.814	85.7 (42.1–99.6)	66.0 (51.7–78.5)	2.52 (1.56–4.09)	0.22 (0.035–1.34)
ALT/Plt	>0.569	0.714	100 (59.0–100)	58.5 (44.1–71.9)	2.41 (1.75–3.32)	0
GGT/Plt	>0.590	0.798	100 (59.0–100)	58.5 (44.1–71.9)	2.41 (1.75–3.32)	0
ALP/Plt	>1.23	0.900	100 (59.0–100)	73.6 (59.7–84.7)	3.79 (2.42–5.93)	0
CRP/Plt	>0.529	0.914	85.7 (42.1–99.6)	88.7 (77.0–95.7)	7.57 (3.36–17.1)	0.16 (0.026–0.99)
IL-6/Plt	>1.77	0.946	85.7 (42.1–99.6)	92.5 (81.8–97.9)	11.4 (4.22–30.6)	0.15 (0.025–0.95)

rates for positive outcomes were observed for D-dimer/Plt (LR+=29.5) and APTT/Plt (LR+=28) (Table 3, Fig. 2).

In the ROC analysis to estimate ICU requirements, D-dimer/Plt (AUC=0.978, cut-off >0.118) and IL-6/Plt (AUC=0.946, cut-off >1.77) showed the highest predictive accuracy with sensitivity

and specificity exceeding 85% in most cases. The highest positivity rates were observed for INR/Plt (LR+=15.1) and D-dimer/Plt (LR+=13.3) (Table 4, Fig. 2).

Similar to the results in patients admitted to the ICU, ROC analysis to estimate mortality risk exhibited high predictive accura-

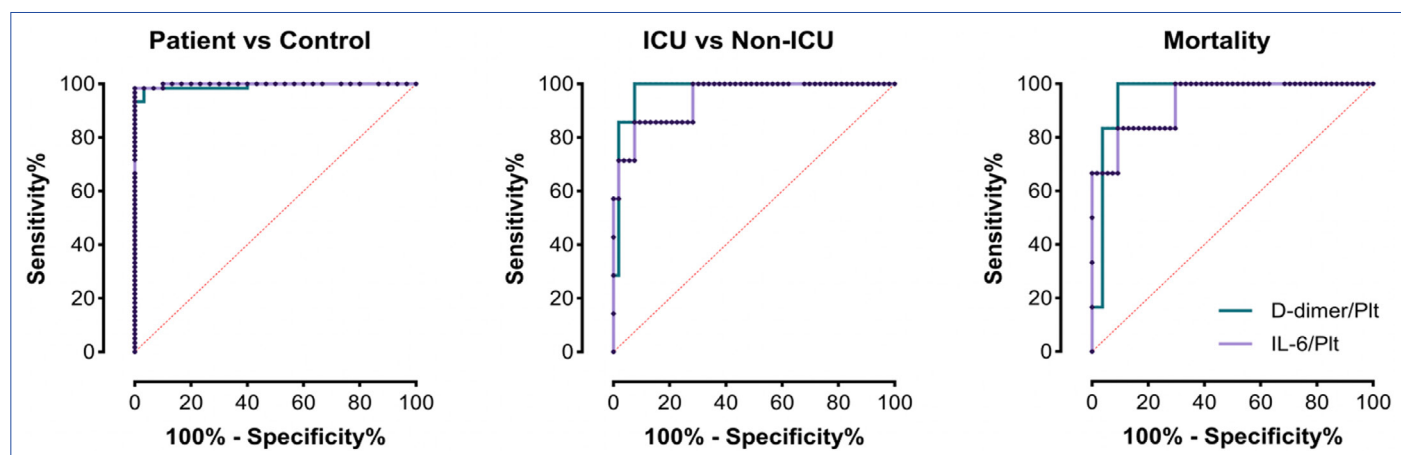


Figure 2. Diagnostic accuracy of D-dimer/Plt and IL-6/Plt levels in CCHF (Crimean-congo hemorrhagic fever) prediction, ICU (intensive care unit) requirements and mortality risk.

cy with sensitivity and specificity exceeding 80% for D-dimer/Plt (AUC=0.960, cut-off >0.118) and IL-6/Plt (AUC=0.935, cut-off >1.77) (Fig. 2). The highest positivity rates were observed for INR/Plt (LR+=11.3) and D-dimer/Plt (LR+=10.8).

Discussion

Viral hemorrhagic fevers are a group of serious, often fatal illnesses caused by several different families of viruses. Among these illnesses is CCHF, which is characterized by systemic inflammation, vascular instability, and coagulation abnormalities, frequently leading to hemorrhage, multiple organ failure, and death [12]. Thrombocytopenia is an important feature of CCHF and plays a critical role in the prognosis of the disease. Platelet count below 20,000/ μ L has been associated with severe bleeding and poor outcomes [13]. An important factor that plays a role in the pathogenesis of CCHF is uncontrolled immune response and cytokine storm [14]. IL-6 is an important proinflammatory cytokine involved in the acute phase response and has been widely studied as a biomarker of disease severity in a variety of infectious and inflammatory conditions [15]. Studies have reported increased levels of IL-6 in patients with CCHF [14, 16]. In a study conducted in Türkiye, serum IL-6 levels were found to be high in CCHF patients and positively correlated with disease severity [16]. Another study highlighted that high levels of IL-6 are associated with disseminated intravascular coagulation (DIC) in CCHF patients, and high levels of IL-6 are observed in fatal cases [3]. Our results in this study show that the IL-6/Plt ratio is significantly higher in CCHF patients compared to healthy controls. We think that thrombocytopenia and an increased level of IL-6 reflect the complex interplay between viral infection, immune activation, and clotting disorders. The elevation of IL-6/Plt level showed high diagnostic accuracy with an AUC of 0.998, specificity of 100%, and sensitivity of 98.3%. These findings suggest that IL-6/Plt ratio is a valuable biomarker in the early detection of CCHF.

Understanding the mechanisms underlying this finding in CCHF is also important for developing targeted therapies to improve the management and outcomes of this life-threatening disease and to mitigate its impact in CCHF.

D-dimers are produced as a result of the breakdown of cross-linked fibrin by plasmin during the fibrinolysis process. D-dimer is elevated in conditions associated with coagulation activation and fibrinolysis, such as DIC. High levels of D-dimers indicate activation of coagulation and fibrinolytic systems, which are hallmarks of viral hemorrhagic fever. In CCHF, elevated D-dimer levels are a common finding and are closely related to disease severity and outcomes. Many studies have documented elevated D-dimer levels in CCHF patients. Büyüktuna et al. [17] found that D-dimer levels were significantly higher in severe CCHF cases compared to mild and moderate groups. Similarly, Ergönül et al. [18] reported that D-dimer levels were strongly associated with coagulopathy and bleeding severity in CCHF patients [18]. Our findings demonstrate that

the D-dimer/Plt ratio is significantly higher among patients requiring ICU care and in those who did not survive, compared with non-ICU and surviving patients. Notably, the high AUC, sensitivity, and specificity values (AUC=0.978 for ICU requirement and AUC=0.960 for mortality) underscore the potential of the D-dimer/platelet ratio as a reliable biomarker for predicting both critical care needs and mortality risk.

This study has some limitations. First, the sample size (60 patients and 30 controls) may restrict the statistical power of subgroup analyses, particularly for rare outcomes like ICU requirement or mortality risk. Second, the single-center design introduces potential selection bias. Third, the cross-sectional nature of the study limits causal inference. Future multicenter studies with larger cohorts are needed to validate these findings.

Conclusion

Our findings highlight the diagnostic and prognostic value of platelet-based ratios in CCHF. A significantly higher IL-6/Plt ratio in patients compared to healthy controls indicates that it can be used as an early detection biomarker with its high sensitivity and specificity. Furthermore, the D-dimer/platelet ratio was found to be higher among patients requiring intensive care and non-survivors, underscoring its potential role in predicting both critical care needs and mortality risk. These results reflect the complex interaction between viral infection, immune activation, and coagulation disorders, highlighting the importance of thrombocytopenia and inflammatory cytokines in the pathophysiology of CCHF. Going forward, mechanistic investigations focusing on the specific pathways by which IL-6 and D-dimer affect coagulation and immune responses in CCHF could guide the development of targeted therapies. The incorporation of these biomarkers into existing clinical protocols can improve early diagnosis, risk stratification, and therapeutic decision-making, ultimately improving patient outcomes in this life-threatening disease.

Ethics Committee Approval: The study was approved by the Siyas Cumhuriyet University Non-interventional Clinical Research Ethics Committee (no: 2025-01/62, date: 16/01/2025).

Informed Consent: Informed consent was obtained from all participants.

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Research Article

Determination of analytical performances of NT-proBNP and aPTT tests with three methods

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Abstract

Objectives: This study aimed to evaluate the analytical performances of N-terminal pro-B-type natriuretic peptide (NT-proBNP), which has not been investigated before, and activated partial thromboplastin time (aPTT), which has been the subject of little research, using the six sigma methodology and to calculate the quality goal index values of low-performing parameters. It was aimed to evaluate the analytical process with three methods by presenting this performance with Operation specification charts, which have been done in few other studies.

Methods: Three consecutive months of internal quality control data obtained from NT-proBNP and aPTT tests, twice daily, and data obtained from a monthly external quality control program were used. Sigma values were calculated using the calculation of $\text{Sigma} = (\text{Total allowable error} - \text{bias}) / (\text{Coefficient of variation})$ and shown with Operation specification charts (OPS Specs). Quality goal index (QGI) was calculated for those with $\text{sigma} < 6$.

Results: The sigma values for levels 1 and 2 of the NT-proBNP test were calculated as 5.06 and 5.65, and the performance status was determined as very good. The sigma values for levels 1 and 2 of the aPTT test were calculated as 4.28 and 3.56, respectively, and this was evaluated as moderate and good performance. The Quality goal index values (QGI) for levels 1 and 2 of the NT-proBNP test were calculated as 0.11 and 0.12, respectively. The Quality goal index (QGI) values for levels 1 and 2 of the aPTT test were calculated as 0.90 and 0.75, respectively.

Conclusion: Both tests had moderate, good and very good performance. It is of great importance to increase quality standards in laboratory tests. In this direction, continuous improvement-oriented initiatives should be implemented to make analytical processes more competent.

