ISSN 2220-315x (online)

World Journal of *Experimental Medicine*

Quarterly Volume 15 Number 1 March 20, 2025

Published by Baishideng Publishing Group Inc

World Journal of Experimental Medicine

Contents

Quarterly Volume 15 Number 1 March 20, 2025

EDITORIAL

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Retrospective Cohort Study

Observational Study

Prospective Study

Basic Study

LETTER TO THE EDITOR

World Journal of Experimental Medicine

Quarterly Volume 15 Number 1 March 20, 2025

Contents

ABOUT COVER

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INDEXING/ABSTRACTING

The *WJEM* is now abstracted and indexed in PubMed, PubMed Central, Scopus, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The *WJEM*'s CiteScore for 2023 is 1.7 and Scopus CiteScore rank 2023: Internal medicine is 109/167.

RESPONSIBLE EDITORS FOR THIS ISSUE

NAME OF JOURNAL
World Journal of Experimental Medicine

ISSN
ISSN 2220-315x (online)

LAUNCH DATE
December 20, 2011

FREQUENCY
Quarterly

PUBLICATION DATE
March 20, 2025

PUBLISHING PARTNER
Department of Clinical Laboratory, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

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https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

PUBLISHING PARTNER's OFFICIAL WEBSITE
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World Journal of Experimental Medicine

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World J Exp Med 2025 March 20; 15(1): 96318

DOI: 10.5493/wjem.v15.i1.96318

ISSN 2220-315x (online)

EDITORIAL

Therapeutic strategies and prognostic challenges in linitis plastica

Grigorios Christodoulidis, Sara Eirini Agko, Konstantinos Eleftherios Koumarelas, Marina Nektaria Kouliou

Specialty type: Surgery

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification

Scientific Quality: Grade B, Grade D

Novelty: Grade B, Grade C

Creativity or Innovation: Grade B, Grade C

Scientific Significance: Grade B, Grade C

P-Reviewer: Shao JK; Zhang JW

Received: May 3, 2024

Revised: October 9, 2024

Accepted: November 29, 2024

Published online: March 20, 2025

Processing time: 236 Days and 16.8 Hours

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Abstract

Gastric cancer ranks fifth as the most common cancer and third as the leading cause of death worldwide. Risk factors include advancing age, low-fiber diets, high salt intake and *Helicobacter pylori* infection. Diagnosis relies on histological examination following endoscopic biopsy with staging accomplished through various imaging modalities. Early gastric cancer is primarily managed *via* endoscopic resection, while non-early operable cases typically undergo surgery. Advanced cases are addressed through sequential chemotherapy lines, with initial treatment usually comprising a platinum and fluoropyrimidine combination. Linitis plastica (LP) is a rare, aggressive form of gastric cancer characterized by diffuse infiltration of the gastric wall, resulting in poor outcomes even after curative resection. The absence of a standardized definition contributes to uncertainty regarding the precise incidence of these tumors. LP is often diagnosed at advanced stages, with a reported median survival rate of approximately 4%-29%, despite "curative resection". Its distinctive biological behavior includes perineural invasion, nodal metastasis, and peritoneal dissemination. The bleak prognosis for LP patients partly stems from delayed diagnosis and its aggressive biological nature, posing significant challenges for clinical management. Currently, no specialized treatment strategy exists for LP, and clinical approaches typically align with those used for general gastric cancer treatment. Surgical resection is the primary treatment, but the optimal surgical approach remains contentious. Recent studies have investigated the efficacy of neoadjuvant chemotherapy and radiotherapy in improving survival outcomes for LP patients. However, controversies persist regarding the role of adjuvant chemotherapy and postoperative radiotherapy. LP requires a multidisciplinary approach and personalized treatment strategies tailored to each patient's condition. Further research is needed to elucidate optimal therapeutic interventions and improve outcomes for LP patients.

Key Words: Linitis plastica; Surgery; Chemotherapy; Radiotherapy; Treatment strategies; Gastric cancer

Core Tip: Linitis plastica (LP) gastric cancer poses a significant challenge due to its aggressive nature and poor prognosis. Early detection and personalized treatment strategies are essential for improving outcomes in LP patients. While surgery remains the mainstay of treatment, the role of adjuvant chemotherapy and postoperative radiotherapy is still under debate. Neoadjuvant chemotherapy, particularly with regimens such as docetaxel plus oxaliplatin and S-1, shows promise in enhancing survival rates for LP patients. Multidisciplinary collaboration and further research are necessary to optimize therapeutic interventions and improve outcomes in this challenging subset of gastric cancer.

Citation: Christodoulidis G, Agko SE, Koumarelas KE, Kouliou MN. Therapeutic strategies and prognostic challenges in linitis plastica. *World J Exp Med* 2025; 15(1): 96318
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/96318.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.96318

INTRODUCTION

Gastric cancer is rated the fifth most common cancer and is the third most frequent cause of death worldwide[1]. Risk factors encompass advancing age, diets low in fiber, high salt intake, and *Helicobacter pylori* infection can lead to this disease. Diagnosis relies on histological examination following endoscopic biopsy, with staging accomplished through computed tomography, endoscopic ultrasound, positron emission tomography, and laparoscopy. Also, the disease exhibits significant molecular and phenotypic diversity. Early gastric cancer is primarily managed *via* endoscopic resection, while non-early operable cases typically undergo surgery. Perioperative or adjuvant chemotherapy enhances survival rates in stage 1B and higher cancers. Advanced cases are addressed through sequential chemotherapy lines, with initial treatment usually comprising a platinum and fluoropyrimidine combination, yielding a median survival of less than a year[2].

Linitis plastica (LP) is a rare form of gastric cancer characterized by diffuse infiltration of the submucosal and mucosal layers, resulting in thickening and rigidity of the gastric wall[3,4]. Due to the absence of a standardized definition, the precise incidence of these tumors remains uncertain. Terms such as "scirrhous adenocarcinoma", "Borrmann type 4" or "large (> 8 cm in diameter) type 3 gastric cancer" are inconsistently employed to depict LP[5]. LP is a characteristic finding of scirrhous gastric carcinoma, is identified on barium studies by the stomach's irregular narrowing and rigidity [6]. As a distinct prognostic factor, signet ring cell carcinoma may aid in risk stratification and optimization of treatment, particularly for patients with locally advanced stages[7]. These tumors are often identified at advanced stages, with a reported median survival rate of approximately 4%-29%, even after "curative resection". Such outcomes are attributed to its distinctive biological behavior, including a heightened propensity for perineural invasion, nodal metastasis, peritoneal dissemination, and infiltration into adjacent tissues[8,9]. Moreover, LP exhibits distinct characteristics, including younger age at diagnosis, higher prevalence among females, elevated incidence of stages 3 and 4, lymph node invasion, and notably reduced overall survival (OS) rates primarily attributed to higher frequency of R1 resection[8]. Metastatic LP can develop through various pathways, including hematogenous spread, lymphatic dissemination, and direct extension. It is clinically indistinguishable from primary scirrhous carcinoma of the stomach[6].

The bleak prognosis for patients with LP partly stems from delayed diagnosis in most cases, compounded by the aggressive and fast-paced growth and invasion of this cancer, posing significant challenges for clinical management. Currently, no specialized treatment strategy exists for gastric LP (GLP), thus clinical approaches typically align with those used for general gastric cancer treatment. While surgical resection stands as the primary treatment for gastric cancer, the optimal surgical approach remains contentious. Non-curative resection may offer potential enhancement of the prognosis in individuals afflicted with GLP[10].

SURGERY AND ADVANCING TREATMENT

Given the complexities associated with GLP, surgical intervention remains a central component in its management strategy. Surgery has long been a cornerstone in treating gastric cancer. Japanese surgical oncologists initially favored a surgery-first approach due to the effectiveness of D2 lymph node dissection and the prevalence of surgically treatable cancer cases[11]. A study conducted by Liang *et al*[12] of patients (36%) that underwent curative resection, patients (40%) that underwent palliative resection and patients (29%) that were judged unresectable, showed that regardless of the tumor stage and grade, OS rates at 1, 2 and 5 years were significantly worse in patients who underwent palliative resection but notably better than the patients who were judged unresectable. Another study included 88 patients who underwent curative surgery, and 80 patients who underwent non-curative surgery. The 3-5 year OS rate in the curative

group was significantly higher than that in the non-curative group. In the curative group the most common area of recurrence was the peritoneum (85.7%) with most recurrences occurring within two years. These findings suggest that the role of surgery is quite limited[13].

Early efforts were made at combining surgery with postoperative chemotherapy, particularly in the Far East where this strategy gained ample evidence for treating stage II/III gastric cancer. However, a significant drawback emerged as many post-gastrectomy patients struggled with adhering to rigorous combination chemotherapy regimens[11]. The results of the study by Luo *et al*[10] were consistent with the conclusion of a study conducted by Aranha *et al*[14] and showed that LP is not surgically curable due to poor postoperative survival. Most researchers argue that the prognosis is notably worse for LP patients who undergo curative resection. Moreover, Luo *et al*[10] found that the 1-year survival rate in the non-resection group was worse than that in the non-curative group.

Despite the central role of surgery in managing LP, there is ongoing debate about the potential benefits of non-curative resection *vs* other treatment modalities. Certain researchers argue that instead of opting for non-curative resection in patients with LP, chemotherapy should be considered a preferable alternative[10]. Adjuvant chemotherapy aims to eliminate micrometastatic tumor cells both before and after curative surgery. Despite numerous phase III trials investigating the efficacy of postoperative adjuvant chemotherapy, many have failed to show statistical significance or high patient compliance. While treatments like S-1 for 1 year or combination therapy with capecitabine and oxaliplatin for 6 months have proven effective, more intensive chemotherapy is deemed necessary to further enhance survival rates. Neoadjuvant chemotherapy offers advantages such as a high rate of R0 resection, tumor regression, high patient compliance, and the avoidance of unnecessary surgeries[15].

A study conducted by Iwasaki *et al*[16], showed that the phase III JCOG0501 trial aimed to establish the superiority of neoadjuvant S-1 plus cisplatin followed by D2 gastrectomy over upfront surgery. However, the study revealed no survival benefit for neoadjuvant S-1 plus cisplatin. In Korea, the PRODIGY study, a phase III trial investigating neoadjuvant docetaxel plus oxaliplatin and S-1 (DOS) for gastric cancer of T2-3N+ or T4Nany, demonstrated a significantly superior progression-free survival in the neoadjuvant DOS arm[17] Consequently, DOS therapy emerges as a promising option for preoperative chemotherapy in cases of LP. In Europe, the standard treatment involves docetaxel, oxaliplatin, fluorouracil, and leucovorin therapy, resulting in a 16% observed rate of pathological complete regression. Conversely, in East Asia, DOS is viewed as a promising triple therapy option[18]. Xu *et al*[19] conducted a study to investigate the efficacy of neoadjuvant chemotherapy (NAC) using non-S-1 plus cisplatin (non-SP) regimens for LP patients with type 3 gastric cancer and type 4 gastric cancer. The result was unbeneficial in terms of the survival rate of LP patients with type 4 gastric cancer. They showed that the 5-year survival rates for patients with LP with type 3 gastric cancer treated with NAC and surgery was 54.5%, and for patients with LP who underwent surgery was 28%. These findings demonstrate that NAC can enhance the prognosis of individuals with LP and type 3 gastric cancer[19].

With regard to other strategies, radiotherapy is a growing area of treatment. Recently, scholars have increasingly emphasized the importance of radiotherapy as a component in the comprehensive treatment of LP. Song *et al*[9] demonstrated that surgery and chemoradiotherapy resulted in a better outcome than surgery and chemotherapy. They indicated that postoperative radiotherapy may offer additional benefits for LP, owing to its extremely aggressive biological nature and potential for metastasis. They conducted a cohort study including 174 patients with non-metastatic GLP and compared the OS between treatment groups. Those patients who received surgery alone had a median survival of 8.38 months, those who received surgery with chemotherapy and/or radiotherapy had a median survival of 13.90 months, those who received chemotherapy and/or radiotherapy had a median survival of 8.94 months and patients that received no treatment had a median survival of 2.50 months[9].

The impact of surgery between the study conducted by Liang *et al*[12] and the study conducted by Kim *et al*[13] showed that they both focused on the role of surgery in LP. The former study showed a general survival benefit with gastrectomy, whereas the latter study provided more granular data, comparing curative and non-curative resections. In this study curative resection significantly improved three to five years OS but non-curative resection also offered better outcomes than no surgery[12,13]. However, the study conducted by Iwasaki *et al*[16] introduced the role of adding NAC before surgery, which further enhanced the survival outcomes in comparison to the study conducted by Kim *et al*[13]. Of utmost importance are the results from the study performed by Kang *et al*[17], which revealed that compared to the results by Iwasaki *et al*[16], NAC followed by surgery is superior to surgery first plus adjuvant chemotherapy.

The results from these studies suggest that while surgery plays a crucial role in managing LP, its efficacy is significantly enhanced when combined with NAC. It appears to be the most effective strategy for improving long-term outcomes in these patient groups. Future studies should focus on understanding the molecular and genetic profile of LP and how to evaluate treatment approaches. Studies should also focus on personalized treatment taking onto account each patient's risk factors.

CONCLUSION

In conclusion, LP is a rare and aggressive form of gastric cancer characterized by diffuse infiltration of the gastric wall, resulting in poor outcomes even after curative resection. Surgery has traditionally been the mainstay of treatment for gastric cancer, with various surgical approaches and adjuvant therapies aimed at improving outcomes. However, controversies persist regarding the optimal surgical strategy and the role of adjuvant chemotherapy.

Overall, the management of LP remains challenging, requiring a multidisciplinary approach and personalized treatment strategies tailored to each patient's unique circumstances. Further research is needed to elucidate the optimal therapeutic interventions and improve outcomes for patients with LP.

FOOTNOTES

Author contributions: Christodoulidis G designed the overall concept and outline of the manuscript; Christodoulidis G, Agko ES, Koumarelas KE and Kouliou MN contributed to the discussion and design of the manuscript; Christodoulidis G, Agko ES, Koumarelas KE and Kouliou MN contributed to the writing, editing the manuscript, and review of literature.

Conflict-of-interest statement: Nothing to disclose.