Keywords: aPTT, NT-proBNP, OPS Specs, quality goal index, six sigma

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The entire testing process in clinical laboratories is divided into three stages: Preanalytical, analytical, and postanalytical. Research indicates that error rates are estimated to range from 30–75% in the preanalytical stage, 4–30% in the analytical stage, and 9–55% in the postanalytical stage [1]. Laboratories need to assess their process performance based on scientifically established quality standards. This assessment involves analyzing the rate of sample errors and rejections during the preanalytical phase, evaluating the accuracy and precision of test results in the analytical phase, and monitoring the reporting of critical values as well as test turn around times in the postanalytical phase [2]. Among

these stages, analytical quality alone is not sufficient as a standalone quality requirement; however, other quality parameters hold no significance unless analytical quality is achieved. Laboratories must ensure accurate test results before addressing other quality criteria [3].

The Six Sigma approach is a technique applied in quality control and process enhancement. It aims to detect defects and minimize mistakes and variations [4]. Six sigma quality management is not just a tool for defining process performance; it is also a methodology aimed at reducing the error rate within the process. In automated analytical systems, it is important to determine the situations where precision error, accuracy error,

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or both errors occur together, which are among the test-specific reasons, in order to ensure the quality improvement of tests. These performance data can also be evaluated by calculating the quality goal index (QGI) [5]. The six sigma method allows for an objective assessment of performance. The sigma level of a process can be determined by using specific equations. The sigma value indicates the frequency of potential errors. A low sigma value suggests that the process is more likely to produce errors. Ideal or world-class performance should have a minimum of 6 sigma values, which translates to fewer than three or four errors per million products [6].

In this study, the research was planned by prioritizing the feedback from clinicians to the laboratory regarding the tests. Considering this situation, it was aimed to evaluate the analytical performances of N-terminal pro-B-type natriuretic peptide (NT-proBNP), which has not been investigated before, and activated partial thromboplastin time (aPTT), which is a subject of little research, using the six sigma methodology and to calculate the quality target index (QGI) values of the parameters showing low performance. It was aimed to evaluate the analytical process with three methods by presenting this performance with Operation Specification Charts (OPSspecs charts), which have been done in few other studies.

Materials and Methods

The study was approved by the Bursa City Hospital Scientific Research Ethics Committee (no: 2024-21/23, date: 11/12/2024), following the principles of the Declaration of Helsinki.

For the six sigma methodology calculations, three consecutive months of NT-proBNP tests performed on the Cobas 8000 Modular Analyzer System (Cobas, Mannheim, Germany) and aPTT tests performed on the Cobas t 711 (Roche Diagnostics Mannheim, Germany) coagulation analyzer, two-level internal quality control (IQC) data per day and data obtained from the monthly external quality control (RIQAS, UK) program were used retrospectively. All stages of the study were carried out conformity the Helsinki Declaration.

Calculation of sigma values of tests

To calculate sigma values; The mean, standard deviation (SD), coefficient of variation (CV%), bias (%) and total analytical error calculations of the tests must be made. The calculations were made as follows:

CV values (%) = (SD/Mean of IQC data)×100
For CVmean (mean %CV) values, 2-level internal quality control results were used.
 $CVmean = (CV1^2 + CV2^2)^{1/2}$
The bias (%) values were calculated using the formula provided below:
 $Bias\ (\%) = [(IQC\ data\ mean\ of\ our\ laboratory - target\ mean\ of\ IQC\ data) / target\ mean\ of\ IQC\ data] \times 100.$
The total analytical error for each parameter and control level was determined using the formula outlined below: Total analytical error = Bias+(1.65x CVmean)
Sigma values were calculated for each parameter and each control level.
 $Sigma = (TEa - Bias) / CV$ formula was used [7].

Evaluation of analytical performance of tests using OPSspecs charts

OPSspecs charts can also be used as quality planning and performance evaluation tools in clinical laboratories [8]. In the study, sigma levels of the tests were shown on OPSspecs charts.

Calculation of quality goal indices of tests

The Quality Goal Index (QGI) is a recent parameter that reflects the extent to which both accuracy and precision align with the applicable quality targets and helps identify which factor may be responsible for the issue [9]. Quality goal index calculation; $QGI = Bias / (1.5 \times CV)$ formula was used [10].

Statistical analysis

All calculations were made using Microsoft Office Excel 2021 software.

Results

In the study, two levels, three-month average %CV, %Bias values, %TEa ratios of NT-proBNP and aPTT tests are shown (Table 1). The mean, standard deviation (SD), and coefficient of variation (CV%) of the tests were calculated using internal quality control data collected over a 3-month period.

In the study, the sigma value of Level 1 for the NT-proBNP test was calculated as 5.06 and the sigma value of Level 2 was calculated as 5.65. For the aPTT test, the sigma value of Level 1 was calculated as 4.28 and the sigma value of Level 2 was calculated as 4.85.

Table 1. CV%, Bias%, total analytical error and TEa values of NT-proBNP and aPTT tests						
Parameter	CV (%)		CVmean	Bias (%)	Total analytical error	TEa (%)
	Level 1	Level 2				
NT-proBNP	5.74	5.13	7.70	0.98	16.19	30
aPTT	2.66	3.20	4.15	3.61	11.91	15

CV: Coefficient of variation; TEa: Total allowable error; NT-proBNP: N-terminal pro-B-type natriuretic peptide; aPTT: Activated partial thromboplastin time; CLIA 2025: Clinical laboratory improvement amendments 2025.

Table 2. Sigma values of NT-proBNP and aPTT tests, performance status of these values and recommended control rules

Parameter	Sigma		Performance		Recommended internal control rules	
	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
NT-proBNP	5.06	5.65	Very good or excellent, individual quality control rules apply	Very good or excellent, individual quality control rules apply	$1_{2.5S}$	$1_{2.5S}$
aPTT	4.28	3.56	Good, multiple quality control rules are applied	Medium requires quality control procedure. More than 1 analytical run and multiple measurements per run	$1_{2.5S}$	$1_{3S}/2_{2S}/R_{4S}/4_{1S}$

NT-proBNP: N-terminal pro-B-type natriuretic peptide; aPTT: Activated partial thromboplastin time.

Table 3. Recommended control rules based on sigma values

Sigma	Performance description	Recommended control rules	R (number of measurements)	N (number of controls)
<3 sigma	Bad, quality improvement plan should be implemented	$1_{3S}/2_{2S}/R_{4S}/4_{1S}$	2 or 4	R=2 for N=4 R=4 for N=2
≥3-<4 sigma	It is fit for purpose but more than one quality study should be done and multiple rules should be used	$1_{3S}/2_{2S}/R_{4S}/4_{1S}$	1 or 2	R=1 for N=4 R=2 for N=2
≥4-<6 sigma	Fit for purpose	$1_{2.5S}$	1	2
≥6 sigma	World class	1_{3S}	1	2

Table 4. Performance criteria according to sigma values

Sigma	Performance criteria
<2- sigma	Unacceptable, not valid as a measurement procedure
>2- sigma ve <3- sigma	Bad, quality improvement plan should be implemented
>3- sigma ve <4- sigma	Medium requires Quality Control (QC) procedure. More than 1 analytical run and multiple measurements per run
>4- sigma ve <5- sigma	Good, multiple quality control rules are applied
>5- sigma ve <6- sigma	Very good or excellent, individual quality control rules apply
≥6- sigma	world class

culated as 3.56. According to these measured sigma values, performance statuses and recommended control rules according to these performances are shown (Table 2).

The quality control Westgard rules used depending on the sigma metric value of the analytes are shown [7] (Table 3).

The performance criteria created depending on the sigma metric value of the analytes are shown [7, 11] (Table 4).

In this study, sigma values of NT-proBNP and aPTT tests are shown with OPSpecs charts [12] (Fig. 1). The sigma of the line closest to the operation point we obtained gives our process sigma level.

The criteria for interpreting the quality goal index ratios of analytes are as follows; <0.8 QGI: Indicates that there is precision error, 0.8–1.2 QGI: Indicates that there is precision and accuracy error, and >1.2 QGI: Indicates that there is accuracy error. In this study, the quality goal indices of the

tests have been calculated and the error types corresponding to these results are shown (Table 5).

Discussion

The six sigma methodology, in addition to identifying the causes of errors, provides recommendations on control measures. The sigma method, which can be applied to every step of the total testing process, is used to evaluate laboratory performance [13]. In this research, the analytical performance of NT-proBNP and aPTT parameters was assessed using the six sigma approach, QGI, and OPSpecs charts.

No study was found in the literature review on the NT-proBNP test. However, a study was found on the BNP (brain natriuretic peptide) test. Accordingly: Üstündag et al. [14] calculated sigma values for the BNP test using the six sigma methodology. They reported that sigma values varied between 0.76 and 2.06 at dif-