Country of origin: Greece

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S-Editor: Gong ZM
L-Editor: Webster JR
P-Editor: Zhao S

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World Journal of Experimental Medicine

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World J Exp Med 2025 March 20; 15(1): 101289

DOI: 10.5493/wjem.v15.i1.101289

ISSN 2220-315x (online)

EDITORIAL

Editorial on amylase and the acini–islet–acinar reflex: A new frontier in metabolic health research

Opeyemi Deji-Oloruntoba, Uchenna E Okpete, Haewon Byeon

Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification
Scientific Quality: Grade C
Novelty: Grade B
Creativity or Innovation: Grade B
Scientific Significance: Grade C

P-Reviewer: Mujahid S

Received: September 11, 2024
Revised: November 5, 2024
Accepted: November 20, 2024
Published online: March 20, 2025
Processing time: 107 Days and 0.3 Hours

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Abstract

This editorial comments on the study by Pierzynowska *et al* investigating the acini-islet-acinar (AIA) reflex, which integrates the exocrine and endocrine functions of the pancreas. The study investigates whether exogenous amylase introduced to the interstitial fluid surrounding pancreatic islets can inhibit insulin release. Historically, high serum amylase levels were associated with pancreatitis, but recent findings suggest that low amylase levels are more linked to metabolic diseases like diabetes and obesity. In their experiment, six pigs were used to examine the effects of amylase infusion on insulin release during an intravenous glucose tolerance test. The pigs received different treatments (amylase, saline, or bovine serum albumin), and blood samples were taken over two hours to measure insulin and glucose levels. The results showed amylase delayed glucose-stimulated insulin release, whereas bovine serum albumin increased insulin levels supporting the existence of the AIA reflex and suggesting amylase as a key metabolic regulator. Enzyme supplementation, particularly with α-amylases, may offer therapeutic benefits in preventing and managing metabolic disorders, including diabetes and obesity. Further research is warranted to explore the full scope of amylase's role in metabolic health and its therapeutic potential.

Key Words: Alpha-Amylase; Insulin secretion; Glucose metabolism; Pancreatic signaling; Metabolic regulation; Acino-insular axis

Core Tip: This editorial emphasizes the critical role of α-amylase, an enzyme essential for starch digestion, in metabolic regulation beyond its digestive function. Recent studies, including that of Pierzynowska *et al*, demonstrate that amylase can inhibit insulin secretion, delaying glucose clearance and increasing blood sugar levels, with effects persisting even after the infusion. This suggests amylase's influence on pancreatic signaling and confirms the existence of the acini-islet-acinar reflex. Understanding the broader metabolic role of amylase may open therapeutic avenues for conditions like diabetes and obesity through enzyme supplementation, highlighting the need for further research into its regulatory mechanisms.

Citation: Deji-Oloruntoba O, Okpete UE, Byeon H. Editorial on amylase and the acini–islet–acinar reflex: A new frontier in metabolic health research. *World J Exp Med* 2025; 15(1): 101289
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/101289.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.101289

INTRODUCTION

The exocrine pancreas, with acinar cells comprising 85%-90% of its mass, produces essential digestive enzymes, primarily amylase (a glycolytic enzyme) and lipase (a lipolytic enzyme). These acinar cells are located near the endocrine islets of Langerhans, which secrete insulin, a hormone crucial for regulating blood glucose and overall metabolic health. An insulin deficiency is associated with pancreatic atrophy and exocrine pancreatic insufficiency, highlighting the interconnected roles of the exocrine and endocrine pancreas[1]. Historically, pancreatic endocrine and exocrine functions have been viewed as distinct; however, recent studies reveal a coordinated relationship between these functions, challenging the traditional view of their independence.

The study by Pierzynowska *et al*[2], published in the *World Journal of Experimental Medicine*, provides new insights with the proposed acini-islet-acinar (AIA) reflex, which connects pancreatic acinar cells to insulin-producing islet cells[2]. This reflex suggests that α-amylase may influence insulin secretion by altering pancreatic interstitial fluid dynamics, linking exocrine activity to endocrine response. Pigs were chosen as suitable models for studying insulin secretion and pancreatic enzyme dynamics due to anatomical and physiological similarities between their pancreas and that of humans, compared to rodents.

After a stabilization period, pigs underwent an intravenous glucose tolerance test with a glucose bolus injected into the jugular vein while simultaneously amylase solution was infused into the pancreatic artery in select pigs. This setup was designed to evaluate the hypothesized AIA reflex by examining how increased amylase in the pancreatic interstitial fluid affects insulin release. Blood samples were then collected at specific intervals to assess glucose and insulin levels over time, tracking the physiological response to glucose. Findings on the AIA reflex thus reveal a more complex interaction between the endocrine and exocrine pancreas, with implications for understanding glucose and lipid metabolism mechanisms, particularly concerning metabolic diseases such as type 2 diabetes and obesity. This study, by exploring the integrative endocrine (hormonal) and exocrine (digestive) roles of the pancreas, aims to advance our understanding of metabolic health, with the potential to enhance future diagnostics and treatments for metabolic diseases.

AMYLASE AND INSULIN RELEASE

α-Amylase is an enzyme that catalyzes the hydrolysis of internal α-1,4-glycosidic linkages in starch, breaking it down into glucose, maltose, and maltotriose. This process begins in the mouth, where salivary α-amylase initiates carbohydrate digestion. The feedback mechanism between amylase activity (carbohydrate digestion) and insulin release (glucose regulation) plays a crucial role in maintaining blood sugar balance. As amylase converts carbohydrates into glucose, rising blood glucose levels prompt insulin release to facilitate glucose uptake and storage, preventing significant fluctuations in blood sugar that could lead to hyperglycemia or hypoglycemia[3].

The AIA axis reflex integrates this feedback mechanism at the pancreatic level, linking acinar cells, which release digestive enzymes, with the islet cells, which release insulin. This cross-talk is vital for efficiently meeting metabolic demands. In cases of insulin resistance or diabetes, this feedback loop is disrupted, as impaired insulin release or action interferes with normal blood glucose management. Even as amylase continues carbohydrate breakdown, elevated glucose levels persist in the bloodstream due to insufficient insulin response, emphasizing the importance of balanced amylase activity and insulin response for metabolic health.

Interestingly, studies show a negative correlation between serum amylase levels and fasting blood glucose in diabetic populations (Table 1), with lower amylase levels associated with greater glucose dysregulation[1,4-6]. This supports the findings that individuals with higher salivary amylase activity often have lower postprandial glucose levels and better starch adaptation, suggesting that enzyme levels in the AIA axis might play a role in metabolic resilience and glucose homeostasis.

Recent research suggest that low amylase secretion may underlie blood sugar abnormalities[7,8]. Enzymatic supplementation with α-amylases could help prevent and treat these undesired physiological disorders. Purified combinations of pancreatic proenzymes/enzymes such as trypsinogen/trypsin, chymotrypsinogen/chymotrypsin, and amylase[9] have been shown to have strong antimetastatic and anticancer properties. Such proenzyme/enzyme combinations have

Table 1 Serum amylase levels and metabolic indicators in different health states[4-6]

Metabolic states/groups	Healthy	Pre-diabetic	Diabetic	Ref.
Amylase levels (IU/L)	25-125	40-80	30-60	Yadav *et al*[4]; Khan *et al*[5]
Fasting blood glucose (mg/dL)	70-99	100-125	126 and above	Khan *et al*[5]; American Diabetes Association Professional Practice Committee [6]
HbA1c (%)	< 5.7	5.7-6.4	6.5 and above	American Diabetes Association Professional Practice Committee[6]
Total cholesterol (mg/dL)	Below 200	180-200	200 and above	Yadav *et al*[4]; Khan *et al*[5]
Triglycerides (mg/dL)	100-150	150-200	200 and above	Yadav *et al*[4]; Khan *et al*[5]

HbA1c: Glycated hemoglobin.

been implicated in inhibiting tumor cell migration at the cellular level. These findings point to the potential health benefits of enzyme supplements and warrant ongoing research and clinical trials.

DISCUSSION

The modulation of pancreatic function is complex, involving neurological and hormonal signals. Acinar cells produce pancreatic enzymes, and insulin regulates exocrine secretion through the AIA reflex. Notably, a fast intravenous glucose infusion in one study significantly reduced amylase secretion, indicating a close interaction between glucose levels and enzyme release[10].

The AIA model posits that pancreatic acini and islets coordinate through biochemical signaling to maintain a balance between digestion and metabolism in response to food intake[11]. This interaction is mediated through paracrine and neuroendocrine pathways, enabling mutual regulation between acinar cells' amylase secretion and beta cells' insulin release[12].

Pierzynowska *et al*'s experiment on pigs investigated the possibility of an AIA reflex, specifically examining the reciprocal amylase and insulin interactions, to confirm both the presence of the reflex and the close anatomical integration of exocrine and endocrine pancreatic components[2]. The study found that exogenous amylase infusion into the pancreas delayed glucose-stimulated insulin secretion, suggesting a functional link between acinar enzyme activity and islet function. Pigs with compromised exocrine function showed delayed insulin responses, and enzyme supplementation improved glucose clearance. These findings imply that pancreatic enzymes might influence blood glucose utilization independently of insulin release[13].

Previous research indicates that amylase may limit insulin secretion by redirecting glucose from the bloodstream to the intestine[14]. In general, gut amylase reduces glucose absorption and insulin release. This shift in glucose utilization, potentially mediated by enterocytes before glucose reaches the bloodstream, could represent an insulin-independent glucose regulation mechanism[10].

In Pierzynowska *et al*'s study, insulin levels remained unexpectedly low under glucose loading, with glucose peaking at 400 mg/dL within 15 minutes[2]. Remarkably, amylase's suppressive effect on insulin secretion persisted for 30 minutes post-infusion, possibly due to altered pancreatic interstitial signaling or slowed beta-cell recovery. In insulin-resistant conditions, AIA reflex feedback may be impaired, allowing glucose to accumulate in the bloodstream, potentially exacerbating hyperglycemia. This chronic hyperglycemia may further strain beta cells, disrupting their role in modulating acinar cell responses during digestion. These insights also point to a complex regulatory network influenced by other metabolic pathways.

Emerging evidence suggests that changes in amylase levels, either due to exogenous or endogenous factors, may affect the gut microbiome, which, in turn, could influence pancreatic insulin release. High amylase levels may deplete beneficial short-chain fatty acid-producing bacteria, disrupting insulin signaling and delaying glucose-stimulated insulin release[7]. Increasing evidence also highlights the microbiome's influence on the development of metabolic disorders, underlined by correlations between the salivary and urinary metabolome and pediatric obesity[14].

Removing pancreatic enzymes from pigs' digestive systems significantly reduced glucose absorption after oral glucose loading, supporting the idea that active salivary amylase in the alimentary canal enhances glucose uptake. Low serum amylase levels are associated with a higher risk of metabolic syndrome, and there is a noted negative correlation between salivary amylase levels and obesity. Genetic studies reveal individuals with high amylase gene copy numbers produce more salivary amylase, which can increase glucose absorption and potentially predispose them to fat accumulation. Interestingly, however, high amylase gene copies have also been linked to a lower risk of obesity, suggesting an inconclusive, gene-dependent relationship with metabolic health[15].

FUTURE DIRECTIONS AND RECOMMENDATIONS

Hyperglycemia has been associated with several pathological conditions like diabetes and obesity. Adequate insulin signaling is essential to counteract these effects. The inhibition of insulin signaling by amylase presents a critical research question that demands an urgent answer to combat metabolic diseases. Further studies are needed to completely understand how amylase affects the broader metabolic network and pancreatic function, possibly *via* gut microbiome interactions. This could provide better insights into the regulation of insulin secretion and its consequences for metabolic health.

Recent developments, including advanced three dimensional imaging of pancreatic innervation, have provided deeper insight into the AIA reflex's anatomy and function. This imaging has demonstrated the intricate innervation pathways that link acinar and islet cells, highlighting their integrated roles in metabolic health and revealing the dysregulated neural signaling often found in metabolic diseases[16]. Additionally, therapies targeting the AIA reflex offer therapeutic strategies for managing blood glucose levels, with α-amylase as a potential modulator. Understanding and potentially manipulating the AIA reflex represents a promising frontier for treating metabolic diseases.

CONCLUSION

The study by Pierzynowska *et al*[2] sheds new light on the integrative functions of the pancreas through the AIA reflex, emphasizing the significant role of amylase in metabolic regulation. The findings suggest that targeted enzyme supplementation could be a promising strategy to enhance metabolic health and mitigate conditions like diabetes and obesity. Beyond digestion, amylase emerges as a potential metabolic biomarker, with reduced levels indicating early dysfunction. By exploring the AIA reflex further, we may advance our understanding of enzyme-based therapeutic interventions, providing novel approaches for early diagnosis and effective management of metabolic diseases.

FOOTNOTES

Author contributions: Deji-Oloruntoba O, Okpete UE and Byeon H contributed to this paper; Byeon H designed the study; Deji-Oloruntoba O, Okpete UE involved in data interpretation and developed methodology; Deji-Oloruntoba O, Okpete UE and Byeon H assisted with writing the article.

Supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, No. NRF-RS-2023-00237287, No. NRF-2021S1A5A8062526; and Local Government-University Cooperation-Based Regional Innovation Projects, No. 2021RIS-003.

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

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S-Editor: Fan M
L-Editor: A
P-Editor: Zhang L

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World Journal of Experimental Medicine

Submit a Manuscript: https://www.f6publishing.com

World J Exp Med 2025 March 20; 15(1): 97404

DOI: 10.5493/wjem.v15.i1.97404

ISSN 2220-315x (online)

REVIEW

Genetic factors that predict response and failure of biologic therapy in inflammatory bowel disease

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Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification
Scientific Quality: Grade C
Novelty: Grade C
Creativity or Innovation: Grade C
Scientific Significance: Grade C

P-Reviewer: Jameel F

Received: May 30, 2024
Revised: October 9, 2024
Accepted: November 14, 2024
Published online: March 20, 2025
Processing time: 209 Days and 23.2 Hours

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Abstract

Inflammatory bowel disease (IBD) represents a significant disease burden marked by chronic inflammation and complications that adversely affect patients' quality of life. Effective diagnostic strategies involve clinical assessments, endoscopic evaluations, imaging studies, and biomarker testing, where early diagnosis is essential for effective management and prevention of long-term complications, highlighting the need for continual advancements in diagnostic methods. The intricate interplay between genetic factors and the outcomes of biological therapy is of critical importance. Unraveling the genetic determinants that influence responses and failures to biological therapy holds significant promise for optimizing treatment strategies for patients with IBD on biologics. Through an in-depth examination of current literature, this review article synthesizes critical genetic markers associated with therapeutic efficacy and resistance in IBD. Understanding these genetic actors paves the way for personalized approaches, informing clinicians on predicting, tailoring, and enhancing the effectiveness of biological therapies for improved outcomes in patients with IBD.

Key Words: Inflammatory bowel disease; Genetic predictors; Inflammatory bowel disease treatment; Biologic therapy; Biologic therapy response; Genetic markers in inflammatory bowel disease; Inflammatory bowel disease treatment failure; Pharmacogenomics; Biologic therapy efficacy; Genetic variability

Core Tip: Understanding the genetic factors that influence the response and failure of biological therapy in inflammatory bowel disease (IBD) is crucial for optimizing treatment strategies. Identifying specific genetic markers can help predict patient outcomes, tailor personalized therapies, and improve efficacy while minimizing adverse effects. This approach enhances clinical decision-making, leading to better management of IBD and improved patient quality of life. Future research should focus on expanding genetic profiling to refine therapeutic interventions.

Citation: Peruhova M, Stoyanova D, Miteva DG, Kitanova M, Mirchev MB, Velikova T. Genetic factors that predict response and failure of biologic therapy in inflammatory bowel disease. *World J Exp Med* 2025; 15(1): 97404
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/97404.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.97404

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition that comprises two entities: ulcerative colitis (UC) and Crohn's disease (CD). The inflammation in UC continuously affects the colonic mucosa, with no granulomas detected on biopsy[1]. On the other hand, CD is characterized by transmural inflammation and granulomas that can affect any part of the gastrointestinal tract, most commonly the terminal ileum[2]. IBD is considered one of the most frequently diagnosed gastrointestinal diseases, with its incidence and prevalence constantly rising since the second half of the 20th century. This is true for both Europe and North America, as well as the newly industrialized countries of Asia, Africa, and South America. The highest incidence of 505 UC cases and 322 CD cases per 100000 persons has been reported in Norway and Germany, respectively[2]. IBD undoubtedly impairs quality of life, with fatigue, lack of energy, and sleep disturbances being the most common complaints. This is predominantly encountered in women, in patients suffering from CD, and in materially deprived persons[3]. IBD poses a significant disease burden characterized by chronic inflammation, pain, and complications that can severely impact patients' quality of life. Effective diagnostic strategies for IBD include clinical assessments, endoscopic evaluations, imaging studies, and biomarker testing to identify and differentiate the disease accurately. Early diagnosis is crucial for managing IBD effectively and preventing long-term complications, emphasizing the need for ongoing advancements in diagnostic approaches[3].

The treatment of IBD includes conventional therapy with 5-American Society of Anesthesiologists, corticosteroids and non-targeted immunosuppressants, and biological therapy. The biological rationale for using biologics in IBD is based on the known aspects of the disease pathophysiology. In patients with IBD, dysregulation of the immune response leads to the infiltration and accumulation of immune cells, which stimulate the release of various cytokines, chemokines, and growth factors[2]. This cascade may further impact the inflammation and carcinogenesis processes. Immune cells such as regulatory T cells (Tregs), type 2 macrophages, CD4+ T helper 17 cells, CD8+ T cells, and natural killer cells can play roles in either sustaining inflammation in IBD or contributing to disease progression[2].

Traditional gold standard methods for diagnosing IBD, such as endoscopy and histological examination, provide critical insights into mucosal inflammation and tissue morphology but can be invasive and uncomfortable for patients. By contrast, advanced techniques such as capsule endoscopy and biomarkers (*e.g.*, fecal, serum, genetic) offer non-invasive alternatives that enhance patient comfort and convenience[4]. While capsule endoscopy allows visualization of the entire small intestine, fecal calprotectin (FC) testing enables the quick assessment of inflammation levels. Each approach has its advantages and limitations, and the choice of diagnostic method should be tailored to individual patient needs and clinical scenarios to ensure accurate and effective diagnosis[4,5].

Conventional therapy of IBD can induce a clinical response and maintain remission mainly in mild to moderate forms [4,5]. However, a recent meta-analysis showed a modest effect in terms of both induction and maintenance of remission in moderate to severe IBD[6]. Hence, biological therapy emerged as a new class of drugs with the potential to influence treatment failure with conventional therapy. The initial drugs, infliximab and adalimumab, showed excellent clinical and endoscopic efficacy. Still, the subsequent follow-up of patients revealed up to 30% response failure, 50% loss of response over time, and 10% surgical treatment requirement[7].

The new drugs available on the market also show incomplete responses. Vedolizumab, an anti-integrin antibody, achieved endoscopic improvement and remission in 51% and 29% of patients with UC in week 52, respectively. In CD, the same treatment goals were observed in 76% and 48% of cases, respectively[8]. For ustekinumab, an anti-interleukin (IL) 12/23 antibody, clinical response and remission at 1 year were seen in 76.8% and 50.6% of patients with UC, respectively[9]. For CD, the percentage of patients in clinical remission in week 44 was approximately 50%[10]. The non-selective Janus kinase (JAK) inhibitor, tofacitinib, which has only been approved for the treatment of UC, achieved clinical remission in 40.6% of cases in week 52[11]. In comparison, the clinical and endoscopic remission rates of the selective JAK1 inhibitor upadacitinib were 33% and 15% for UC *vs* 41% and 24% for CD, respectively[12]. In keeping with those mentioned above, there seems to be wide interindividual variation in the efficacy of biological treatment, which can be genetically determined. A recent systematic review published in 2024 by Plaza *et al*[13] showed that single nucleotide polymorphisms (SNPs) may be associated with a different treatment response towards anti-tumor necrosis factor (TNF),

anti-integrins, and anti-IL-12/23 inhibitors. Therefore, a more individualized approach is needed for every patient with IBD.

In this review article, we identify and analyze genetic factors that predict patient response and failure to biologic therapy in IBD, facilitating personalized treatment strategies and improving clinical outcomes for the patients.

SEARCH STRATEGY

A comprehensive literature search was conducted across multiple databases, including PubMed, MEDLINE, Scopus, Web of Science, and Google Scholar, covering the period to May 2024. The search terms used were combinations of key words and Boolean operators: "Inflammatory Bowel Disease" AND ("Genetic Predictors" OR "Genetic Markers") AND ("Biologic Therapy" OR "Biologic Therapy Response" OR "Biologic Therapy Failure") AND ("Pharmacogenomics" OR "Genetic Variability") AND "IBD Treatment". Approximately 500 papers were retrieved, and relevant articles were selected based on their relevance to the topic, focusing on studies that explored the genetic factors influencing the efficacy and failure of biological therapies in IBD.

GENETIC FACTORS INFLUENCING RESPONSE TO BIOLOGICAL THERAPY IN IBD

Role of genetic variations in drug metabolism

Cytochrome P450 (CYP) enzymes are involved in drug metabolism, and genetic polymorphisms in CYP2C19 significantly influence drug metabolism. Variations can result in different enzyme activity levels, categorizing individuals into poor, intermediate, extensive, and ultra-rapid metabolizers. This impacts drug efficacy and safety. For example, poor metabolizers may have higher drug levels, increasing the effectiveness or risk of toxicity for medications metabolized by CYP2C19[14]. Variations in CYP3A4 also affect the metabolism of many drugs used in IBD, contributing to variability in treatment outcomes[15].

Regarding the impact on drug levels and efficacy, thiopurine S-methyltransferase (TPMT) polymorphisms influence the metabolism of thiopurines. Low TPMT activity leads to higher active metabolite levels, increasing efficacy but also the risk of toxicity. TPMT genotyping helps tailor dosing to improve outcomes and reduce side effects[16]. Similarly, N-acetyltransferase 2 polymorphisms can affect the metabolism of certain IBD drugs, impacting their levels and effectiveness[17].

Pharmacogenetics of drug receptors and targets

The relevance of genetic variations in drug targets is described for several genes. Polymorphisms in TNF receptors (TNF receptor superfamily member 1A [TNFRSF1A] and TNFRSF1B) can affect the binding and efficacy of anti-TNF therapies such as infliximab and adalimumab. Specific polymorphisms are associated with better or worse responses to these treatments[18].

Variations in the IL-23 receptor (IL-23R) gene influence responses to biologics targeting the IL-23 pathway, such as ustekinumab. Specific IL-23R genotypes are linked to improved treatment responses[19].

When we discuss the implications for treatment outcomes, we should focus on the human leukocyte antigen (HLA)-DQA1*05 allele associated with developing anti-drug antibodies (ADA) against infliximab and adalimumab, reducing their efficacy. Patients with this allele may need closer monitoring and therapy adjustment[20]. Variants in Fc gamma receptor 3A (FCGR3A) can also affect the response to anti-TNF agents by altering drug binding to immune cells, impacting clinical outcomes[18].

GENETIC MARKERS ASSOCIATED WITH BIOLOGICAL THERAPY RESISTANCE IN IBD

To date, more than 240 non-overlapping genetic loci have been identified as significant risk factors of IBD[21-23]. Among them statistically significant genes include autophagy-related 16-like 1 (*ATG16L1*), E-cadherin, *HLA*, hepatocyte nuclear factor 4 alpha, *IL-10*, *IL-10RA*, *IL-10RB*, *IL-23R*, leucine-rich repeat kinase 2, nucleotide oligomerization domain 2 (*NOD2*), protein tyrosine phosphatase non-receptor type 2, TNF superfamily member 15, immunity-related GTPase family M, caspase recruitment domain protein 9, and RING finger protein 186, which are linked to innate and adaptive immunity, autophagy, epithelial barrier, innate mucosal defense, Tregs, oxidative stress, IL-10 and IL-23 signaling, and cell apoptosis, among others[22-24].

Identification of genetic variants linked to treatment resistance

First, we start by describing the genetic mutations in drug targets. A critical point in IBD treatment is identifying genetic variants associated with an individual's drug response. Mutations in genes encoding drug targets can significantly impact drug efficacy and contribute to treatment resistance. IBD-causing alleles are rich in non-synonymous mutations in their coding region, modulating the protein structure and function and thus affecting drug binding affinity or downstream signaling pathways. Furthermore, 80%-90% of IDB loci are non-synonymous variants due to mutations in their non-coding regions exerting pathogenic effects by modulating the gene expression[25].

Extensive meta-analyses combined with genome-wide association studies (GWAS) have identified specific variants and polymorphisms associated not only with the onset and severity of IBD but also with a role in treatment response in patients undergoing drug therapy[26]. The clinical trials and real-life practice demonstrate the association of some genetic variants with no or limited response (primary non-response or secondary loss of response) to drug treatment[21,26,27] and, in some cases, even worsen it[28].

Altered pathways leading to reduced drug efficacy

Genetic variants leading to altered target structure or gene expression are not the only cause of reduced drug effectiveness, as additional causes include drug interactions, development of resistance, and drug quality. For example, changes in physiological conditions, such as the potential of hydrogen and blood flow, can influence drug distribution and metabolism, affecting drug efficacy. The use of multiple drugs can lead to the inhibition or induction of drug-metabolizing enzymes, competition for binding sites, or synergistic or antagonistic effects on drug targets[29].

INFLUENCE OF GENETIC POLYMORPHISMS ON IMMUNOGENICITY OF BIOLOGICAL THERAPY IN IBD

Some biological drugs can induce immune reactions, leading to the formation of antibodies that neutralize the therapeutic effects of the drug. Immunogenicity with the formation of ADA to biological products is one of the causes of treatment failure in IBDs. The drug concentration, inadequate drug exposure, and high drug clearance can also be responsible for undesirable therapeutic outcomes in patients with IBD. Other factors besides immunogenicity can accelerate the clearance of biologics such as increased body weight, low serum albumin, and even disease status and medications[30]. Biologics have long been used to treat IBD, but guidelines regarding their optimal use are still being researched and developed. Over the past few decades, IBD-related costs have significantly increased due to the frequent administration of TNF-α antagonists and other biological products for treatment[31]. This gives reason to assume that the optimal use of these products is essential to improve the efficacy of therapy and reduce adverse effects.

Various strategies to prevent ADA formation have been investigated. Combining a biological product with an immunomodulator was found to preclude the formation of ADA[32,33]. ADAs decrease by adding or changing immunomodulators[34,35]. It has also been shown that fewer ADAs are detected at higher anti-TNF dosing[36].

The TNF-α antagonists infliximab, adalimumab, golimumab, and certolizumab pegol are used as anti-TNF therapies in the clinical setting of IBD[37,38]. They have different pharmacological profiles and efficacy and can improve remission [39]. However, some patients with IBD either do not respond or have loss of response to treatment over time. Genetic factors are responsible for this inability. Genetic profiling techniques and GWAS have enabled the identification of genetic variants that can influence the treatment response and development of adverse effects[24].

A study revealed genetic associations with primary non-response[40]. The authors found that SNPs in loci DENN domain containing 1B (rs2488397) and aryl hydrocarbon receptor (rs1077773) are most strongly associated with primary non-response. Similarly, they observed genetic associations with time to loss of response. In addition to a number of known IBD susceptibility loci, SNPs in PR domain zinc finger protein 1 (rs62421049), chromosome 21q22.2 (rs2836866), cluster of differentiation 28 (rs3116494), *SMAD3* (rs17293632), and interferon (IFN) induced with helicase C domain 1 (rs1990760) were associated[40].

Usually, SNPs with long-term responses are associated with responsiveness to infliximab or adalimumab therapy in patients with IBD. However, some SNPs such as rs396991-GG (*FCGR3A*), rs6100556-TT (phosphatase and actin regulator 3 [*PHACTR3*]), rs2241880-AA, rs10210302-CC, and rs2241880-GG (*ATG16L1*) have shown a reduced clinical response at the end of treatment in pediatric patients with IBD (pIDB)[27,41].

In some cases, the presence of certain variants in the genotype of patients with IDB may worsen the drug treatment. In their research, Zapata-Cobo *et al*[28] demonstrated that specific SNPs such as rs6908425 (cyclin-dependent kinase 5 regulatory subunit-associated protein 1-like 1), rs2241880 (*ATG1L1*), rs2188962 (IFN regulatory factor 1 antisense RNA 1), and rs6100556 (*PHACTR3*) were associated with long-term worse response to anti-TNF drugs in children with IBD.

SNPs with short-term responses do not show a response to drug treatment. For example, rs976881-AA+GA (*TNFRSF-1B*), which is related to the TNF-α pathway, and rs1813443-CC and rs1568885-TT (contactin 5) from the immunoglobulin superfamily are associated with non-response to infliximab, and rs4645983-GG (caspase-9 [*CASP9*]) is associated with non-response to adalimumab[27]. Studies on the association between some variants and drug treatment response have shown controversial outcomes, probably due to population or age differences or insufficient analyses. For example, the SNP rs1061624-AA+GA in *TNFRSF1B* in Spanish patients with CD is related to beneficial long-term response to infliximab, whereas in Italian patients, it is linked to a short-term non-response[27].

BIOMARKERS FOR PREDICTING THE BIOLOGICAL THERAPY RESPONSE

Overview of biomarkers in IBD

The International Organization for the Study of IBD STRIDE-II recommendations from 2021 confirmed that the most critical long-term achievable treatment targets for patients with IBD are clinical remission, endoscopic healing, restoration of quality of life, and absence of disability. With accumulating clinical evidence, serum and fecal biomarkers have been validated as intermediate- or medium-term feasible treatment goals, meaning that at times, treatment could be revisited solely based on these tests to facilitate care in the clinical setting[42].

Based on their low cost and availability, FC and C-reactive protein (CRP) are the two most widely used classical biomarkers in IBD. A meta-analysis that summarized the performance of FC when using all available data, whatever the cutoff values used, showed a pooled sensitivity of 82%, specificity of 72%, and area under the curve of 0.84 for FC in reflecting endoscopic disease activity in CD[43]. The evidence suggests that a reduction in FC and target below a certain threshold have clear prognostic significance, justifying the utilization of this biomarker as a treatment target. FC predicted long-term clinical outcomes when measured 12 weeks after initiating medical treatment[44]. A meta-analysis of six studies showed that patients with elevated FC had a 53% to 83% probability of relapse during the subsequent 2 months to 3 months[45]. Different studies that confirmed FC as a predictor of relapse at the time of anti-TNF discontinuation, predicted subsequent relapse at cutoff values of 50 mg/g to 150 mg/g[46].