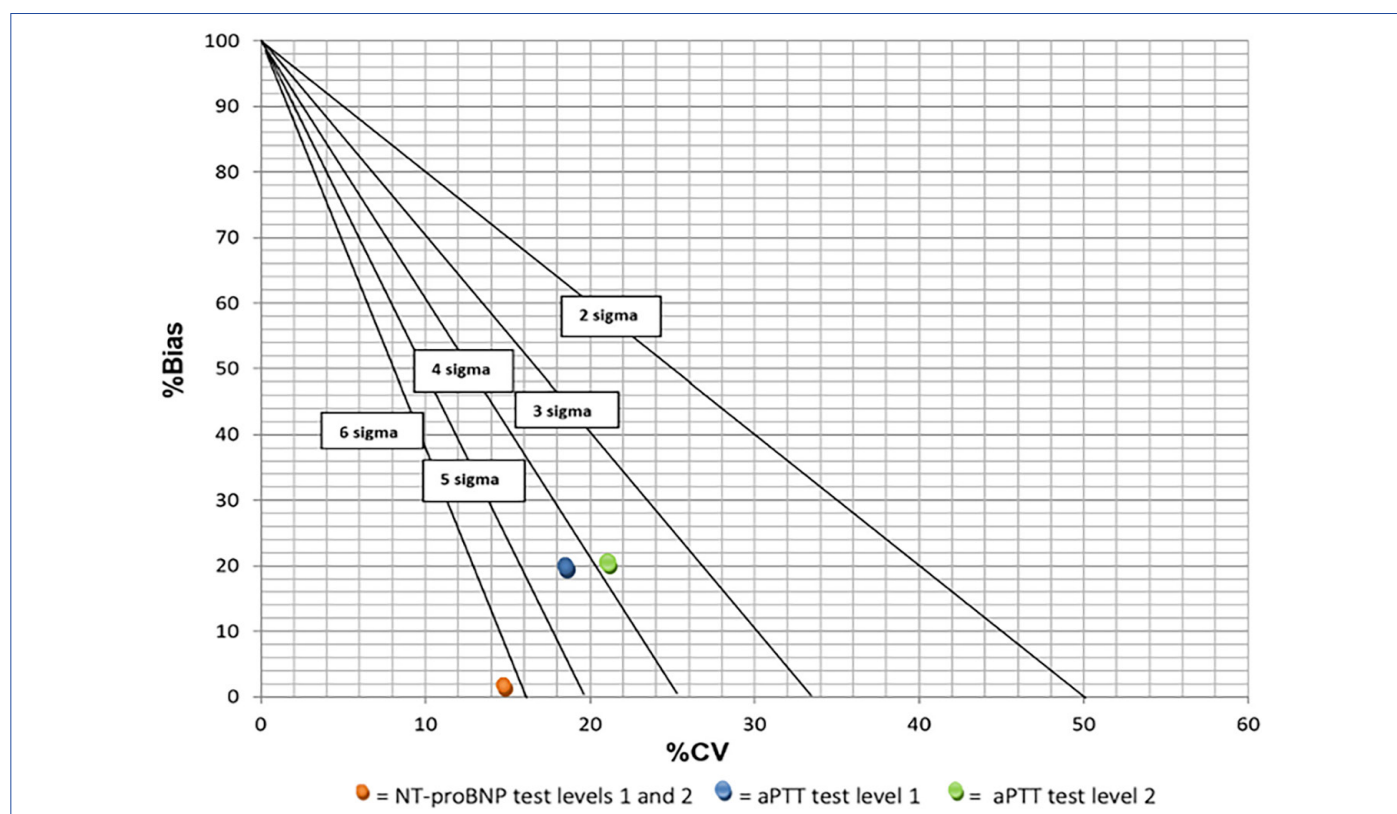


Figure 1. OPSpecs chart for NT-proBNP and aPTT parameters for levels 1 and 2.

CV: Coefficient of variation; aPTT: Activated partial thromboplastin time; OPSpecs: Operation Specification Charts; NT-proBNP: N-terminal pro-B-type natriuretic peptide.

ferent quality control levels and that the problem in the BNP study was uncertainty according to the calculated QGI levels.

Studies evaluating aPTT performance are as follows. El-Neanaey et al. [15] calculated sigma values of aPTT tests in a study they conducted and found sigma values of the test to be >3 at normal and pathological levels, according to their findings. Hollestelle et al. [16] showed that sigma values for aPTT in two laboratories were higher than 3. Aksit et al. [17] found level 1 and level 2 sigma values for aPTT to be 5.27 and 4.31, respectively. Üge et al. [11] calculated the normal and high level sigma values of the aPTT test as 4.51 and 4.31, respectively. They reported that they found the QGI calculation for the aPTT test as 0.41 and 0.36, respectively, at normal and high levels.

Total allowable error (TEa) refers to the maximum acceptable difference between the actual concentration of an analyte

and the value reported by the laboratory, ensuring the result is considered accurate and trustworthy [18]. The TEa values for NT-proBNP and aPTT parameters were sourced from the CLIA 2025 database [19]. In this study, the total analytical error rate for the NT-proBNP test was determined to be 16.19, which is below the permissible total error rate set by CLIA (30%). Likewise, the total analytical error rate for the aPTT test was calculated as 11.91, which is also lower than the CLIA allowable total error rate of 15% (Table 1).

Since the sigma values of the NT-proBNP test in this study were calculated as 5.06 and 5.65 for levels 1 and 2, respectively, its performance was evaluated as very good or excellent and it was recommended to apply single quality control rules in the form of the 12.5S rule. Since the sigma value of the level 1 control for the aPTT test was found to be 4.28, its performance was; It was evaluated as good and the 12.5S

Table 5. Sigma values of NT-proBNP and aPTT tests and QGI ratios for sigma <6

Parameter	Sigma		QGI		Performance	
	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
NT-proBNP	5.06	5.65	0.11	0.12	Precision error	Precision error
aPTT	4.28	3.56	0.90	0.75	Precision and accuracy error	Precision error

NT-proBNP: N-terminal pro-B-type natriuretic peptide; aPTT: Activated partial thromboplastin time; QGI: Quality goal index.

rule was recommended to be applied as multiple quality control rules. The sigma value of the level 2 control of the aPTT test was found to be 3.56, accordingly its performance was evaluated as moderate and it was recommended to perform multiple analytical runs as 13s/22s/R4s/41S and multiple measurements per run (Table 2, 3).

The sigma values calculated in this study were calculated as 5.06 for level 1 of the NT-proBNP test and 5.65 for level 2. For the aPTT test, it was calculated as 4.28 for level 1 and 3.56 for level 2. When evaluated in terms of performance criteria according to sigma values, a sigma value less than 3 is an indicator of a poor performance procedure. Good performance is shown by a sigma level higher than 3 [20] (Table 4).

OPSpecs charts describe the deviations from the allowable precision and accuracy for a method and specify the internal quality control rules required to monitor the performance of the method. The inaccuracy plot is shown on the y-axis, while the imprecision plot is represented on the x-axis. The operating point is the combination of the deviations in both precision and accuracy [21]. The sigma values are plotted on the OPSpecs charts (Fig. 1). OPSpecs charts assist in evaluating the quality of an analytical process by offering a sigma value. For each sigma metric, the appropriate Westgard rule (along with the optimal number of QC levels) can be selected to maximize error detection while minimizing false rejections. It is evident that the sigma metric can be enhanced in two ways: By decreasing bias or by reducing the CV [12].

The quality goal index (QGI), introduced by Westgard, incorporates both repeatability (precision) and accuracy elements. It is used to pinpoint the source of error in measurements with a sigma value less than 6. A QGI score of <0.8 indicates that precision needs to be improved, a QGI score of >1.2 indicates that accuracy needs to be improved, and a QGI score between 0.8 and 1.2 indicates that both precision and accuracy need to be improved [19]. For the NT-proBNP test, the QGI values were determined to be 0.11 and 0.12 for levels 1 and 2, respectively. Similarly, the QGI values for the aPTT test were calculated as 0.90 and 0.75 for levels 1 and 2, respectively. The QGI values for the NT-proBNP test point to precision errors at both levels, while the aPTT test values indicate precision and accuracy errors at level 1 and accuracy errors at level 2 (Table 5). These results highlight the need for improvements in both precision and accuracy.

Conclusion

The process performance of laboratories should be evaluated in accordance with internationally accepted scientific quality criteria. In order to ensure higher accuracy, reliability and repeatability, it is of great importance to increase quality standards in laboratory tests. In this direction, continuous improvement-oriented initiatives should be implemented to make analytical processes more effective.

Ethics Committee Approval: The study was approved by the Bursa City Hospital Scientific Research Ethics Committee (no: 2024-21/23, date: 11/12/2024).

Informed Consent: Informed consent was obtained from all participants.

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Research Article

Adult references intervals for thyroid hormones using beckman coulter from Türkiye

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Abstract

Objectives: In this study, we aimed to establish reliable reference intervals (RIs) for free triiodothyronine (fT3), free thyroxine (fT4), and thyroid stimulating hormone (TSH) in our population using the Beckman Coulter UniCel Dxl 800.

Methods: We followed the Clinical Laboratory Standards Institute (CLSI) C28-A3 guidelines to calculate RIs through both direct (DRM) and indirect methods (IDM) and compared them with the RIs provided by Beckman Coulter UniCel Dxl 800 Access® immunoassay system. High sensitive (h)TSH reagent used for TSH analyses. For DM, we excluded anti thyroid peroxidase (anti-TPO) or antithyroglobulin (anti-TG) positive samples, outliers, and samples with insufficient serum, resulting in final sample sizes of 420 for TSH, 411 for fT4, and 407 for fT3. For IDM, anti-TPO or anti-TG-positive samples, repeated samples, and outliers were excluded, resulting in final sample sizes of 2874 for TSH, 2072 for fT4, and 1163 for fT3.

Results: Our study included 450 participants (225 females, 225 males) over the age of eighteen for DRM and utilized data from the Laboratory Information System (LIS) between March 1, 2018, and February 28, 2020, for IDM. After excluding certain samples and outliers, the final sample sizes were determined. The reference intervals (RIs) for TSH, fT4, and fT3 were 0.42-4.18 mIU/L, 0.41-4.45 mIU/L, 0.57–1.08 ng/dL, 0.63–1.14 ng/dL, 2.62–4.01 pg/mL, and 2.72- 4.41 pg/mL for DRM and IDM, respectively.

Conclusion: In conclusion, the RI that we will use for thyroid hormones in our laboratory is different from that provided by the manufacturer.

Keywords: Reference intervals, thyroid diseases, thyrotropin, thyroxine, triiodothyronine

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The clinical manifestations of thyroid diseases, especially subclinical hypothyroidism (SCH) or subclinical hyperthyroidism, are not specific. SCH is a more commonly encountered condition [1], and the disease ranges between euthyroidism and overt hypothyroidism [2–4].

The symptoms associated with hypothyroidism typically include general and mental fatigue and cognitive difficulties, referred to as brain fog, which involve problems with concentration, motivation, memory, and reasoning, thereby impairing an individual's quality of life [3]. These patients are often prone to high levels of anxiety, and depression [4]. All of these symptoms prompt clinicians to inquire about other diseases, and the symptoms are not specific to the thyroid.

Due to the nonspecific nature of clinical symptoms, the diagnosis of thyroid dysfunction relies on laboratory results. The most sensitive and specific indicator of systemic thyroid function is thyroid-stimulating hormone (TSH). Changes in serum TSH levels act as an 'early warning system' while attempting to maintain thyroid hormone levels within healthy ranges. Thus, SCH is diagnosed by elevated TSH levels above the reference interval (RI) accompanied by normal levels of free thyroxine (fT4) and free triiodothyronine (fT3) [2].

However, reliable RIs are crucial for interpreting TSH measurements. Reporting test results with well-defined RIs is critical for correct interpretation, especially when diagnosing subclinical thyroid dysfunction [5–7].

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For instance, a recent study was conducted in a region of China considered to have sufficient iodine levels to determine the reference intervals (RIs) for thyroid hormones using the Abbott Architect analyzer. In this study, the RIs obtained from the healthy population were inconsistent with the RIs provided by the manufacturer [8].

The laboratories typically use the RIs provided in kit inserts. However, RIs can vary depending on socioeconomic status, geographic location, exposure to environmental factors, and race. For these reasons, it is recommended by the Clinical and Laboratory Standards Institute (CLSI) and The International Federation of Clinical Chemistry (IFCC) that the suitability of the RI provided in kit inserts for the regional population be tested or that each laboratory establish its own reference ranges [7].

Especially when developing clinical guidelines for thyroidal diseases, the RIs used should be tailored to accommodate differences in the analyzer platform on which the test will be performed. Since TSH levels are used as the basis for initiating thyroid hormone therapy and adjusting treatment doses for thyroidal diseases, the use of device- and population-specific RIs ensures that the most accurate decisions are made in managing the disease [6, 9].

The aim of this study was to calculate the RIs for fT3, fT4 and TSH using both the direct method (DRM) and the indirect method (IDM) according to the Clinical Laboratory Standards Institute (CLSI) C28-A3 guidelines and to compare them with the RIs provided by the Beckman Coulter Dxl 800 [10].

Materials and Methods

This study aimed to determine the reference intervals (RIs) for fT3, fT4, and TSH in patients with DRM and IDM. All procedures performed in studies involving human participants were in accordance with the ethical standards of national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards for patient with DRM. Chief medical officer's approval was received for retrospective data (patient with IDM). This study was conducted with the approval of the Kocaeli University Ethical Committee (No: KOU GOAEK 2019/196, Date: 08/05/2019).

Reference individuals for DRM were selected among volunteers who visited our hospital's Central Laboratory Blood Collection Unit and Primary Health Care Centers in our province. Volunteers who agreed to participate were administered a questionnaire with exclusion criteria.

The questionnaire included questions about family history of thyroid disease, recent history of illness, presence of chronic disease, recent blood transfusion or donation, pregnancy and breastfeeding, drug addiction, surgery in the last 3 months, fasting for 8–12 hours, and obesity, weight gain or loss, loss of appetite, smoking (>20/day), alcohol abuse, hypotension or hypertension, vitamin use, oral contraceptive use, fatigue, irritability, insomnia, exhaustion, muscle weakness, intolerance to cold or heat, dull mood, difficulty remembering, and trem-

or. Individuals who were considered unsuitable for the study according to the exclusion criteria were excluded. Informed consent was obtained from individuals eligible to participate in the study. Blood samples were collected between 08:30 and 10:00, after the participants had fasted for 8–12 hours. Samples were collected using red-capped biochemistry tubes without anticoagulants or any other active substances (Plain tube, BD vacutainer, lot number 8306831). Samples were allowed to clot for 1 hour and then centrifuged at $1800 \times g$ for 15 minutes to obtain the serum. Equal numbers of male and female reference individuals were carefully selected to ensure age and gender homogeneity. An equal number of individuals of both genders were included in each age group (1st group: 18–28 years, 2nd group: 29–38 years, 3rd group: 39–48 years, 4th group: 49–58 years, 5th group: 59 years and older).

Although participants were assumed to be healthy in terms of thyroid function, adhere to exclusion criteria, and have no history of thyroid dysfunction in themselves or their families, it is known that subclinical thyroid dysfunction may still exist. To overcome this issue and identify and exclude these individuals, thyroid antibody (TAb), anti-thyroid peroxidase (anti-TPO) and antithyroglobulin (anti-TG) antibody tests were conducted on reference individuals. Individuals with anti-TPO >9 IU/mL or anti-TG >4 IU/mL results, i.e., all individuals with positive antibody tests, were excluded from the study. Therefore, the study began with 450 individuals to ensure that results were obtained from at least 400 individuals for calculation purposes.

For IDM, data were downloaded from the Laboratory Information System (LIS) for patients who underwent simultaneous fT3, fT4, and TSH tests between March 01, 2018, and February 28, 2020. According to the CLSI's EP28-A3C guidelines, patients from oncology and intensive care units were not included in the study. Since individuals with multiple test results are likely to be healthy, the latest result in the LIS was included in the study [6].

In our study, serum fT3, fT4, TSH, anti-TPO and anti-TG data obtained from both DRM and IDM groups were analyzed with various reagents using chemiluminescence method on Beckman Coulter UniCel Dxl 800 Access[®] immunoassay system (Beckman- CLIA, Brea, CA, USA). Access provides standardization to the World Health Organization 3rd International Standard (IS) for human TSH analysis (IRP 81/565). In our study, the TSH 3rd IS method was used for TSH analysis and hypersensitive TSH (hTSH) test results were obtained. The Access TSH (3rd IS) assay provides high functional sensitivity and precision. Calibration of anti-TPO (Access calibrator ref: A18227) and anti-TG (Access calibrator ref: A36920) were conducted using original calibrators and controls provided by Beckman Coulter. Internal quality controls were performed three times daily with SeronormTM Immunoassays (207005 Liq L-1 12×3 mL, 207105 Liq L-2 12×3 mL, and 207205 Liq L-3 12×3 mL) for all tests (Sero AS, Hvalstad, Norway). External quality controls were performed monthly using the External

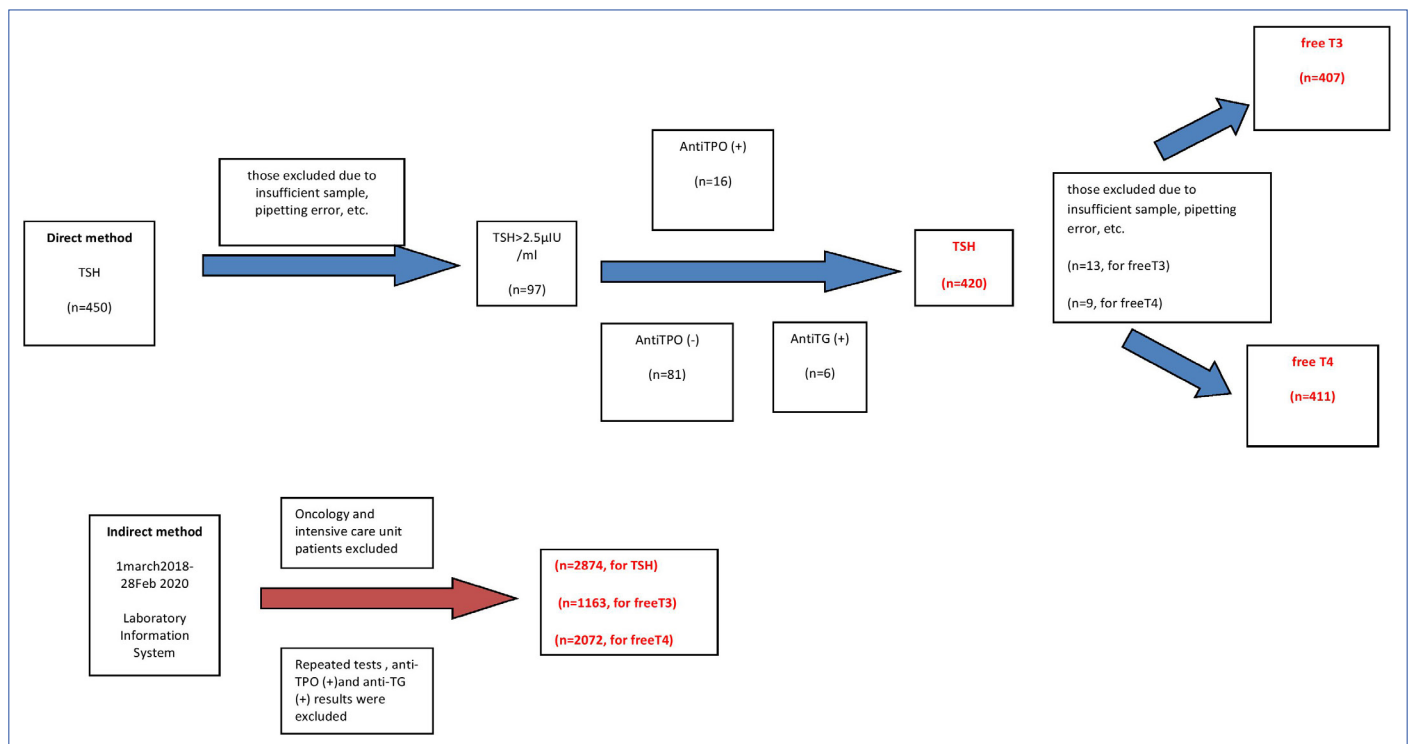


Figure 1. The algorithm graph for the number of included and excluded individuals for each parameter.

TSH: Thyroid stimulating hormone; ft4: Free thyroxine; ft3: Triiodothyronine; anti-TPO: Anti-thyroid peroxidase; anti-TG: Antithyroglobulin.

Quality Assurance Services (EQAS) Immunoassay Program. No outlier values were found in TSH, ft3 and ft4 evaluations in the EQAS reports of our laboratory.

At the end of the study, the data obtained from the DRM and IDM were transferred to the IBM SPSS Statistics 24.0 program. The histograms of the data were evaluated. The normality of the data was assessed using the Kolmogorov–Smirnov test. Outliers values were identified through histogram evaluation and by applying the Dixon method. After removing outlier values, the distribution of the remaining data was re-evaluated as visual. Independent sample t tests were conducted to determine whether there were significant differences between sexes for tests showing a normal distribution, while the Mann–Whitney U test was used for tests not conforming to a normal distribution.

The reference intervals for tests showing a normal distribution were calculated using the standard deviation (SD) (lower limit: mean - 1.96 × SD, upper limit: mean + 1.96 × SD). For tests not showing a normal distribution, the reference interval boundary values were found using the formula lower value = 0.025 × (n+1) and upper value = 0.975 × (n+1), where 'n' represents the number of data points (noninteger values were rounded to the nearest integer).