Whereas FC has high sensitivity and lower specificity in identifying mucosal inflammation, CRP has the opposite characteristics of higher specificity but low sensitivity[47]. Consequently, high CRP values determined at the time of anti-TNF discontinuation are associated with a higher risk of relapse[48]. CRP normalization at 8 weeks to 14 weeks after treatment predicts remission at 1 year with vedolizumab[49] and anti-TNF success at 2 years[50]. Similarly, CRP > 5 mg/dL in week 22 has been shown to predict secondary loss of response to anti-TNF[51].

Although newer biomarkers are emerging and being tested in practice, none have been successfully validated or proved reliable as a sole predictive tool in personalized medicine in IBD.

Genetic biomarkers for predicting response

Identifying predictive genetic markers is the first step in predicting the response to IBD biological treatment. Although genetic studies are not universally applicable in the clinical field, they can be used to diagnose IBD, predict therapeutic or toxic responses to drugs, and assess the risk, thereby enabling precision medicine for patients. Precisely understanding the underlying immunopathogenic mechanisms of IBD will lead to the development of targeted therapies. Effective and careful consideration of underlying factors, including immunogenicity potential, treatment safety profile, and optimal therapeutic duration in these patients, is needed.

Over the past decade, a range of predictive biomarkers has been identified that promise to provide personalized and effective treatments for patients[52]. There are also some limitations and clinical applications of these biomarkers in monitoring optimized patient outcomes and providing personalized care. Some of the predictive genetic markers are associated with predicting the response to biological treatment in patients with IBD. A favorable clinical response may be associated with polymorphisms in genes such as *FCGR3A*, *TNFRSF1A*, *IL-6*, and *IL-1B*; conversely, variants of Toll-like receptor 2 (*TLR2*) and *TLR9* show a negative correlation[53].

Some genetic variants in *TNFRSF1B* and nuclear factor kappa B (*NF-kB*) genes can affect TNF-α production or the binding of TNF-α blockers to the TNF-α receptor. These, in turn, may influence the primary response to anti-TNF therapy in patients with CD[54] or UC[55].

Polymorphisms in the IL-23R are associated with response in patients with UC, and a polymorphism in the *NOD2/CARD15* gene is associated with patients with CD[56]. These data[54] and the genetic variants described above are related to predicting positive, negative, or no response to biological treatment in patients with IBD[55-59].

It is also mandatory to integrate clinical and other biomarkers into clinical practice. Protein markers can provide valuable information for monitoring and therapeutic responses to anti-TNF therapy[60]. Markers such as CRP, human angiopoietin 1 (ANG1), ANG2, carcinoembryonic antigen-related cell adhesion molecule 1, extracellular matrix metalloproteinase inducer, transforming growth factor alpha, matrix metalloproteinases 1-3 (MMP 1-3), MMP-9, IL-6, and some apolipoproteins have been identified as predictive[61]. It should be noted that proteomics has great potential, but various factors influence protein levels and are individual in patients. Therefore, protein markers alone are not sufficient to be universal markers of therapeutic response in IBD patients.

FC, lactoferrin, and other fecal biomarkers can also be used as potential markers in patients with CD and UC on anti-TNF therapy[62,63]; however, the results of the studies are contradictory. In some cases, high calprotectin levels correlated with better treatment response[64], others inversely[65] or failed to confirm the data[66,67].

The correlation between the gut microbiome and anti-TNF therapy is complex. Still, there is evidence that some microbial markers may be associated with treatment response[68]. Patients with a more diverse gut microbiome respond better to anti-TNF-α therapy, whereas the presence of other species is associated with a negative response[69,70]. In dysbiosis, there is often no or poor response to anti-TNF therapy[71,72]. A recent study by Caenepeel *et al*[73] investigated different combinations of clinical and microbial data to predict the efficacy of TNF-α treatment. The authors examined certain clinical parameters and microbial dysbiosis, achieving a 73.9% accuracy rate in predicting treatment responses.

Fungi and viruses are also being studied for their correlation with responses to therapy[74-76]. The diversity of these different populations cannot be completely ruled out as misleading in clinical practice, as their amount and types also depend on different factors. Recently, microRNAs (miRNAs) have also been considered potential biomarkers for therapeutic responses in patients with IBD[77].

One study found significant changes in the expression of several miRNAs after anti-TNF treatment in patients with pIDB[78], but another study did not confirm the correlations[79]. Additional studies on miRNAs as possible predictive markers in patients with IBD are needed. Changes in blood or mucosal parameters can also be assessed for anti-TNF therapy's effectiveness. If there is a reduction in TNF-α and IFN-g levels and reduced inflammation at the mucosal level, then anti-TNF therapy is effective[65,80]. Some cytokines have also shown potential as candidate biomarkers in patients with IBD[81].

Many identified biomarkers indicate inflammation and are not specific to IBD alone. Various factors such as age, sex, genetics, biochemical profile, microbial composition, and mucosal conditions influence the therapy response, which may explain why none of these biomarkers have been included in routine clinical practice. For this to happen, future efforts

should focus on the robust validation of certain biomarkers in large numbers of patients with IBD.

CLINICAL APPLICATIONS OF GENETIC PREDICTORS

Personalized medicine approaches in IBD treatment

It is a well-established fact that one-third of patients with IBD are primary non-responders to inceptive treatment despite new targeted therapies that have been available recently in clinical practice. Another troubling fact is that half of patients on therapy lose treatment response with time[82]. Presently, there is an insufficient number of biomarkers that can be useful in predicting treatment failure. In clinical practice, validating equitable biomarkers, which can predict treatment response or failure, would greatly help clinicians tailor personalized therapeutic algorithms for managing patients with IBD.

Personalized medicine is the idea that the appropriate medication may be given to the relevant patient at the proper time. This process could only be possible with precise knowledge of the underlying molecular processes causing IBD. Better rates of patient outcomes, reduction of morbidity due to improper treatment, and decline in healthcare costs would all be made possible by such an approach. Creating a personalized medicine approach in IBD is connected with identifying, developing, and validating novel biomarkers to support individualized treatment[83].

Tailoring therapy based on genetic profiles

A study by Park and Jeen[22] established 240 susceptibility loci for IBD. Understanding the role of these genes in IBD pathogenesis will help to identify novel therapeutic targets. Recently, a plethora of data on the connection between genetic markers and therapeutic response has been published. For example, a study by Jürgens *et al*[56] demonstrated that therapeutic responses to infliximab were detected in adult patients with CD who were homozygous for the high-risk IL-23R variant compared to low-risk IL-23R variants. Unfortunately, a low percentage of patients have these IL-23R variants; thus, using this marker in clinical practice is unreliable[56].

The *CASP9* gene regulates activation of the caspase cascade and the process of cell apoptosis. Thus, polymorphism in *CASP9* could affect the process of apoptosis in peripheral blood lymphocytes in patients with IBD. It has been established that polymorphism in the *CASP9* gene (rs4645983) is related to short-term non-response to adalimumab[84].

Some data have shown a better response to infliximab in patients with CD with polymorphisms in the *CASP9* gene and FAS ligand gene[85]. According to some GWAS, there has been conflicting evidence about the relationship between treatment response and polymorphisms in TNF-encoding genes. Two polymorphisms in the *TNF* promoter were linked to the responsiveness of patients with IBD to TNF inhibition, according to a 2013 meta-analysis: More often occurring alleles were linked to higher response rates[86].

Better clinical responses have been found to be positively correlated with polymorphisms in the *FCGR3A*, *TLR4*, *TNFRSF1A*, *IFN-g*, *IL-6*, and *IL-1B* genes, whereas variants of *TLR2* and *TLR9* have shown a negative correlation[87]. In individuals with IBD receiving anti-TNF medication, polymorphisms in TNF, NF-kB, and other cytokine pathways have been connected to better outcomes. For instance, a study by Bank *et al*[88] demonstrated that in patients with IBD receiving anti-TNF therapy, polymorphisms in TNF, NF-kB, and other cytokine pathways were correlated with a better response to treatment.

A study by Koder *et al*[84] investigated SNPs in genes that regulate the cell division cycle (cyclin Y; rs12777960 CC), chromatin organization (chromosome 11 open reading frame 30; rs7927894 CC), and synthesis of some proinflammatory cytokines (*IL-13*; rs1295686 TT). The authors established that these SNPs are related to long-term response to adalimumab [84]. Furthermore, in patients with CD receiving anti-TNF therapy, the HLA-DQA1*05 allele, HLA-DRB1 allele, and polymorphisms at the *FCGR3A* locus (encoding immunoglobulin G Fc receptor IIIa) have been linked to a higher risk of ADA production[89-91]. Monoclonal antibodies represent large, complex proteins; they can lead to the synthesis of ADA, which are linked to therapy inefficacy. One such chimeric antibody is infliximab[92]. Finding patients at high risk of developing ADA would be very beneficial since concurrent immunosuppression (with thiopurines and methotrexate) lowers the likelihood of developing them[93].

IL-13R alpha 2 is another marker that has been previously discovered by gene array investigations in the mucosal biopsies of patients with IBD[94]. The biomarker, assessed as mRNA expression in the mucosa of patients with IBD before therapy, was recently found to be particularly predictive of the absence of response to anti-TNF in terms of mucosal healing at 6 months. The area under the curve for infliximab and adalimumab was 0.90 and 0.94, respectively, with $P < 0.001$[95].

The *NOD2* gene, which encodes a protein involved in inducing the immune response and connected to the TNF-inflammatory pathway, is linked to both a more aggressive course of the disease and susceptibility to CD[96,97]. According to particular research, *NOD2* mutations are linked to a poorer response to anti-TNF therapy[98-100]. In patients with CD receiving TNF antagonist treatment, polymorphisms in the *ATG16L1* gene have been linked to improved response rates and prolonged benefits[84]. In actual therapeutic practice, the genetic variants that confer vulnerability to ADA development could be quite helpful in identifying individuals who could benefit from biological therapy. Few studies revealed certain genetic polymorphisms[101-105] and gene variants[106-110] associated with various responses to biological therapy in IBD.

An overview of the currently known genetic factors that influence the response to biological therapy for patients with IBD is presented in Table 1[28,41,53,56,58,84,86-91,98-110].

Table 1 Genetic factors that influence the response to biological therapy for patients with inflammatory bowel disease

Genetic markers	Patients	Clinical consequences	Ref.
Polymorphisms in TLR2, rs11938228, TLR4, TLR9, TNFRSF1A, IFNG, IL-6, and IL-1B (rs4848306, NOD-like receptor thermal protein domain associated protein 3, Janus kinase 2)	IBD	Clinical response in to anti-TNF	Bek *et al*[53], 2016; Salvador-Martín *et al*[87], 2019; Steenholdt *et al*[101], 2012; Medrano *et al*[102], 2013; Bank *et al*[88], 2014
Polymorphisms in TNF-α promoter (-308 A/G and -857 C/T)	IBD and spondylarthritis	Clinical response to anti-TNF	Tong *et al*[86], 2013; Song *et al* [103], 2015
Polymorphisms implicated in NF-kB pathway: TLR2, TLR4, TLR9, LY96 (MD-2), CD14, mitogen-activated protein kinase 14 (NIK), TNF-α, TNFRSF1A, TNFAIP3 (A20), IL-1B, IL-1RN, IL-6, IL-17A, IFNG	IBD	Clinical response to anti-TNF (adalimumab)	Bank *et al*[88], 2014; Song *et al* [103], 2015; Bek *et al*[53], 2016
Polymorphisms in IL-23R	UC	Early response to infliximab	Jürgens *et al*[56], 2010
HLA-DQA1*05	CD	Development of ADA against infliximab and adalimumab	Sazonovs *et al*[89], 2020; Salvador-Martín *et al*[87], 2023
HLA-DRB1	IBD	Development of ADA against infliximab	Billiet *et al*[90], 2015
Polymorphisms in FcγRIIIa	CD	Development of ADA against infliximab	Louis *et al*[91], 2004
Polymorphisms in NOD2	CD	Clinical response to anti-TNF	Niess *et al*[98], 2012
Polymorphisms in NOD2	CD	Loss of response to anti-TNF	Juanola *et al*[99], 2015
Polymorphisms in NOD2	CD	Lower anti-TNF trough levels	Schäffler *et al*[100], 2018
Polymorphisms in ATG16L1 (C11orf30; rs7927894 CC, CCNY; rs12777960 CC) (rs10210302)	IBD and CD	Clinical response to adalimumab	Koder *et al*[84], 2015; Zapata-Cobo *et al*[28], 2023
Polymorphisms in FAS, FASL, and CASP9 (apoptotic pharmacogenetic index)	CD	Clinical response to infliximab and adalimumab	Hlavaty *et al*[104], 2007; Koder *et al* [84], 2015
Multiple polymorphisms (combined clinical-genetic model)	CD and UC	Short-term and long-term to clinical response anti-TNF	Barber *et al*[58], 2016; Burke *et al* [105], 2018
Polymorphisms in TNFSF4/18, perilipin 2, rs762787, rs9572250, rs144256942, rs523781	IBD	Clinical response to anti-TNF	Wang *et al*[106], 2019
PHACTR3 (rs6100556)	UC and CD	Response to anti-TNF in children	Zapata-Cobo *et al*[28], 2023
CNTN5	CD		Thomas *et al*[107], 2014
FCGR3A	CD and UC	Antibody-dependent immune responses	Curci *et al*[41], 2021
PTGER4 (rs10512734)	CD	Response to adalimumab	Koder *et al*[84], 2015
IL-27	CD	Response to adalimumab	Koder *et al*[84], 2015
NR12	CD	Response to adalimumab	Koder *et al*[84], 2015
FASL (rs763110)	CD	Clinical response in patients with CD treated with infliximab	Zapata-Cobo *et al*[28], 2023; Steenholdt *et al*[101], 2012
IRF1-AS1	UC	Response to anti-TNF in children	Zapata-Cobo *et al*[28], 2023
Polymorphisms in IL-1B	IBD	Response to anti-TNF-α	Bank *et al*[88], 2014
Polymorphisms in IL-18	IBD	Response to anti-TNF-α	Bek *et al*[53], 2016
TLR2 (rs1816702 CC and rs3804099 TT)		Clinical response to infliximab	Salvador-Martín *et al*[87], 2019
Polymorphisms in CXCL12	IBD	Response to anti-TNF-α in children	Zapata-Cobo *et al*[28], 2023
Polymorphisms in IL-10	IBD	Response to anti-TNF-α in children	Salvador-Martín *et al*[108], 2020; Salvador-Martín *et al*[109], 2023
Polymorphisms in IL-17	IBD	Response to anti-TNF-α in children	Salvador-Martín *et al*[108], 2020; Salvador-Martín *et al*[109], 2023; Bank *et al*[88], 2014
Polymorphisms in IL-6	IBD	Response to anti-TNF-α in children	Salvador-Martín *et al*[108], 2020

Gene protein tyrosine phosphatase non-receptor type 2 (rs7234029 AG + GG, CASP9)	IBD	Non-response to anti-TNF and ustekinumab	Hoffmann *et al*[110], 2021

ADA: Anti-drug antibodies; ATG16L1: Autophagy-related 16-like 1; CASP9: Caspase-9; CCNY: Cyclin Y; CD: Crohn's disease; CD14: Cluster of differentiation 14; CNTN5; Contactin 5; CXCL12: C-X-C motif chemokine ligand 12; FASL: Fas ligand; FCGR3A: Fc gamma receptor 3A: FcγRIIIa: Immunoglobulin G Fc receptor IIIa; HLA: Human leukocyte antigen; IBD: Inflammatory bowel disease; IFNG: Interferon gamma; IL: Interleukin; IL-1RN: Interleukin 1 receptor antagonist; IRF1-AS1; Interferon regulatory factor 1 antisense RNA 1; LY96: Lymphocyte antigen 96; NF-kB: Nuclear factor kappa B; NOD: Nucleotide-binding oligomerization domain-containing protein; NR12: Hemopoietin receptor gene; PHACTR3: Phosphatase and actin regulator 3; PTGER4: Prostaglandin E receptor 4; TLR: Toll-like receptor; TNF: Tumor necrosis factor; TNFAIP3: Tumor necrosis factor alpha-induced protein 3; TNFR: Tumor necrosis factor receptor; TNFRSF1A: TNF receptor superfamily member 1A; UC: Ulcerative colitis.

FUTURE DIRECTIONS IN GENETIC RESEARCH FOR IBD THERAPY

Genomics and precision medicine advancements are revolutionizing the approach to treating IBD. Integrating multi-omics data, including genomics, transcriptomics, proteomics, and metabolomics, allows for a comprehensive understanding of the complex genetic and molecular mechanisms underlying IBD. By analyzing this vast array of data, researchers can identify specific genetic markers and pathways associated with the disease, leading to more personalized and effective treatment strategies.

Artificial intelligence (AI) is crucial in predicting patient response to biological therapies. Machine learning algorithms can process and analyze large datasets to uncover patterns and predict outcomes based on genetic and clinical information. This predictive capability can significantly enhance clinical decision-making, allowing for selecting the most suitable biological therapy for each patient, thereby improving treatment efficacy and reducing the risk of adverse reactions[111,112].

The potential for novel therapeutic targets based on genetic insights is immense. Emerging therapies, such as those targeting specific genetic pathways implicated in IBD, are showing promise. For example, therapies designed to modulate the immune response or repair intestinal barrier function are developing based on genetic findings. The future landscape of IBD treatment will likely see a shift towards these targeted therapies, which offer the potential for improved patient outcomes and a reduction in the burden of disease. Continued research in this field is essential to fully realizing the benefits of precision medicine in IBD therapy.

CONCLUSION

In summary, understanding the genetic factors that influence the response and failure of biological therapy in IBD is crucial for advancing treatment approaches. Key genetic factors, such as specific gene polymorphisms, mutations, and epigenetic modifications, play significant roles in determining how patients respond to biological therapies. Identifying these genetic markers enables a more precise prediction of treatment outcomes, paving the way for personalized medicine. Integrating multi-omics data and the application of AI in this field are poised to revolutionize IBD treatment. These advancements will allow for the development of novel therapeutic targets and the optimization of existing treatments, ultimately improving patient outcomes. As we move towards a future where treatment plans are tailored to the individual genetic makeup of patients, the potential for reducing the burden of IBD and enhancing the quality of life for patients is immense. However, the implications for personalized medicine are profound. By leveraging genetic insights, healthcare providers can offer more targeted and effective therapies, minimizing adverse effects and maximizing therapeutic benefits. Continued research and technological advancements will be essential to fully harness the potential of precision medicine in IBD treatment, transforming the clinical management of this complex disease.

FOOTNOTES

Author contributions: Peruhova M, Stoyanova D, and Miteva D were involved in conceptualizing the study and writing the manuscript; Peruhova M and Velikova T created the table; Kitanova M, Mirchev M, and Velikova T wrote additional sections of the manuscript; Velikova T was responsible for the critical revision of the manuscript for relevant intellectual content; All authors approved the final version of the manuscript prior to submission.

Supported by The European Union-Next Generation EU, through the National Recovery and Resilience Plan of the Republic of Bulgaria, No. BG-RRP-2.004-0008.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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S-Editor: Luo ML
L-Editor: Filipodia
P-Editor: Zhang XD

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World Journal of Experimental Medicine

Submit a Manuscript: https://www.f6publishing.com

World J Exp Med 2025 March 20; 15(1): 100402

DOI: 10.5493/wjem.v15.i1.100402

ISSN 2220-315x (online)

REVIEW

Alcohol and alcoholism associated neurological disorders: Current updates in a global perspective and recent recommendations

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Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification
Scientific Quality: Grade B
Novelty: Grade B
Creativity or Innovation: Grade B
Scientific Significance: Grade B

P-Reviewer: Huang EP

Received: August 16, 2024
Revised: November 27, 2024
Accepted: December 16, 2024
Published online: March 20, 2025
Processing time: 132 Days and 19.1 Hours

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Abstract

Alcohol use disorder (AUD) is a medical condition that impairs a person's ability to stop or manage their drinking in the face of negative social, occupational, or health consequences. AUD is defined by the National Institute on Alcohol Abuse and Alcoholism as a "severe problem". The central nervous system is the primary target of alcohol's adverse effects. It is crucial to identify various neurological disorders associated with AUD, including alcohol withdrawal syndrome, Wernicke-Korsakoff syndrome, Marchiafava-Bignami disease, dementia, and neuropathy. To gain a better understanding of the neurological environment of alcoholism and to shed light on the role of various neurotransmitters in the phenomenon of alcoholism. A comprehensive search of online databases, including PubMed, EMBASE, Web of Science, and Google Scholar, was conducted to identify relevant articles. Several neurotransmitters (dopamine, gamma-aminobutyric acid, serotonin, and glutamate) have been linked to alcoholism due to a brain imbalance. Alcoholism appears to be a complex genetic disorder, with variations in many genes influencing risk. Some of these genes have been identified, including two alcohol metabolism genes, *alcohol dehydrogenase 1B gene* and *aldehyde dehydrogenase 2 gene*, which have the most potent known effects on the risk of alcoholism. Neuronal degeneration and demyelination in people with AUD may be caused by neuronal damage, nutrient deficiencies, and blood brain barrier dysfunction; however, the underlying mechanism is unknown. This review will provide a detailed overview of the neurobiology of alcohol addiction, followed by recent studies published in the genetics of alcohol addiction, molecular mechanism and detailed information on the various acute and chronic neurological manifestations of alcoholism for the Future research.

Key Words: Alcohol; Alcoholism; Neurotransmitter; Neurological disorders; Alcohol metabolism

Core Tip: This review delves into the neurobiology of alcohol use disorder (AUD), highlighting the role of neurotransmitter imbalances, genetic factors like *alcohol dehydrogenase 1B gene* and *aldehyde dehydrogenase 2 gene*, and the associated neurological disorders. It explores the complex mechanisms underlying neuronal degeneration and blood brain barrier dysfunction in AUD, offering insights for future research into the acute and chronic neurological effects of alcoholism.

Citation: Sahu P, Verma HK, Bhaskar L. Alcohol and alcoholism associated neurological disorders: Current updates in a global perspective and recent recommendations. *World J Exp Med* 2025; 15(1): 100402
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/100402.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.100402

INTRODUCTION

Alcohol (ethanol) is an easily accessible, legal, and widely consumed drug in our society. It is used by a large number of people worldwide. Alcohol is a simple two-carbon molecule that rapidly diffuses through almost every biological compartment in our body upon ingestion. In small amounts, alcohol can have some beneficial effects, such as a reduced risk of cardiovascular infections and all-cause mortality among middle-aged and older individuals[1]. However, excessive consumption costs a lot of major issues, including physical, psychological, and social issues[2]. The levels of alcohol in the brain rise within minutes of consumption, and signs of intoxication can be observed shortly after administering a high dose. At low blood concentrations, alcohol functions as a central nervous system (CNS) depressant, leading to reduced anxiety, feelings of euphoria, and behavioral excitation[3]. While at higher blood concentrations it may result in acute intoxication, which can lead to sluggishness, ataxia, slurred speech, stupor and coma. When intake is stopped, blood alcohol levels start to decline. This decline occurs at a consistent rate (zero-order) of roughly 0.016 g/dL/hour for men and 0.018 g/dL/hour for women[4]. When administered the same amount of alcohol per gram of body weight, women tend to experience higher peak blood alcohol levels compared to men[5]. This is because women have larger levels of body fat than men.

Men are more prone than women to regularly consume large quantities of alcohol, a behavior that is linked to substantial risks to their health and safety. Furthermore, these risks escalate in proportion to the amount of alcohol consumed[6]. Further, unsafe alcohol consumption (40-60 g/day of alcohol in females or 60-100 g/day in males) can create clinical changes linked to various diseases[7,8]. The amount and intensity of alcohol consumed distinguish between an alcohol addict and a nonaddict[9]. There is no ideal meaning of alcoholism; however, most judgments require people to drink vigorously throughout an all-encompassing timeframe and have endured numerous significant life issues because of their liquor/alcohol utilization. A subset of alcohol consumers develops problems because of alcohol use disorder (AUD)[10]. Alcoholic cirrhosis, alcoholic pancreatitis, malignancies of the upper gastrointestinal tract and liver, cardiovascular disease, breast cancer, diabetes, and fetal alcohol syndrome are all risk factors for AUD and can exacerbate results (alcohol intake during pregnancy raises the likelihood of congenital defects in the unborn child)[11]. Brain plasticity events contribute to the development of AUD and result in cravings and habitual alcohol-seeking behavior. Furthermore, chronic or high-dose alcohol intake causes adverse or adaptive reactions in the CNS as well as in nearly every organ system[12].

Chronic alcohol exposure induces brain plasticity changes, particularly in the reward system, reinforcing alcohol cravings and compulsive alcohol-seeking behaviors[13]. These neuroadaptive changes involve alterations in neurotransmitter systems such as gamma-aminobutyric acid (GABA), dopamine (DA), and glutamate, impacting brain regions responsible for reward, stress, and executive function[14]. Additionally, alcohol's neurotoxic effects contribute to structural and functional damage in the CNS, which can impair cognition, decision-making, and emotional regulation, further perpetuating dependence[15]. These findings underline the critical role of CNS adaptations in AUD progression.

The purpose of this review is to demonstrate the various brain manifestations of alcoholism. Alcohol intake is linked to additional inhibitory and excitatory neurotransmitter systems, as well as genes that protect drinkers from future clinical obstacles.

MEDICAL BURDEN OF ALCOHOL ABUSE

AUDs impact an estimated 76.3 million people worldwide, resulting in nearly 1.8 million deaths each year. A study shows that up to 42% of patients treated to general hospitals and 33% of patients admitted to intensive care units have AUD[16]. Alcohol withdrawal syndrome (AWS) is a well-known condition that occurs in around 8% of hospitalized AUD inpatients following abrupt cessation of excessive or persistent drinking[17]. According to the National Institutes of

Health, 28% of persons aged 18 and older consume alcohol on a regular basis at amounts that put them at risk of developing alcoholism, liver disease, and other medical and psychological issues[10].

In 2016, the global average yearly alcohol intake per individual over 15 was 6.4 liters, specifically around 1 liter of wine every week[18]. Alcohol use is responsible for about 5.1 % of the worldwide disease burden and over 3.3 million fatalities per year[19]. AUDs are most frequent in Europe (7.5%) and are least prevalent in the eastern Mediterranean region, which includes Afghanistan, Bahrain, and Egypt. Fifty percent of deaths due to liver cirrhosis, 30% of deaths due to oral and pharyngeal malignancies, 22% of fatalities due to interpersonal violence, 22% of deaths due to self-harm, 15% of deaths due to traffic accidents, 12% of tuberculosis fatalities, and 12% of liver cancer deaths occur globally[19,20].

According to the National Mental Health Survey of India 2015-2016, the prevalence of AUDs in adult men in India was 9%. In India, the alcohol-attributable fraction of all-cause mortality was discovered to be 5.4%. Alcohol was responsible for roughly 62.9% of all fatal liver cirrhosis cases[21].

ALCOHOL DEFINITIONS

Alcoholism is an ongoing sickness described by a physical and mental reliance on alcohol. Individuals with alcohol addiction need to drink to work. Signs that might be battling with alcohol dependence include.

Unit of alcohol

In the United Kingdom, this implies a beverage with 8 g of ethanol—for instance, a large portion of 16 ounces of brew or a little (125 mL) glass of wine[22].

Hazardous drinking

It is described as an amount or pattern of alcohol use that puts people at risk for adverse health consequences[23]. It refers to drinking more than 4 units each day for men and 2 units for ladies. These figures are also expressed as the week-by-week aggregates of 21 units each week for men and 14 units for ladies[24].

Alcohol dependence

A chronic disease wherein individuals crave alcohol drinks and can't handle their drinking. Likewise, an individual with this disease needs to drink more prominent sums to have a similar impact and have withdrawal side effects after stopping alcohol use[25]. Alcohol dependence influences physical and mental health and can cause family, companions, and work issues. Normal heavy alcohol consumption builds the danger of a few kinds of malignancy, like alcohol addiction or Alcoholism[26].

Alcohol tolerance

One expects to drink more significant amounts of alcohol to get similar brain-changing impacts. Alcohol tolerance is expanded by ordinary drinking[27]. This diminished affectability to the actual effects of alcohol utilization necessitates that higher amount of liquor be consumed to accomplish similar impacts as before resistance was set up. Reverse tolerance refers to the natural responses to the positive effects of ethanol found in alcoholic beverages. This includes direct tolerance, the rate at which one recovers from intoxication, and the ability to resist or protect against the development of AUD[28].

Reverse tolerance to alcohol

It happens when the liver is no longer able to produce the necessary enzymes to break down and metabolize alcohol, individuals may experience a condition known as reverse tolerance. This phenomenon is typically observed in individuals with liver damage[29]. Since the liver cannot handle alcohol, it makes people intoxicated more rapidly[30].

Alcohol withdrawal

Being without alcohol for any timeframe can cause one to feel genuinely physically sick[31]. On the off chance that one drinks alcohol heavily for quite a long time, months, or years, one may have mental and actual issues when he stops or truly cut back on the amount he drinks. This refers to alcohol withdrawal. Side effects can go from gentle to genuine[32].

Alcohol abuse

The individuals who keep on drinking regardless of repetitive social, relational, wellbeing, and legitimate issues because of their alcohol use[33]. It's a global issue, comprising the seventh driving danger factor for death also, disability. Harmful drinking or alcohol abuse upsets the system[34,35]. It causes hormonal disturbances that may bring about different issues, such as stress intolerance, reproductive dysfunction, thyroid issues, immune abnormalities, and mental and behavioral problems[36].

Compulsion

One experiences serious cravings/yearnings to drink alcohol and gets oneself incapable of quitting drinking in any event, when needed to.

Alcohol addiction

It is a chronic disease caused by uncontrolled drinking, both mentally and physically, such as a biopsychosocial problem defined by determining the use of drugs (alcohol) despite significant harm and adverse outcomes[37,38].