Results

After excluding inadequate samples or pipetting errors resulting in failure to obtain results, as well as data from individuals with positive Anti-TPO or Anti-TG test results and identified

outliers, the following number of data points were obtained for hTSH: 420, ft3: 407, and ft4: 411 from the reference individual group formed by DRM. For IDM, following exclusion of samples according to CLSI's EP28-A3C guidelines and after removing outliers, the following number of data points were obtained for hTSH: 2874, sT3: 1163, and sT4: 2072 from the LIS. The algorithm graph for the number of included and excluded individuals for each parameter is shown in Figure 1.

When testing for normality, data with a significance level (p value) greater than 0.05 in the Kolmogorov–Smirnov test indicated a normal distribution, while those with a significance level less than 0.05 indicated a nonnormal distribution. Statistical evaluations of the data obtained from the DRM and IDM in our study revealed that the ft3 test results exhibited a normal distribution, while the hTSH and ft4 test results did not. For ft3, which showed a normal distribution, an independent sample t test was conducted to assess the significance of sex differences. The groups had a homogeneous distribution, and there was a statistically significant difference between DRM (for ft3 patients with DRM, $p < 0.001$; for ft3 patients with IDM, $p < 0.001$).

When examining the significance of sex differences for hTSH and ft4, which were not normally distributed, a statistically significant difference was found for both tests (for DRM, hTSH $p < 0.001$, ft4 $p = 0.033$; for IDM, hTSH $p = 0.024$, ft4 $p < 0.001$). Based on this information, RIs were calculated. The RIs at the 95% confidence intervals obtained without sex differences from the DRM and IDM data, as well as the RIs recommended

Table 1. The reference intervals obtained through direct and indirect methods for TSH, fT4, fT3, and the reference intervals provided by the manufacturer

Test	Unit	DRM		MRI		IDM	
		LL	UL	LL	UL	LL	UL
fT3	pg/mL	2.62	4.01	2.5	3.9	2.72	4.41
fT4	ng/dL	0.57	1.08	0.61	1.12	0.63	1.14
hTSH	mIU/L	0.42	4.18	0.34	5.60	0.41	4.45

hTSH: High sensitive thyroid stimulating hormone; fT4: Free thyroxine; fT3: Triiodothyronine; DRM: Direct method; MRI: The reference intervals provided by the manufacturer; IDM: Indirect method, LL: Lower limit; UL: Upper limit.

Table 2. The presentation of reference intervals obtained through DRM and IDM according to genders

Test	Gender	Unit	DRM		IDM	
			2.5 th percentile	97.5 th percentile	2.5 th percentile	97.5 th percentile
fT3	Female	pg/mL	2.54	3.99	2.62	4.29
	Male	pg/mL	2.72	4.15	2.88	4.49
fT4	Female	ng/dL	0.57	1.09	0.63	1.12
	Male	ng/dL	0.58	1.08	0.65	1.15
hTSH	Female	mIU/L	0.52	4.33	0.42	4.43
	Male	mIU/L	0.41	3.95	0.40	4.57

by the manufacturer, are presented in Table 1. The RIs obtained at the 95% confidence intervals with sex differences between the DRM and IDM groups are presented in Table 2.

Discussion

In our study, we found that gender-specific RIs were narrower than the RIs determined by DRM and IDM in all cases and that the RIs we obtained differed from those provided by the manufacturer.

For hTSH in our study, we obtained narrower RIs in females with DRM (0.52–4.33 mIU/L) than in those with IDM (0.42–4.43 mIU/L). In males, we found the RI to be particularly lower at the upper limit compared to the IDM (0.41–3.95 mIU/L for DRM versus 0.40–4.57 mIU/L for IDM). The RI provided by the manufacturer for hTSH without sex differentiation was 0.34–5.60 mIU/L. In a multicenter study conducted in Italy with IDM using the same kit and autoanalyzer as our study (Access TSH 3rd IS, using UniCel Dxl), the hTSH RI without sex differences was found to be different from that in our study (0.36–5.28 mIU/L) [10].

In an RI determination study conducted with DRM in a different region of Turkey in 2010 using the same device as ours (Beckman Unicel Dxl, although the generation of the kit was not emphasized), a TSH RI of 0.41–4.25 mIU/L was found [11]. Despite using the same brand of kit and autoanalyzer, there are some differences in the RIs found among regions.

In fact, according to data obtained from the Global Iodine Network in 2021, Turkey appears to be among the countries with sufficient iodine intake [12]. However, there may still be differences in dietary habits among regions. Therefore, determining RIs for a specific region or city's population is

important. In particular, determining RIs with DRM ensures more reliable RI determination [13].

In a region of China reported to have sufficient iodine levels, the RIs for TSH determined with DRM using the Abbott Architect autoanalyzer were 0.67–4.62 mIU/L for males and 0.72–5.15 mIU/L for females. The RIs obtained for this study without sex differences were 0.70–4.93 mIU/L. The RI provided by the manufacturer for TSH without sex differences was 0.35–4.94 mIU/L. In particular, there was a significant difference in the lower limit of the RI compared to what was provided by the manufacturer in this study [8].

In a study conducted with IDM using the Siemens Advia Centaur XP analyzer, the RIs for TSH were found to be 0.71–4.92 mIU/L, which differs from the manufacturer's recommended TSH range (0.55–4.78 mIU/L) [14].

In another study conducted in the United Kingdom in 2017 with DRM, researchers determined RIs for TSH and fT4 through the four most commonly used analytical platforms in their country. As seen in this study, it is obvious that the same RI cannot be used for thyroid hormones analyzed on all analytical platforms and different RIs should be used. In this study, the Beckman Unicel Dxl RI for TSH was found to be 0.57–3.60 mIU/L [15].

According to the guidelines of the European and American Thyroid Associations, the serum TSH level is the most reliable test for the diagnosis of all forms of hypothyroidism and hyperthyroidism [16–18]. However, it should be noted which analytical platform the test is performed on when creating guidelines [9, 15]. Additionally, the region where the RI will be applied is also important. Reporting patient test results with RIs not determined according to regional populations may lead to the di-

agnosis of thyroid dysfunction in many individuals who do not have thyroid dysfunction, resulting in unnecessary medication use. Similarly, it may also lead to the misclassification of many individuals with thyroid dysfunction as healthy individuals. Considering the possible harmful effects of unnecessary medication use or the vital risks of not treating patients who require treatment, it is essential for each laboratory to determine its own RI to minimize these critical issues for every individual [7].

In our study, the RIs obtained for fT4 differed between DRM and IDM for females (0.57–1.09 ng/dL with DRM and 0.63–1.12 ng/dL with IDM) and males (0.58–1.08 ng/dL with DRM and 0.65–1.15 ng/dL with IDM), especially at the lower limit. The RIs obtained with both methods were also different from the values provided by the manufacturer (0.61–1.12 ng/dL). In a study conducted in a different region of Turkey with the same autoanalyzer as ours in 2010, the RI for fT4 with DRM was also different from ours (0.61–1.06 ng/dL) [11].

Another study conducted with IDM on the Siemens Advia Centaur XP analyzer showed apparent differences in RIs for fT4, with ranges of 12.2–20.1 pmol/L for males and 11.9–18.9 pmol/L for females, compared to the manufacturer's RI of 11.5–22.7 pmol/L [14]. In a study conducted in the UK in 2017 with 261 participants from the same region, different RIs were obtained for fT4 patients with DRM using four different analyzers. One of the analyzers used in this study was the same as that used in our study, and the RI for fT4 (7.9–13.0 pmol/L) was different from that used in our study [15].

As observed, different populations and different analyzers yielded different results. However, another factor to consider may be the units used. Unit differences can also lead to problems in result interpretation.

In our study, the RIs for fT3 differed between DRM and IDM, with ranges of 2.54–3.99 pg/mL for females and 2.62–4.29 pg/mL for males with DRM and 2.72–4.15 pg/mL for females and 2.88–4.49 pg/mL for males with IDM. The manufacturer's kit insert indicated a range of 2.5–3.9 pg/mL, and particularly, the upper limits of the RIs we established for males differed from those of the manufacturer.

In an RI determination study conducted with DRM in another region of our country, the RI for fT3 was found to be 2.62–3.84 pg/mL, although separate RIs for sex were not determined [11]. With IDM on the Siemens Advia Centaur XP analyzer, the RIs for fT3 were 3.9–6.0 pmol/L, while the manufacturer recommended 3.5–6.5 pmol/L for fT3. The RIs for fT3 were notably different for males (4.3–6.2 pmol/L) and females (3.8–5.5 pmol/L) [14].

The CLSI and IFCC have recommended that RI determination studies be conducted either with DRM or, if not feasible, with IDM. Therefore, many laboratories tend to prefer IDM due to concerns such as cost, time, and labor.

In our study, the RIs determined for fT3, fT4, and TSH using DRM and IDM showed statistically significant differences between sexes. As a result, separate RI values were provided for each gender in our study, as in previous similar studies [18]. The population in our study comprised individuals aged be-

tween 18 and 88 years. In our DRM-based RI determination study, care was taken to ensure an equal number of male and female reference individuals in each age group. Despite attempts to establish age-specific RIs by setting age boundaries, a definitive age cutoff could not be reached, and statistically significant differences were not obtained; therefore, age-specific RIs could not be determined. Our results were in line with the recommendation in the CLSI's EP28-A3c guidelines, emphasizing the necessity for each laboratory to establish its own RI. When comparing the RIs determined by both DRM and IDM with those provided by the manufacturer, although the RIs for fT3 and fT4 were close, the RIs we established had a narrower range. For the TSH test, while the lower limit of our RI was similar to that set by the manufacturer, there was a significant difference between the upper limit set by the manufacturer (5.60 mIU/L) and ours (DRM: 4.18 mIU/L, IDM: 4.45 mIU/L).

Subclinical thyroid dysfunction is defined by fT4 levels within the RI and TSH levels outside the RI. Studies have demonstrated an association between SCH and increased risks of hypertension, hyperlipidemia, atherosclerosis, and coronary artery disease, while subclinical hyperthyroidism has been linked to increased risks of atrial fibrillation and coronary artery disease [19].

Due to the greater number of reference individuals obtained with the IDM than with the DRM, we may have obtained wider RIs with the IDM. Additionally, the reliability of the health status of the reference individuals determined with DRM may not be ensured with IDM; therefore, we believe that the RI determined with DRM is more sensitive.

Conclusion

In conclusion, using the RI determined by us instead of the RI provided by the manufacturer for the TSH test revealed that many individuals considered normal in terms of thyroid function may actually have SCH. Therefore, we are of the opinion that the manufacturer's RI may not be suitable for our region, and it would be more appropriate to use the RI determined by our laboratory instead of the RI provided by the manufacturer.

Ethics Committee Approval: The study was approved by the Kocaeli University Non-interventional Clinical Research Ethics Committee (no: 2019/196, date: 08/05/2019).

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Research Article

Evaluation of the analytical performance of the access vitamin B12 II assay with the new calibrator

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Abstract

Objectives: We aimed to compare the analytical performance of the Access Vitamin B12 assay with the new B12 II calibrator to the current Access and Abbott assays and determined the method-specific reference interval.

Methods: The new B12 II was assessed for imprecision, accuracy, analytical sensitivity, linearity, and carryover. Bland-Altman, Passing Bablok, and concordance correlation coefficient (CCC) analyses were performed on 650 samples. Vitamin B12 tests were performed using the UniCel DxI 800 (Beckman Coulter, USA), and Alinity i System (Abbott Laboratories, Abbott Park, IL, USA) analyzers.

Results: The Access new B12 II assay demonstrated acceptable analytical performance; however, its reference range (138–787 pg/mL) was lower than the manufacturer's recommendation. The Access Vitamin B12 assay showed significant negative differences of 45.8% and 37.0% relative to the Abbott and new B12 II assays, respectively, while the new B12 II assay showed a smaller difference of 9.4% against Abbott. Significant proportional and constant errors were observed between Access and new B12 II (slope: 0.780, intercept: -21.95) and Access and Abbott (slope: 0.707, intercept: -18.95). Abbott and new B12 II demonstrated lower proportional and constant errors (slope: 0.902, intercept: 6.388). Concordance analysis indicated poor agreement of the Access assay with both Abbott and new B12 II (CCC: 0.806, 0.879), whereas Abbott and new B12 II demonstrated substantial agreement (CCC: 0.958).

Conclusion: The new B12 II assay demonstrated appropriate analytical performance and improved consistency with the Abbott assay. The reference interval we established differed from the manufacturer's suggested range, highlighting the importance of determining population-based reference intervals.

Keywords: Calibration, reference standards, vitamin B12, vitamin B12 deficiency

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Vitamin B12, or cobalamin, is a water-soluble vitamin that is critical for key physiological processes, including DNA synthesis, fatty acid metabolism, and myelin production. It is predominantly obtained from animal-derived sources such as red meat, dairy products, and eggs [1]. Absorption of vitamin B12 occurs in the terminal ileum and requires intrinsic factor, a glycoprotein secreted by parietal cells in the stomach. Disruptions in this absorption mechanism—resulting from dietary insufficiency, malabsorption syndromes, or intrinsic factor de-

fiency—can lead to significant clinical consequences, including hematologic abnormalities and neurological dysfunction. Although excess vitamin B12 is stored in the liver, prolonged disruption in B12 absorption—due to factors such as dietary insufficiency, malabsorption, or a deficiency of intrinsic factor—can deplete liver stores, resulting in a deficiency [1–3].

Vitamin B12 deficiency is a significant global health problem and vitamin B12 levels naturally decline with age [4, 5]. Sub-clinical B12 deficiency is notably more prevalent among the

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elderly, with reported prevalence rates ranging from 6% to 40% [6–9]. However, younger populations are also at an elevated risk of vitamin B12 deficiency and high-risk groups include vegetarians [10], individuals with gastrointestinal disorders [11], those suffering from depression [12], heavy drinkers [13], and individuals with renal dysfunction [11].

Despite its high prevalence, diagnosing vitamin B12 deficiency remains complex due to inconsistencies in assay methods and the absence of universally accepted reference standards [11]. At present, no definitive reference method exists for investigating suspected vitamin B12 deficiency. Diagnosis is primarily based on measuring serum or plasma vitamin B12 concentrations [14–16]. According to the World Health Organization (WHO), a serum level greater than 221 pmol/L (300 pg/mL) indicates adequate vitamin B12 status, while levels between 148–221 pmol/L (200–300 pg/mL) are considered low. A serum level below 200 pg/mL is classified as vitamin B12 deficiency [17, 18]. However, the lack of standardized reference materials and methods has prevented the establishment of uniformity in current measurement techniques. This results in variability between different vitamin B12 assays [19].

In December 2024, Beckman Coulter launched the Access Vitamin B12 II Calibrators for use with the Access Vitamin B12 assay (new B12 II) on Access Immunoassay Systems. These calibrators offer enhanced precision and accuracy in vitamin B12 detection, with a total imprecision of $\leq 12.0\%$ across the measuring range. Standardized to the WHO International Standards (IS 03/178), the calibrators ensure greater confidence in patient test results. The analyte in the Access Vitamin new B12 II Calibrators (REF D06116) is traceable to the manufacturer's working calibrators, in accordance with the traceability guidelines outlined in EN ISO 17511. The Access Vitamin B12 assay demonstrated an average recovery rate of 111% compared to the WHO IS 03/178 assigned value of 480 pg/mL [20]. While the initial claims highlight improved diagnostic reliability, independent validation is necessary to confirm these advancements and assess the analytical performance against existing methods.

In this study, we aimed to evaluate the analytical performance of the Access Vitamin B12 assay using the newly introduced new B12 II calibrators, focusing on imprecision, accuracy, LoB, LoD, LoQ, linearity, and carryover. Additionally, we compared the new B12 II to the current Access Vitamin B12 assay on the DXI 800 system and the Abbott Vitamin B12 assay on the Alinity i System.

Materials and Methods

Study design and subjects

To conduct the analytical performance studies for the new B12 II assay, remnant serum samples from patients who visited our hospital for various reasons and had blood drawn and sent to the laboratory were utilized. For the method comparison study, samples were selected from patients aged 18 to 99 years who had Vitamin B12 tests requested from the outpa-

tient clinics of our hospital. A total of 650 patient samples (350 females and 300 males) with sufficient volume for additional Vitamin B12 testing were included in the study. The initial Vitamin B12 concentrations, as determined by the current Access Vitamin B12 assay, ranged from 63 to 1,491 pg/mL. These selected samples were reanalyzed on the same day using the new B12 II assay on the DXI 800 analyzer and the Abbott Vitamin B12 assay on the Alinity i System. The samples were carefully chosen to ensure their concentrations were within the analytical ranges of the alternative systems and represented a broad distribution of Vitamin B12 levels.

Blood Sampling

Blood samples were collected in the morning, between 8:00 and 10:00 AM, following an overnight fast. Venous blood was drawn from the antecubital vein into 5 mL Greiner Bio-One GmbH Samplic®. Blood samples were centrifuged at 2000 x g for 10 minutes. All studies were done according to the Clinical & Laboratory Standards Institute (CLSI) Evaluation Protocols (EP) specific to each parameter. Measurements were performed in the biochemistry laboratory of Dr. Lütfi Kırdar Kartal City Hospital between December 2024 and January 2025. This study was approved by the Ethical Committee of our institution (No: 2025/010.99/12/34, Date: 24/01/2025). This study was conducted in accordance with the principles of the Declaration of Helsinki.

Method

Serum Vitamin B12 analysis was performed using both the Dxl 800 Unicel and the Alinity i Systems. Both methods are based on competitive protein binding, utilizing chemiluminescence immunoassay (CLIA) as the detection method. In the Dxl 800 Unicel (Beckman Coulter, USA), chemiluminescence is generated from enzymatic reactions, while the Alinity i System (Abbott Laboratories, Abbott Park, IL, USA) employs chemiluminescence microparticle immunoassay (CMIA).

Assay performance studies

Imprecision

Imprecision (both within-run and within-laboratory) was analyzed using control samples with four different vitamin B12 concentrations: 185.2, 374.7, 608.7, and 804.8 pg/mL. Two commercial controls were tested at these concentration levels to calculate imprecision, expressed as CV%. Precision evaluation followed the Clinical and Laboratory Standards Institute (CLSI) EP15-Ed3-IG1 guidelines, involving measurements over five consecutive days, with five replicates performed each day [21]. The predefined acceptable imprecision limit was set at CV $\leq 12\%$.

Accuracy

Two samples from the Randox International Quality Assessment Scheme (RIQAS) monthly immunoassay external quality control program were used to assess accuracy. These samples, taken from Cycle 22, were tested using new B12 II in a single

Table 1. Analytical performance characteristics of vitamin B12 assays by manufacturer claims

	Access Vitamin B12 assay	New access Vitamin B12 II assay	Abbott Vitamin B12 assay
Test name	VitB12	B12II	Alinity i system B12
Imprecision (total CV %)	CV<12% across measuring range	CV<12% across measuring range	CV<7.9 % across measuring range
Analytical sensitivity (pg/mL)	LoB (not given) LoD<50 LoQ<50	LoB<78 LoD<105 LoQ<105	LoB<83 LoD<109 LoQ<148
Linearity (pg/mL)	50–1,500	105–2,100	148–2000
Reference intervals (pg/mL)	180–914	222–1,439	187–883

CV: Coefficient of variation; LoB: The limit of blank; LoD: The limit of detection; LoQ: The limit of quantification

analytical run. The percentage deviation from the reported target mean was calculated using the formula: ((Measured value – target mean) / target mean) × 100. The acceptable accuracy limit set by RIQAS was 16.9%.