RELATIONSHIP BETWEEN ALCOHOLISM AND NEUROTRANSMITTER LEVEL

The impacts of alcohol in the CNS are mediated through activities on various Neurotransmitters[39]. There is a complicated interplay between excitatory and inhibitory systems. The numerous neurotransmitters involved in the action of alcohol explain its diverse effects as well as the wide spectrum of pharmacological interactions with both prescribed and illegal medicines (Table 1)[31,40-45]. Alcohol is a powerful substance that affects various neurological pathways and causes major alterations in the brain[46]. Some of the brain pathways impacted by alcohol consumption include the dopaminergic, serotoninergic, aminobutyric acid (GABA), and glutamate pathways[47]. Detailed mechanism depicted in Figure 1

DA pathway

DA is a neurotransmitter primarily involved in a mesolimbic system circuit[48]. It is projected from the brain's ventral tegmental area to the nucleus accumbens and regulates emotional and motivational behavior *via* the mesolimbic dopaminergic pathway. According to studies, ethanol injection into the nucleus accumbens causes local DA release in a dose-dependent manner[49]. Ma and Zhu[50] observed a dose-related increase in extracellular DA levels in the amygdala after ethanol injection. They also noted a delayed increase in DA following ethanol injection in the central amygdaloid nucleus, indicating the critical role of the amygdala in the alcohol-induced effects on the brain[50]. Other research has discovered that ethanol can indirectly raise DA levels in the nucleus accumbens by altering GABAergic neurons and opioid receptors[40]. Alcohol appears to enhance the action of endogenous opioid peptides. In the striatum and substantia nigra, opioid agonists efficiently affect DA release, reuptake, and metabolism, lowering DA production[41].

DA synthesis, release, receptor activation, reuptake, and catabolism are all mechanisms involved in the dopaminergic system[51]. Alcohol has the capacity to suppress the function of the protein monoamine oxidase, which is responsible for the breakdown of DA in the synaptic cleft. This inhibition stops DA from being fully digested, resulting in extended activity on the postsynaptic neuron and heightened feelings of pleasure. Individuals may want to continue experiencing the heightened pleasure generated by DA, which can lead to persistent alcohol intake and, eventually, addiction[52]. Because DA is a pleasure chemical, any decrease in its levels causes reward deficit, resulting in aberrant substance-seeking behavior[53]. Detailed mechanism depicted in Figure 2.

Serotonin pathway

Serotonin is an inhibitory neurotransmitter produced by neurons in the raphe nuclei. It is also known as 5-hydroxytryptamine or 5-HT. Reduced serotonin neurotransmission has been linked to higher alcohol use and susceptibility to alcoholism[54-56]. There is an increase in extracellular 5-HT levels after acute alcohol intake. Chronic alcohol consumption, on the other hand, causes a general decrease in 5-HT neurotransmission, as demonstrated by reduced levels of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite of 5-HT, in heavy drinkers' cerebrospinal fluid (CSF)[42]. This decrease in extracellular 5-HT in the context of chronic alcohol exposure could be attributed to either increased reuptake of 5-HT from the extracellular space *via* the serotonin transporter (5-HTT) or defective 5-HT release in the raphe nuclei [57]. Additionally, Research shows that acute alcohol intake initially increases extracellular serotonin (5-HT) levels, temporarily enhancing mood and reinforcing use[43]. However, with chronic alcohol consumption, there is a marked reduction in 5-HT neurotransmission, indicated by decreased CSF levels of 5-HIAA, the main 5-HT metabolite, in heavy drinkers. This reduction may stem from increased serotonin reuptake *via* the 5-HTT or impaired 5-HT release in the raphe nuclei, leading to diminished serotonergic signaling and potentially exacerbating alcohol dependence[58]. These neurobiological changes suggest a critical role of serotonin in AUD vulnerability.Detailed mechanism depicted in Figure 3.

GABA pathway

GABA is the brain's primary inhibitory neurotransmitter. When alcohol binds to a GABA receptor on a neuron, it allows the entry of negative chloride ions or the exit of positive ions, resulting in a more negative charge within the cell. This inhibits the neuron's ability to generate an action potential[59]. GABA acts through two receptor subtypes known as GABA A and GABA B[60].

Alcohol affects GABA activity in the brain in two ways. Firstly, it can act on the presynaptic neuron responsible for GABA release, leading to increased GABA release. Secondly, it can act on the postsynaptic neuron, interacting with the GABA A receptor alcohol's effects on GABA transmission are regulated by particles that interfere with GABA A receptor activity (GABA A receptor antagonists) and compounds that stimulate the GABA B receptor (GABA B agonists) in specific brain regions such as the nucleus accumbens, ventral pallidum, bed nucleus of the stria terminalis, and amygdala.

Research has demonstrated that both acute and chronic alcohol exposure increase GABA transmission in these regions [61].

Glutamate pathway

Glutamate is the primary excitatory neurotransmitter in the brain and exerts its effects through several receptor subtypes, including the N-methyl-D-aspartate (NMDA) receptor[44]. It has long been known that the glutamate system is involved in the reinforcing effects of alcohol. By using NMDA receptor antagonists, researchers can mimic the effects of alcohol on

Table 1 Major neurotransmitters involved in alcoholism

Name	Primary function	Location and distribution	Receptor	Disease-related	Comments	Ref.
Dopamine	Reward pathway; voluntary motions; motor circuit, cognitions	Hypothalamus, ventral tegmental area (mesolimbic area); most regions: Short medium and long axonal projections	D1, D2, D3, D4, D5	Parkinson's disease, schizophrenia	Alcohol increases its use in nucleus accumbens, mediating its pleasurable impacts	Adermark *et al* [40]; Burns *et al* [41]
Serotonin (5-HT)	Mood regulation: Depression, aggression; intestinal movement control appetite; sleep; muscle control	Raphe nuclei in CNS; most regions: Project from pons and brainstem	5-HT1, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT4, 5-HT6, 5-HT7	Schizophrenia, depression, anxiety	Alcohol usage stimulation gives nausea, may also be linked to the pleasant effects of drinking	Bauer *et al*[44]
Gamma-Aminobutyric acid	Inhibits CNS	The limbic system, hippocampus, thalamus, basal ganglia; supraspinal interneuron	GABA A, GABA B	Anxiety disorder, seizures, epilepsy	Alcohol potentiates GABA activity, amnesia and sedation	Elholm *et al*[31]; Alasmari *et al* [45]
Glutamate	Long-term potentiation; learning; memory	CNS, peripheral nervous system; long neuron	NMDA, others	Seizures, schizophrenia	Alcohol blocks excitatory NMDA receptors, restricting it, causing amnesia, depressant impact	Marcinkiewcz [42]; Müller *et al* [43]

CNS: Central nervous system; GABA: Gamma-aminobutyric acid; NMDA: N-methyl-D-aspartate; 5-HT: 5-hydroxytryptamine.

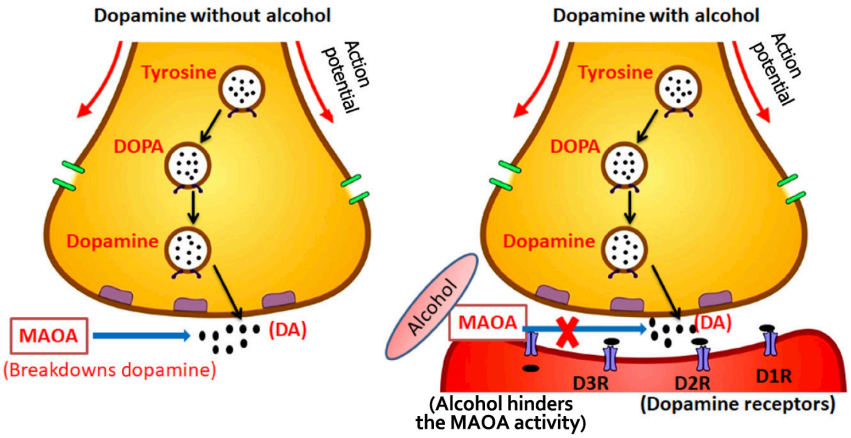

Figure 1 Schematic representation of the molecular mechanism of alcohol with gamma-aminobutyric acid and glutamate neuroreceptors. The electrical voltage across a membrane determines the responsiveness of a neuron. A cell with a higher positive charge is more responsive. When gamma-aminobutyric acid (GABA) binds to GABA receptors, ligand-gated Cl^- ions enter the neuron, making the inside more negative and less likely to respond to new stimuli. Furthermore, alcohol activates GABA receptors, which allows the channels to remain open for longer periods, exaggerating the inhibitory effect. On the other hand, glutamate opens to allow positively charged ions into the cell, causing it to become more positive and more likely to generate an electrical signal. DA: Dopamine; DOPA: Dihydroxyphenylalanine; MOAO: Multi-Object Adaptive Optics.

an organism[45].

Alcohol suppresses the release of glutamate, which leads to a slowing down of neural activity in the brain[62]. It inhibits glutamate activity in the brain[63]. This can be observed in the reduction of extracellular glutamate levels in the brain's striatum, including the nucleus accumbens and other structures, following acute alcohol exposure. These changes undoubtedly impact glutamate transmission involving both ionotropic (NMDA) receptors and another receptor subtype known as metabotropic glutamate subtype 5 receptors[64]. Maintaining a balance between excitatory glutamate and inhibitory GABA neurotransmitters, by increasing excitatory activity and decreasing inhibitory activity, is crucial for proper brain development and functioning[65-67].

GENETIC CONTRIBUTION TO ALCOHOLISM

Environmental and genetic factors, as well as biological variables, influence drinking habit. Recent studies in both human and animal models have shown that genes play a role in the development of alcoholism as well as other social or biological reactions to alcohol[10,68]. Polymorphisms in *alcohol dehydrogenase* (*ADH*) and *aldehyde dehydrogenase* (*ALDH*) *genes*, which alter alcohol metabolism, have been linked to a lower chance of developing alcoholism (Table 2)[69-72].

Table 2 Summary of *alcohol dehydrogenase* and *aldehyde dehydrogenase* family gene

Enzyme	Gene name	Allelic variants	Amino acid differences between allele	Chromosomal location	Subunit components or protein name	Class
ADH	*ADH1A*			4q21-q23	$\alpha_1\alpha_1$	I
	ADH1B	ADH1B 1	Arg48, Arg370 (previously Arg47, Arg369)		$\beta_1\beta_1$	I
		ADH1B 2	His48, Arg370		$\beta_2\beta_2$	
		ADH1B 3	Arg48, Cys370		$\beta_3\beta_3$	
	ADH1C	ADH1C 1	Arg272, Ile350		$\gamma_1\gamma_1$	I
		ADH1C 2	Gln272, Val350		$\gamma_2\gamma_2$	
	ADH4				$\pi\ \pi$	II
	ADH5				$\chi\ \chi$	III
	ADH6				$\mu\ \mu$	IV
	ADH7				$\sigma\ \sigma$	V
ALDH	*ALDH1A1*			9q21.13	Cytosolic aldehyde, dehydrogenase 1	
	ALDH2	ALDH2 1		12q24.2	Mitochondrial aldehyde dehydrogenase	

ADH: Alcohol dehydrogenase; ALDH: Aldehyde dehydrogenase.

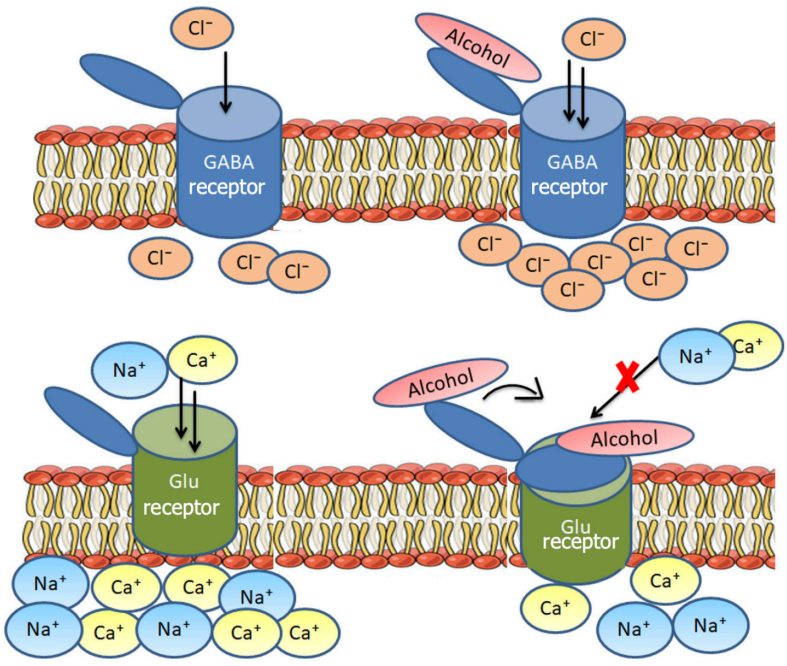

Figure 2 Schematic representation of dopaminergic reward pathway with alcohol. Alcohol inhibits the activity of monoamine oxidase, a protein that is responsible for the breakdown of dopamine. If dopamine is not degraded, it is transferred to the next neuron, confining its pleasurable effect. GABA: Gamma-aminobutyric acid.

Although some ethanol metabolism can occur in other organs and produce localized harm, the liver is the principal location for ethanol metabolism[71]. The primary mechanism of ethanol metabolism involves its conversion into acetaldehyde, which is mediated by ADHs. Acetaldehyde is subsequently further oxidized to acetate by ALDH enzymes in a second step[72]. The genes *ADH 1B gene* (*ADH1B*) and *ALDH 2 gene* (*ALDH2*), particularly mitochondrial ALDH, have the greatest impact on the risk of alcoholism and alcohol intake[73].

ADH

Seven closely similar ADHs are found along chromosome 4, which codes for medium-chain ADHs[73]. The ADH enzymes they encode function as dimers, with the active forms consisting of two components. These seven ADH types

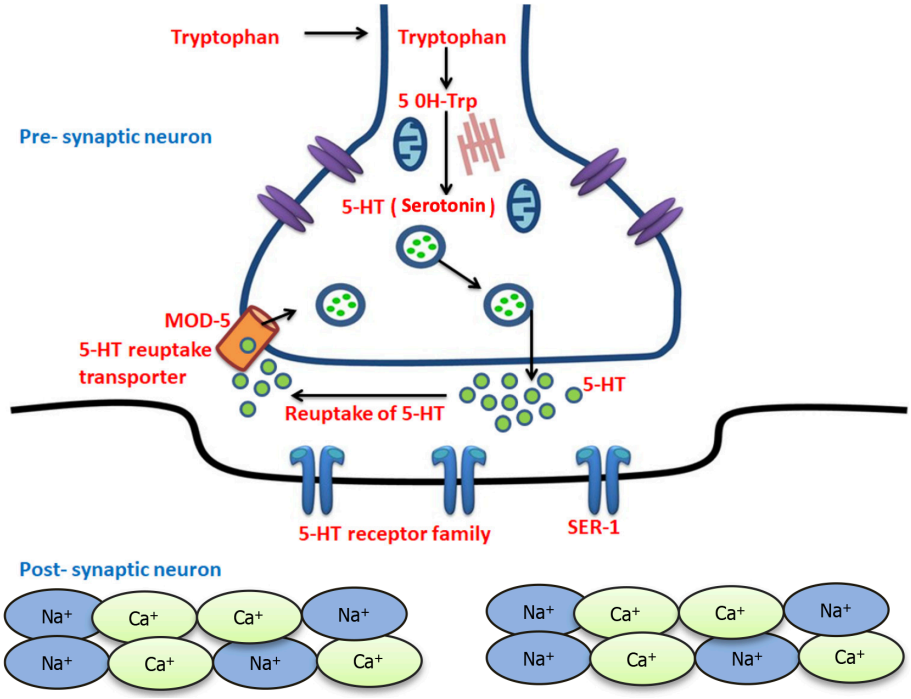

Figure 3 Schematic representation and molecular mechanism of serotonin pathway in the presence of alcohol. During acute alcohol exposure, there is an increase in the 5-hydroxytryptamine (5-HT) extracellular level. Whereas during chronic alcohol exposure, there is a reduction in extracellular 5-HT level. This happens due to its reuptake from extracellular space through serotonin transporter. MOD: Moderate dysplasia; SER-1: Spherical equivalent refraction-1; 5-HT: 5-hydroxytryptamine.

have been divided into five classes based on similarities in amino acid sequences and kinetic properties[35]. *ADH1* genes encode subunits, which join together to create homodimers or heterodimers that account for the majority of the liver's ethanol oxidizing activity[74]. ADH4 generates-ADH, which is required for the oxidation of ethanol at higher doses. ADH5 encodes-ADH, a formaldehyde dehydrogenase with a moderate affinity for ethanol that is extensively expressed. Although ADH6 mRNA is detected in both fetal and adult livers, the enzyme has not been isolated from any tissue. ADH7 produces-ADH, which participates in the oxidation of both ethanol and retinol[75]. *In vitro,* some studies reveal that the enzymes encoded by ADH1B × 48His and ADH1B × 370Cys metabolize ethanol at 30-40-fold more excellent rates than β1–ADH[76].

Furthermore, research indicates that variations in these genes affect alcohol metabolism rates, influencing acetaldehyde accumulation and contributing to individual differences in alcohol tolerance and dependence[77]. Variants of ADH1B (such as ADH1B × Arg47His) and ALDH2 (particularly ALDH2 × Glu504 Lys) have been shown to significantly reduce alcoholism risk[78], highlighting their substantial protective effects through acetaldehyde-mediated aversive responses. More recent studies of genome-wide association suggest that these genetic differences can modulate susceptibility to alcoholism through interactions with other genetic and environmental factors[79-81].

ALDH

Acetaldehyde is a toxic intermediate that affects the entire system accumulation, causing an unpleasant sensation of dizziness, nausea, and tachycardia. Two significant ALDH proteins utilize the acetaldehyde created during ethanol oxidation[81-83]. *ALDH1*, *ALDH1A1* is the gene that encodes ALDH2, which is found in the mitochondrial DNA and is encoded by the *ALDH2 gene*[84]. The mitochondrial ALDH2 is most important in removing acetaldehyde from the body to maintain its low level[85]. The *ALDH1A1 gene* stretches out over 52 kb on chromosome 9, and ALDH2 reaches out more than 43 kb on chromosome 12[86]. The ALDH2 × 2 allele results in the substitution of lysine for glutamate at position 504. The ALDH2 × 2 SNP rs671 (Glu504 Lys) influences how people metabolize acetaldehyde at a much slower pace. The delayed metabolism of acetaldehyde provides an unpleasant alcohol flushing sensation[87]. When both the ADH and ALDH2 variations are present, they give significant protection against the development of AUD[88]. The exact balance of ethanol and acetaldehyde oxidation rates may be critical in defining acetaldehyde concentrations within cells, and even modest changes in the relative activity of ADH and ALDH can have an effect[89].

NEUROLOGICAL MANIFESTATION OF ASSOCIATED WITH THE ALCOHOLISM

Acute complications

Alcohol intoxication (alcohol poisoning): Acute alcohol intoxication is a condition caused by consuming excessive alcohol in a short period[90]. It is the most common of the various alcohol-related diseases affecting both adults and

teenaged[91]. In certain circumstances, persons with this disease may have used household goods containing alcohol, like mouthwash, aftershave, vanilla essence, or shampoo by mistake or on purpose[92,93]. In addition to the amount of alcohol consumed, individual body weight, tolerance to alcohol, and the percentage of alcohol in the beverage, the duration of alcohol intake also appears to be particularly relevant in determining the level of acute alcohol intoxication. Alcohol intoxication occurs due to alcohol's inhibitory effect on nerve cells in the brain and spinal cord[7,94]. As alcohol consumption increases, this inhibitory effect spreads to cortical, brain stem, and spinal neurons.

According to the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and the World Health Organization's International Classification of Diseases criteria, alcohol poisoning is diagnosed clinically based on the presence of clinical or psychiatric problems accompanied by slurred speech, reduced awareness, and coma with respiratory failure[95,96].

Symptoms are generally linked to the amount of alcohol in one's blood alcohol concentration (BAC) of more than 300 mg/dL (65.1 mmol/L), which increases the risk of respiratory depression and arrest[97]. A BAC of more than 400-500 mg/dL (108.5 mmol/L) is usually associated with death from acute alcohol intoxication; however, the fatal alcohol dosage might vary[98]. These effects may be decreased in alcohol-dependent people who acquire tolerance to alcohol due to repetitive exposure to ethanol[99]. In this process, compensating variations in excitatory NMDA and inhibitory GABA appear to be involved[97].

The first significant difficulty in alcohol intoxication is transient anterograde amnesia (commonly called "black-out") [100] when the individual cannot recall a portion of everything that happened during one intoxicated drinking episode [101]. Impairment of judgment and understanding is another typical side effect of alcohol intoxication[102]. The scent of alcohol on the patient's breath is the first indication of alcohol poisoning[103]. Usually, the diagnosis may be determined through history and physical testing. Information regarding the time of the last drink is essential to avoid and treat withdrawal symptoms, which may emerge 6-8 hours after drinking is stopped[96]. Breath analysis or saliva dipstick can also assess the alcohol level; however, these procedures are less accurate.

The treatment of an alcohol poisoned patient involves support and symptomatic therapy. Management begins with the evaluation of cardiac and respiratory systems and the inspection of the airway. Metadoxine (pyridoxal L-2-pyrrolidone-5-carbohydrate) is thought to speed up ethanol metabolism *via* increasing acetaldehyde dehydrogenase activity[104]. Dihydromyricetin, a natural flavonoid, is beneficial in combating acute symptoms of alcohol poisoning[105]. Recently, an alternative alcohol-borne antidote and to use biomimetic nano complexes such as oxidase and catalase, which lower blood alcohol levels, as a prophylactic measure have been developed[106].

AWS

AWS or abstinence syndrome is a sudden stop to or considerably decreased alcohol consumption in patients with tolerance and dependency on alcohol[107]. AWS can develop intentionally when a person stops drinking freely or unintentionally when abstinence is required due to sickness or injury. Alcohol works primarily through two neural receptors. One way alcohol affects the CNS is by modulating the GABA type A receptor, a neurotransmitter receptor that reduces neuronal excitability. This mechanism helps explain the sedative and hypnotic properties of alcohol. However, alcohol also increases the expression of glutamate NMDA receptors, leading to enhanced glutamate activity and promoting hyperexcitation[108].

Patients in mild withdrawal are always aware and have intact orientation. Symptoms appear 6 hours after cessation or reduction in consumption and can persist up to 48 hours (early withdrawal), like irritability, agitation, anxiety, headache, insomnia, nausea and vomiting, and tremors[10]. Moderate withdrawal symptoms begin after 12-14 hours of cessation and include hallucinations of visual, tactile, or auditory characteristics, as well as illusions experienced when awake. They can persist for up to six days[17]. Seizures from alcohol withdrawal usually start 24–48 hours after stopping drinking [109]. Delirium tremens (DT) (onset 48–72 hours/5 days after removal of drinking) is a severe withdrawal syndrome that can last up to two weeks (late withdrawal)[110]. It is recognized by agitation, disorientation, visual hallucinations, and autonomic symptoms such as hyperventilation, tachycardia, and diaphoresis[111]. It can lead to death due to death respiratory or cardiovascular collapse.

The ideal AWS medication would have a fast onset and extended duration to decrease withdrawal symptoms and a very simple metabolism that is not dependent on liver function[112]. Benzodiazepines (BZDs) are now considered the 'gold standard' in AWS treatment[113]. BZDs are the only family of medicines that effectively avoid the development of complex forms of AWS, with an 84% reduction in the incidence of seizures, DT, and the accompanying risk of death[114]. There is more robust evidence for chlordiazepoxide and diazepam, as long-acting medications can produce a smoother withdrawal; propofol potentiates the activity of GABA receptors and can also inhibit NMDA receptors from reducing withdrawal symptoms on multiple receptors[16,115,116].

Wernicke's encephalopathy

Wernicke's encephalopathy (WE) and Korsakoff Syndrome (KS), previously considered distinct diseases, are now recognized as the acute and chronic phases of Wernicke-KS, respectively. WE is an acute neuropsychiatric condition caused by a deficiency of vitamin B1 (thiamine), which serves as a critical coenzyme in carbohydrate metabolism through the Krebs cycle and the pentose phosphate pathways, involving enzymes such as transketolase, α-ketoglutarate dehydrogenase, and pyruvate dehydrogenase[117-119]. A lack of thiamine can cause damage to the brain because these enzymes are known to regulate energy metabolism in the brain, particularly in areas with high metabolic demand, including the thalamic and hypothalamic paraventricular areas, the mammillary bodies, the cerebellar vermis, the floor of the fourth ventricle, and the periaqueductal gray[120]. Other variables that contribute to WE in alcoholics include poor thiamine storage and metabolization in the liver[121]. WE manifests as a slew of symptoms, including ophthalmoparesis (impaired eye movement), altered mental status, gait ataxia (uncoordinated movements), and oculomotor abnormalities[122].

However, only 10% of individuals display all three symptoms, with altered mental status and, in severe cases, coma being the most prevalent clinical findings[15]. Symptoms of WE include reduced attention, memory loss, disorientation, and abulia.

Thiamine blood tests will indicate thiamine serum levels as well as transketolase enzyme activity in peripheral blood. This test, on the other hand, usually takes a long time and is of little use. When it comes to brain imaging exams, magnetic resonance imaging (MRI) is the most important supplemental test for confirming diagnosis. Increased signals in the bilateral medial thalamus, surrounding the third ventricle, and periaqueductal grey matter are shown in T2W and fluid-attenuated inversion recovery imaging in the early phase[123].

The treatment consists of thiamine replacement as soon as possible. Early intravenous thiamine[124] is essential for maintaining an osmotic gradient in the cell membrane, glucose metabolism, and neurotransmitter production[125], and it is usually given before or together with glucose. The average daily thiamine requirement for people is 1.4 mg, or 0.5 mg thiamine should be taken for every 1000 kcal consumed[122]. WE are treated with a high dosage of IV thiamine[126]. Delays in treatment, particularly to pursue diagnostic tests, can be deadly, with a 20% fatality rate[127].

CHRONIC COMPLICATIONS

KS

KS, mainly caused by malnutrition in conjunction with prolonged drinking, typically manifests itself in the aftermath of WE[128]. But it can occur in people with no history of WE or with subacute, with unexplained episodes. DSM-5 defines KS as "alcohol-induced major neurocognitive disorder, amnestic confabulatory type". The 80% of WE patients go on to develop KS[15]. Confabulation, a compensatory response to the inability to recall and retrograde and anterograde amnesia, are all symptoms of KS[129-131]. The confabulatory elements of KS are generally treated symptomatically, but the amnestic results are more challenging to reverse[127]. Clinically, Wernicke-KS is characterized by memory impairment that is disproportionate to other cognitive abilities in a patient who is awake, alert, and responsive.

In most cases, recent memory is more damaged than remote memory[132]. In addition, the KS study has shown that diencephalic regions play a crucial part in the memory function[133], thereby promoting the quest for distinctive and independent brain structure and neuronal circuits underpinning the mnemonic processes[134]. Confusion, lack of muscular coordination, and visual difficulties are other symptoms. The KS occurs slower. Double vision, eyelids may fall, or eyes may be moving fast are some other symptoms[135].

A brain MRI can display changes in brain tissue. But therapy should begin promptly if Wernicke-KS is suspected. The clinical evaluation of those who have KS calls for historical and physical analysis[136]. However, there is no evidence that pharmaceutical treatment is beneficial in KS. Several case reports studies in fluvoxamine, clonidine, reboxetine, or rivastigmine were used to treat KS. These trials did not generate consistent evidence for the effectiveness of any of these interventions. As a result, we can ensure that no effective pharmacological therapy for KS is available[128]. Stopping the usage of alcohol can help to avoid further loss of brain function and nerve damage. A nutritious, well-balanced diet can assist[137].

Marchiafava-Bignami disease

Marchiafava-Bignami is a neurologic disorder that predominantly affects myelin and is associated with persistent alcohol consumption[138]—originally known as "red wine drinker's encephalopathy"[10]. Marchiafava E and Bignami A, two Italian pathologists, discovered it in 1903. They described men with an alcohol use disease who died of convulsions and comas, with necrosis of the corpus callosum identified on autopsy[139]. Marchiafava-Bignami Disease (MBD) is a rare disease characterized by demyelination/necrosis of the corpus callosum's myelinated fiber's central part (middle lamina) and adjacent subcortical white matter disease[140]. It is a degenerative neurological disorder that most commonly affects middle-aged (45 years) or older alcoholic men[141,142]. MBD illness is hallmarked by corpus callosum demyelination. Demyelination of the corpus callosum, especially the splenium, is the major cause[143]. However, demyelination can affect the optic chiasm and tracts, cerebellar peduncle, subcortical area, adjacent white matter, and, in rare cases, cortical grey matter. An interhemispheric disconnection syndrome develops over time[144], presenting with dementia, limb apraxia, tactile and unilateral agraphia, and hemialexia.

The disease can manifest itself in two primary clinical forms: (1) Acute and chronic; and (2) The latter of which can be fatal[127]. There is no well-defined clinical syndrome; it causes altered mental state, ataxia, mood disorders (depression and mania), and psychotic symptoms (paranoia); also the clinical course varies, some patients will become comatose and die, while others can live with dementia for several years, while others will only recover partially[145].

Brain imaging investigations, particularly MRI, are required to confirm a diagnosis (demyelination, inflammation, or necrosis of corpus callosum)[146]. Marchiafava—Bignami illness has no particular treatment; however, abstinence and vitamin supplements are advised. Some studies have also found a positive response to large dosages of corticosteroids [147]. According to some clinicians, thiamine, folate, vitamin B complexes may be useful in delaying the course of Marchiafava-Bignami syndrome[148].