Analytic sensitivity

Studies were conducted in accordance with CLSI EP17 guidelines [22]. The limit of blank (LoB) was determined by analyzing 20 replicates of the manufacturer's zero calibrator and calculated using the formula:

$$\text{LoB} = \text{Mean (blank)} + 1.645 \text{ SD (blank)}.$$

The limit of detection (LoD) was established using the lowest non-zero calibrator (153 pg/mL), which was diluted by half and analyzed in 20 replicates. The LoD was calculated with the formula:

$$\text{LoD} = \text{LoB} + 1.645 \text{ (SD low-concentration sample)}.$$

The limit of quantification (LoQ) study was performed by analyzing samples with concentrations ranging from 76.5 to 174.5 pg/mL over three consecutive days, with three replicates per concentration. To assess precision and accuracy, five samples near the manufacturer's stated LoQ of 102.5 pg/mL were evaluated to calculate coefficients of variation (CVs) and total errors. Total error was determined using the formula: TE = %BIAS + (1.96 × %CV). The LoQ was established as the concentration at which the calculated total error was below the minimum acceptable total error (23%) defined by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM).

Linearity: Linearity testing was conducted according to CLSI EP6 guidelines [23]. A patient serum with a high Vitamin B12 level was diluted to generate seven concentrations ranging from 80 to 2400 pg/mL. Each concentration was tested three times within the same run. The recovery range was acceptable if it fell within ±15% of the target value.

Carryover

Carryover assessment involved testing three replicates of a high-concentration sample (labeled as a1, a2, and a3) followed by three replicates of a low-concentration sample (labeled as b1, b2, and b3). The carryover effect was determined using the formula: (b1–b3)/(a3–b3). A carryover value below 2% was considered insignificant [24].

Method comparison

Vitamin B2 concentrations from 650 patient samples were first measured using the Access Vitamin B12 assay. Subsequently, the same samples were reanalyzed with both the new B12 II and Abbott assays. The Abbott system was chosen for comparison purposes as it was the routine system in our laboratory at the time of the study.

All measurements for method comparison were performed simultaneously on the same serum samples by the same experienced operator, within the analytical range of the systems, processed in duplicate as a single batch with consistent freeze/thaw cycles, and in accordance with CLSI EP09-A3 guidelines [25].

Statistical analysis

The distribution of data was evaluated using the Kolmogorov–Smirnov test, and the results are presented as the median and interquartile range. Imprecision, LoB, LoD, LoQ, and linearity were calculated using EP Evaluator Release 9 software (David G. Rhoads Association, Kennett Square, PA). To assess method comparison, Bland–Altman plots, Passing–Bablok regression, and the concordance correlation coefficient (CCC) were used, with analysis performed using MedCalc Statistical Software (version 12, MedCalc Software, Mariakerke, Belgium). A systematic error was considered significant if the 95% confidence interval excluded 1.0 for the slope (indicating proportional error) or 0 for the y-intercept (indicating constant error).

Results

The analytical performance characteristics of Vitamin B12 assays, as claimed by the manufacturers, are summarized in Table 1. The median values (2.5–97.5 percentiles; pg/mL) for the 650 samples analyzed were as follows: 140 (78.7–714.7) for the Access Vitamin B12, 206 (134.0–949.6) for the new B12 II, and 237 (152–1020) for the Abbott Vitamin B12 assay. The reference interval was calculated from 400 patients whose Vitamin B12 levels were within the Abbott assay's normal range (187–883 pg/mL) and who had normal hemoglobin, hematocrit, and folic acid levels, with no clinical or laboratory evidence of Vitamin B12 deficiency. Following the CLSI EP28-A3c guideline, the non-parametric method was used, and the 2.5th and 97.5th per-

Table 2. Analytical performance characteristics of access Vitamin B12 assay with the new B12 II calibrator

Performance criteria	Study result
Within-run CV (%)	
Level 1 (185.2 pg/mL)	5.41
Level 2 (375.7 pg/mL)	4.07
Level 3 (608.7 pg/mL)	2.80
Level 4 (804.8 pg/mL)	4.01
Within-laboratory CV (%)	
Level 1 (185.2 pg/mL)	7.18
Level 2 (375.7 pg/mL)	6.40
Level 3 (608.7 pg/mL)	7.75
Level 4 (804.8 pg/mL)	5.09
Accuracy (deviation %)	
Riqas 1 (637 pg/mL)	12.5
Riqas 2 (951 pg/mL)	3.3
LoB (pg/mL)	15.84
LoD (pg/mL)	80.82
LoQ (pg/mL)	102
Linearity (pg/mL)	102–2060
Carry-over (%)	0.74

CV: Coefficient of variation; LoB: The limit of blank; LoD: The limit of detection; LoQ: The limit of quantification.

centiles of the distribution were taken as the lower and upper limits, respectively. Using the Beckman new B12 II assay, the calculated interval was 138–787 pg/mL, which is lower than the manufacturer's proposed range of 222–1,439 pg/mL.

The new B12 II assay demonstrated acceptable performance in terms of imprecision, LoB, LoD, LoQ, linearity, and carry-over. The analytical performance characteristics of the new B12 II assay are presented in Table 2. Bland-Altman analysis revealed notable differences between the three systems. The Access Vitamin B12 assay showed significant negative differences of 45.8 % and 37.0 % relative to the new B12 II and Abbott assays, respectively while the new B12 II showed a smaller negative difference of 9.4% against the Abbott. Notably, only the difference between the new B12 II and Abbott assays satisfied the EFLM allowable bias threshold of 14.1%. The comparison

results between the methods are shown in the Bland-Altman plot (Fig. 1). Significant proportional and constant errors were observed between the Access Vitamin B12 and new B12 II assays, with a slope of 0.780 (0.766–0.794) and an intercept of -21.95 (-23.58 to -18.6). Similarly, the Access Vitamin B12 and Abbott assays demonstrated significant proportional and constant errors, with a slope of 0.707 (0.691–0.723) and an intercept of -18.95 (-23.58 to -14.51). The Abbott and new B12 II assays exhibited smaller proportional and constant errors, with a slope of 0.902 (0.883–0.920) and an intercept of 6.388 (0.660–11.613). The Passing-Bablok regression analyses are presented in Figure 2. The Access Vitamin B12 and Abbott, as well as the Access Vitamin B12 and new B12 II assays, exhibited poor agreement, with CCC values of 0.806 (0.787–0.824) and 0.879 (0.866–0.891), respectively. However Abbott and new B12 II showed substantial agreement, with a CCC value of 0.958 (0.952–0.964). Method comparison data are shown in Table 3.

Discussion

This study is the first method evaluation of the newly introduced Access new B12 II calibrator, launched in December 2024. The new B12 II assay demonstrated strong analytical performance with the new calibrator, providing improved traceability, consistency, and reliability when compared to the Abbott assay. Bias analysis revealed that the current Access Vitamin B12 assay showed a significant negative difference of 37% compared to the Abbott assay. However, with the introduction of the new B12 II calibrator, this difference was significantly reduced to -9.4%, indicating improved alignment between the two assays. Additionally, the observed negative difference of 48% between the current Access Vitamin B12 assay and the new B12 II assay indicates that the new calibrator produces higher results than the current assay. While ongoing standardization efforts continue, the reference range determined by the new B12 II assay (138–787 pg/mL) still differs from that of the Abbott assay (187–883 pg/mL). This highlights the need for method-specific reference ranges, rather than relying on a universal cut-off value, such as 200 pg/mL, to define deficiency criteria. Establishing the appropriate reference range for each method is crucial for accurate clinical diagnosis.

Table 3. Method comparison results of Vitamin B12 Assay with the new B12 II calibrator

Method	Passing-bablok regression analysis		Concordance correlation analysis			Bland-altman analysis
	Slope (CI)	Intercept (CI)	CCC (CI)	P	C _b	Bias (%)
Access Vitamin B12 new B12 II	0.780 (0.766–0.794)	-21.95 (-23.56– -18.62)	0.879 (0.866–0.891)	0.984	0.893	-37.0
Access Vitamin B12 abbott	0.707 (0.691–0.723)	-18.95 (-23.58– -14.51)	0.806 (0.787–0.824)	0.973	0.828	-45.8
New B12 II abbott	0.902 (0.883–0.920)	6.388 (0.660–11.613)	0.958 (0.952–0.964)	0.970	0.987	-9.4

CI: Confidence interval; CCC: Concordance correlation coefficient; P: Pearson correlation coefficient; C_b: Bias correction factor

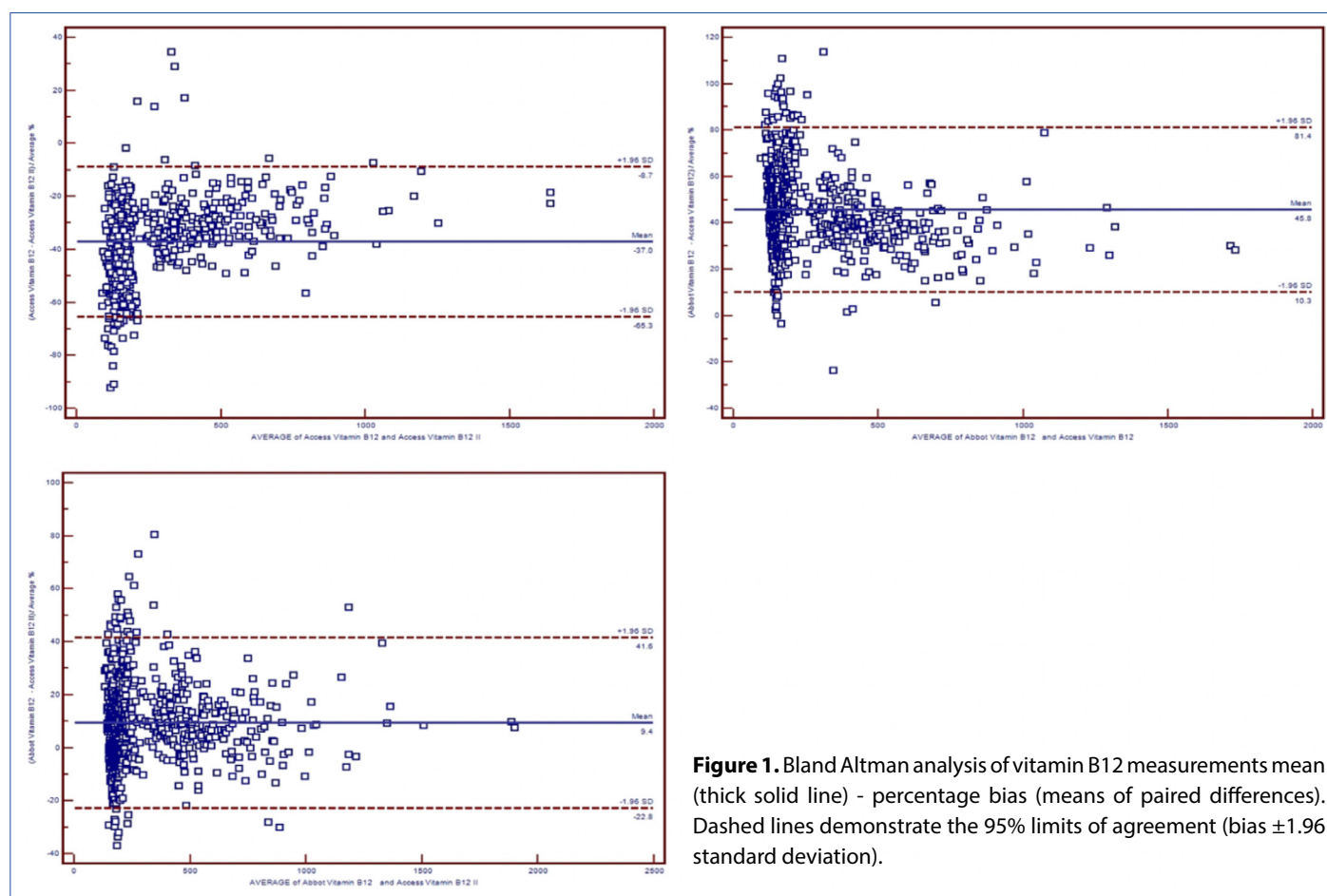


Figure 1. Bland Altman analysis of vitamin B12 measurements mean (thick solid line) - percentage bias (means of paired differences). Dashed lines demonstrate the 95% limits of agreement (bias ± 1.96 standard deviation).

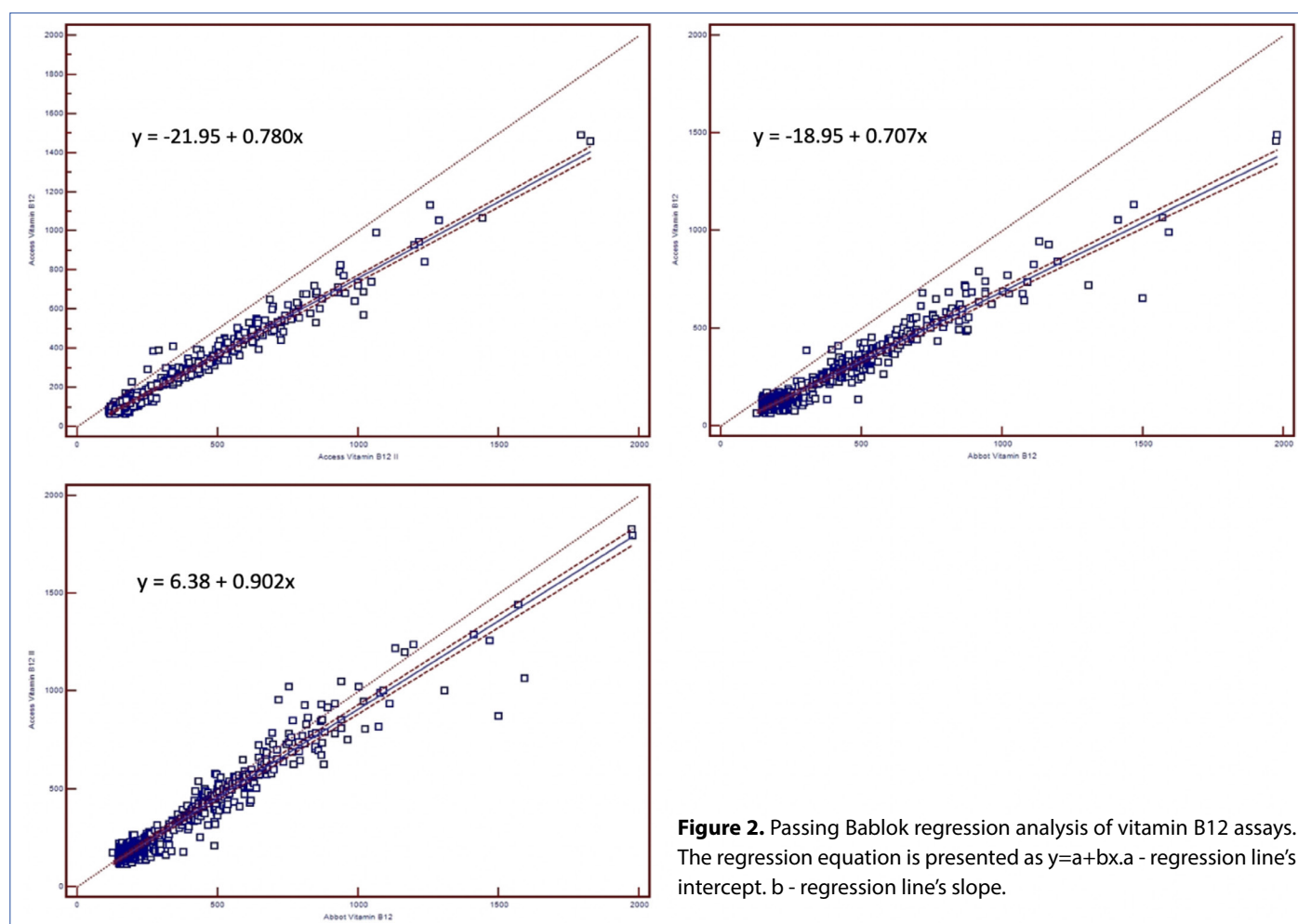
Vitamin B12, the largest of all vitamins, exists in various forms and is present in very low concentrations in serum. It binds strongly to serum proteins [24]. The unique biochemical characteristics of vitamin B12, coupled with the complexities involved in producing pure reference materials and the absence of a universally standardized reference method, present significant challenges to achieving consistent and reliable standardization of vitamin B12 assays. The serum vitamin B12 assay methods have not yet been fully standardized. To address this issue, the World Health Organization (WHO) Expert Committee on Biological Standardization introduced the material 03/178 as an International Standard (IS) for serum vitamin B12 assays. This material was assessed in 24 laboratories across seven countries to evaluate its applicability as a reference standard for both vitamin B12 and folate assays. The findings revealed that employing this standard material reduced variability between laboratories. However, the standard material, produced through the lyophilization of pooled human serum, may lead to challenges concerning its commutability [26].

The National Institute of Standards and Technology (NIST) currently does not provide a certified reference material (SRM) for vitamin B12 or methylmalonic acid (MMA). However, NIST is in the process of developing SRM 3951 for serum vitamin B12, which includes target pools with concentrations of 74 pmol/L (100 pg/mL), 148 pmol/L (200 pg/mL), and 332 pmol/L (450

pg/mL). Among these, the 332 pmol/L pool represents “normal” serum, while the two lower pools consist of a mixture of normal serum and serum that has been stripped of its naturally occurring vitamin B12 [27, 28].

Several comparative studies have been conducted to evaluate the performance of different vitamin B12 assays. In the study by Ispir et al. [14], four vitamin B12 assays—Dxl 800 Unicel, ADVIA Centaur XP, Roche Cobas E601, and Architect i2000sr—were compared. The results showed strong correlations between the assays, with the weakest correlation between Dxl 800 Unicel and ADVIA Centaur. Dxl 800 Unicel produced lower results compared to the others. MMA and homocysteine showed similar correlations with vitamin B12 levels across all methods. The study concluded that while the assays performed well, vitamin B12 assay standardization is still incomplete and requires further efforts.

In the study conducted by Ihara et al. [15], vitamin B12 and folate levels were measured using three different methods: Access, Advia Centaur, and Elecsys. The results revealed significant correlations between the assays; however, serum vitamin B12 levels measured by Elecsys were consistently higher compared to those obtained from the other two methods. Similar to our findings, their study concluded that, in the absence of reliable reference materials and standardized methods, reference values for vitamin B12 and folate remain method-depen-



dent. For instance, certain assays have established lower reference values of 200 pg/mL for vitamin B12, but these values are not applicable to all automated immunoassay methods. Therefore, it is crucial to determine reference values that are specific to each method. This approach ensures accurate diagnosis and consistency across different testing platforms, leading to more dependable clinical results.

In another study, reference intervals for plasma vitamin B12 concentration were established using three different immunoassays in the North Denmark Region. The findings showed that results from different methods were not interchangeable, with significant variation in the frequency of vitamin B12 levels below the cut-off when similar thresholds were applied [29].

Our study demonstrated a stronger correlation and reduced difference between the newly developed new B12 II calibrator and the Abbott system compared to the current Access Vitamin B12 assay. However, there were still concerns regarding clinical interpretation, suggesting that full standardization may not have been achieved. Among the 650 patients, the current Access Vitamin B12 assay identified 405 (62.3%) patients as deficient (below 200 pg/mL), while the Abbott system detected 235 (36.1%) patients below this threshold. With the new B12 II calibrator, the assay classified 288 (44.3%)

patients as deficient under the same cutoff, indicating higher vitamin B12 levels than the previous Access Vitamin B12 assay. These results show that, while correlation between the new B12 II and Abbott assays has improved, differences between the methods remain, emphasizing the continued need for method-specific reference ranges.

The study did not assess potential interference factors, such as hemolysis, lipemia, or elevated bilirubin levels, which may represent a limitation. Additionally, the calculated reference range for the new B12 II assay, based on Vitamin B12 levels according to the Abbott system's normal range, was lower than the manufacturer's proposed values. This indicates that the current reference range for the new B12 II assay does not align with the manufacturer's suggested values for our population, emphasizing the need for population-specific reference range studies in larger and more diverse groups.

Conclusion

The new B12 II assay demonstrated appropriate analytical performance and improved consistency with the Abbott assay. The reference interval we established differed from the manufacturer's suggested range, highlighting the importance of determining population-based reference intervals.

Ethics Committee Approval: The study was approved by the Dr. Lütfi Kırdar Kartal City Hospital Scientific Research Ethics Committee (no: 2025/010.99/12/34, date: 24/01/2025).

Informed Consent: Informed consent was obtained from all participants.

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