Alcoholic cerebellar degeneration

Cerebellar degeneration is a pathological condition that refers to the progressive accumulation of abnormalities in the cerebellum due to alcohol toxicity[149]. Cerebellar degeneration occurs in both alcoholics deficient in micronutrients and those who are not[127]. When neurons in the cerebellum degenerate and die due to the harmful effects of alcohol, this syndrome arises. The cerebellum is the portion of the brain that is in charge of coordination and balance. Alcoholic

cerebellar degeneration (ACD) is characterized by stance and gait ataxia[10]. Persons with cerebellar degeneration can adopt a wide-based gait with short steps, compensating for their balance losses. Other problems may include nystagmus, poor handwriting, upper extremity inconsistency, and moderate dysarthria. Cerebellar ataxia is the clinical manifestation of cerebellar degeneration and can manifest in various ways[150]. Truncal ataxia depicts trunk instability and unbalances that generate corporal oscillations during sitting and causes cerebellar vermis damages[124]. According to some physicians, the length of alcohol consumption is the most critical risk factor for developing clinically severe toxic[151]. It is the most frequent CNS consequence of persistent alcohol consumption, affecting 10% to 25% of alcoholics[152].

While all neuronal cells and the white matter suffer from the injury, Purkinje cells are most affected. Some authors proposed a concept to explain phenomena in which increased gut permeability produced by alcohol-induced intestinal mucosa lesions seen in alcoholic patients might enhance the immune system[153]. After being exposed to harmful antigens (including gliadin peptides), the impairment of the blood-brain barrier caused by chronic alcohol consumption would allow these antibodies to enter the brain *via* previously unknown pathways, causing the brain to degenerate like gluten-induced cerebellar ataxia[153,154].

Diagnosed clinically, anatomopathological and neuroimaging analyses both indicate degeneration of all microcellular components of the cerebellar cortex, notably Purkinje cells on the anterior and superior vermis surfaces. Cerebellar atrophy is seen on computed tomography and MRI images of the brain[155]. No particular therapy has been established; however, vitamin supplements administration and alcohol abstinence are suggested. Although there is no treatment for these diseases, limited studies indicate that some medicines like Riluzole and physical therapy can help with ataxia symptoms[156].

Alcoholic dementia

The phrase "alcohol-related dementia" refers to a type of dementia caused by the direct effects of persistent alcohol use on the brain. Dementia is a clinical condition defined by a gradual decline in cognitive ability and the ability to live and function independently[157]. Dementia impairs memory, reasoning, behavior, and the capacity to do daily tasks[158], and it is a significant cause of impairment in elderly individuals. In observational and imaging investigations, heavy alcohol consumption was linked to structural alterations in the brain and cognitive and executive deficits[159]. The global prevalence of dementia has been estimated to be between 5% and 7% among persons aged 60 and older[160]. According to one research, males who drank ≥ 36 g/day of alcohol had a quicker 10-year decrease in all cognitive areas, with an impact size equivalent to 1.5 to 5.7 additional years of cognitive decline[161]. The CNS shrinkage associated with alcoholic neurodegeneration is produced by myelin breakdown, dendritic connection loss, and neuronal death[15].

Early neuropsychological investigations generally reveal frontal subcortical cognitive impairment, mental slowness, attention deficit, immediate or short-term memory changes, reduced visual-spatial capacity, and decreased management responsibilities, including planning and organization[162]. Imaging studies of simple alcoholics (no nutritional deficit, hepatic failure, or brain damage) have shown structural abnormalities, including alterations to the corpus callosum, pons, and cerebellum[34]. Given that the number of individuals living with dementia is predicted to triple around 2050 and there is currently no treatment, prevention is crucial[163]. The primary mechanism underlying healing from white matter injury is the restoration of myelination and axonal integrity[164]. Abstinence leads to improvements in motor skills and cognition and a reversal of white matter shrinkage. However, if the drinking is restarted, it becomes subject to disturbance once more.

Alcoholic polyneuropathy

Polyneuropathy, often known as peripheral neuropathy, occurs when numerous peripheral nerves are injured. The most common consequence in alcoholic individuals is chronic polyneuropathy[127], caused by prolonged alcohol use. Paresthesia, pain and ataxia are common symptoms. We don't know how many people are afflicted by alcohol neuropathy, but studies suggest that at least 66% of chronic alcoholics have neuropathy[165]. It is thought to be the consequence of a multifactorial process mainly driven by direct toxic effects of ethanol or its metabolites impact and regulated by other variables, including genetic susceptibility, malnutrition, thiamine deficiency, and other systemic illnesses[166]. This is a sensory polyneuropathy with distal, symmetric characteristics that is mainly axonal. The longer axons are more prone to be affected initially[165]. The development of symptoms is gradual and symmetric, mostly sensory, manifesting as dysesthesia, burning feeling, and burning pain on the soles of the feet, toes, arm[167], which progresses to cramping in the calves and hands[168]. Muscle weakness and atrophy, particularly in the distal muscles of the upper or lower limbs, are common motor symptoms that appear later. Trophic skin alterations such as glossiness, hair loss, thinning, hyperpigmentation, and reduced sweating are frequent in affected distributions. Compared to males, women have a greater rate of alcoholic polyneuropathy. Chopra and Tiwari[169] showed that alcohol-induced neuropathy in female rats had a faster start and was more severe than in male rats in preclinical tests, confirming the findings.

Diagnosis includes electrodiagnostic testing and physiological findings that reveal typical axonal sensory neuropathy symptoms, with reduced densities of nerve fibers. Except in people with a long history of neuropathic complaints and significant axonal sprouting, the density of tiny myelinated and unmyelinated axons was lower than the density of large myelinated fibers[170].

In some situations, therapies suppress symptoms rather than treating the underlying illness. Alpha-lipoic acid, benfotiamine, acetyl-L-carnitine, and methylcobalamin have all been the subject of extensive investigation. Myo-inositol, vitamin E, topical capsaicin, and N-acetylcysteine are some other botanical or nutritional treatments. The use of current therapy and nutrition can help to reduce morbidity[165,169]. A balanced diet with vitamin supplements, rehabilitation, and alcohol abstinence are all part of the treatment. Recovery, on the other hand, is gradual and frequently incomplete. Drugs like gabapentin and amitriptyline can be used to treat patients with neuropathic pain[171].

RECOMMENDATIONS

When asked about how alcoholism is treated, many people often think of 12-step programs or 28-day inpatient rehab, but they may be unaware of other available options. In reality, there are several therapy options currently accessible. It is important to recognize that there is no one-size-fits-all approach, and what works for one individual may not work for another. Therefore, understanding the various alternatives can be a crucial first step.

Therapies like Cognitive-behavioral therapy with a therapist or in small groups can be carried out alone. The main aim of this type of treatment is the identification of feelings and situations. The objective is to modify the thinking processes leading to alcohol abuse and build the abilities required to face daily situations. Motivational enhancement therapy is carried out over a short period to motivate and enhance drinking behavior. Family and marital counseling involves spouses and other family members in the therapy process and can play a major part in the rehabilitation and development of family ties[172].

Medications like, naltrexone can aid people in drinking heavily. Acamprosate makes abstinence simpler to sustain [173]. Disulfiram inhibits the body's alcohol breakdown and causes disagreeable sensations, including nausea and skin flushing. People may avoid consuming alcohol while taking disulfiram because of these unpleasant side effects[174]. BZDs, such as diazepam and chlordiazepoxide, are preferable for treating all types of alcohol withdrawal symptoms, including DT, if the liver function test is normal.

Nutrition: During healing, one should consume a diet that balances serotonin (a hormone that aids in relaxing) levels in the brain. This requires consuming carbohydrate-rich meals (grains, fruits, and vegetables), particularly complex carbs found in starchy foods such as legumes (*e.g.*, beans, lentils, and peas), root vegetables (potatoes and carrots), pasta, and bread. Consuming these items in conjunction with protein in daily meals will maintain users at peak performance.

Rediscover hobbies: Many individuals drink to pass the time when they are bored. Pleasurable activities keep one from wanting to drink, but they also help relax, which everyone needs to do.

Most withdrawal symptoms or other alcohol-related issues may be treated well with medicines coupled with proper vitamins, exercise, and sleep[175].

CONCLUSION

Chronic alcohol abuse can result in various neurological symptoms, including both central and peripheral neurologic problems. Polyneuropathy, cerebellar degeneration, and dementia are the most common, whereas WE, KS, and Marchiafava Bignami are the most dangerous. Because alcohol is highly prevalent, and alcohol is complicated. Due to its significant morbidity and mortality often masked by other medical complexities associated with aging or alcoholicity, it is essential to have a thorough knowledge of this disclosure and quickly recognize its scope. Alcohol primarily interacts with GABA A and NMDA receptors, but it also induces various signaling events within well-defined brain pathways. These events lead to adaptive changes in gene expression, resulting in two main states: (1) Addiction; and (2) Toxicity. A significant biological factor underlying susceptibility to AUD and other neurological consequences of chronic alcohol consumption may involve genetically determined features of myelin structure and alcohol's impact on myelin gene expression. Since alcohol does not selectively affect a single region of the nervous system, it is crucial to identify any cerebellar or motor impairments in individuals with cognitive issues. Early detection and intervention are essential steps that healthcare professionals can take to mitigate the neurological consequences of chronic alcohol abuse. In cases where the condition has already been diagnosed, nutritional supplementation and cessation efforts are important in preventing further harm and may lead to some symptom relief.

FOOTNOTES

Author contributions: Sahu P and Verma HK performed the literature search, collected and assembled the data, and analyzed the obtained articles; Verma HK and Bhaskar L designed the review; Bhaskar L supervised the complete article; Sahu P, Verma HK, and Bhaskar L wrote the manuscript and revised the manuscript critically; all authors have read and agreed to the published version of the manuscript.

Conflict-of-interest statement: All authors declare no conflict of interest in publishing the manuscript.

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S-Editor: Luo ML

L-Editor: A
P-Editor: Zhang XD

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World Journal of Experimental Medicine

Submit a Manuscript: https://www.f6publishing.com

World J Exp Med 2025 March 20; 15(1): 100275

DOI: 10.5493/wjem.v15.i1.100275

ISSN 2220-315x (online)

MINIREVIEWS

Impact of curcumin on gut microbiome

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Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification

Scientific Quality: Grade D

Novelty: Grade D

Creativity or Innovation: Grade B

Scientific Significance: Grade C

P-Reviewer: Sardar H

Received: August 12, 2024

Revised: October 12, 2024

Accepted: November 6, 2024

Published online: March 20, 2025

Processing time: 135 Days and 21.4 Hours

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Abstract

The intricate interplay between natural compounds like curcumin and the gut microbiome has gained significant attention in recent years due to their potential therapeutic implications in various health conditions. Curcumin, a polyphenolic compound derived from turmeric, exhibits diverse pharmacological properties, including anti-inflammatory, antioxidant, and anticancer effects. Understanding how curcumin modulates gut microbiota composition and function is crucial for elucidating its therapeutic mechanisms. This review examines the current literature on the interactions between curcumin and the gut microbiome. A systematic search of relevant databases was conducted to identify studies investigating the effects of curcumin on gut microbial diversity and abundance. Key findings from studies exploring curcumin's efficacy in neurological disorders, gastrointestinal diseases, and metabolic dysfunction are synthesized and discussed. Studies have demonstrated that curcumin supplementation can

modulate gut microbiota composition and function, leading to beneficial effects on gut health and homeostasis. Mechanisms underlying curcumin's therapeutic effects include immune modulation, neuroprotection, and inflammation regulation. However, challenges such as poor bioavailability and safety concerns remain significant hurdles to overcome. The interactions between curcumin and the gut microbiome hold promise for therapeutic interventions in a diverse range of health conditions. Further research is needed to optimize curcumin formulations, improve bioavailability, and address safety concerns.

Key Words: Gut microbiome; Curcumin; Neuroprotection; Bioavailability

Core Tip: Curcumin, derived from turmeric, interacts with the gut microbiome and has a significant impact on health. Studies have revealed that curcumin modulated gut microbial composition, immune responses, and inflammation. Challenges such as bioavailability persist, but curcumin holds promise for diverse therapeutic applications.

Citation: Balaji S, Jeyaraman N, Jeyaraman M, Ramasubramanian S, Muthu S, Santos GS, da Fonseca LF, Lana JF. Impact of curcumin on gut microbiome. *World J Exp Med* 2025; 15(1): 100275
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/100275.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.100275

INTRODUCTION

Understanding the complex interactions between natural compounds and the gut microbiome has become increasingly significant in recent years as the importance of gut bacteria composition and function in maintaining human health has become apparent. The gut microbiome, comprising trillions of microorganisms, plays a crucial role in various physiological processes, including metabolism, immune function, and neurobehavioral regulation[1]. Dysbiosis, the imbalance of microbial communities within the gut, has been linked to a plethora of chronic diseases, ranging from metabolic disorders to neurodegenerative conditions[2].

Among the many natural compounds under investigation, curcumin has emerged as a promising candidate for modulating microbial composition and function within the gut. Curcumin, a polyphenolic compound derived from the rhizome of *Curcuma longa*, commonly known as turmeric, has garnered considerable attention due to its diverse pharmacological properties, including anti-inflammatory, antioxidant, and anti-carcinogenic effects[3]. Moreover, curcumin has been shown to exert significant effects on gut microbial communities, making it an intriguing subject of study in the context of microbiome modulation[4].

Investigations into the effects of curcumin on mental health have unveiled its ability to influence gut microbiota composition, thereby implicating its role in neurobehavioral regulation[5]. Several preclinical studies have demonstrated the potential of curcumin in modulating gut microbial composition to mitigate the progression of atherosclerosis, a chronic inflammatory condition characterized by the buildup of plaque within arterial walls[6]. Curcumin supplementation has been associated with reduced plaque burden and favorable alterations in gut microbiota composition, suggesting its therapeutic potential in the management of atherosclerosis[6].

Clinical studies have provided further insights into the impact of curcumin on gut microbial communities. Research investigating the effects of turmeric and curcumin on human gut microbiota composition has revealed personalized responses, with curcumin potentially driving the observed changes in microbial diversity and abundance[7]. Culinary spices like turmeric have been shown to induce beneficial alterations in gut microbial communities, promoting digestive health through increased production of short-chain fatty acids, such as butyrate[8].

Curcumin, also known as diferuloylmethane, is the primary curcuminoid found in turmeric (*Curcuma longa* L.). Its chemical designation is 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, and it possesses a molecular formula of $C_{11}H_{20}O_6$ with a molecular weight of 368.38 g/mol. The chemical structure of curcumin consists of two ferulic acid residues that are linked by a methylene bridge. The molecule exists in tautomeric forms, with the enol form being the dominant structure in solution. This distinctive configuration contributes to curcumin's characteristic yellow hue and its reactivity as a Michael acceptor in various chemical reactions. Within curcumin's structure, several key functional groups are present, which are fundamental to its biological activities and its interactions with a range of molecular targets. These include two aromatic rings containing ortho-methoxy phenolic groups, two α, β-unsaturated carbonyl groups, and a β-diketone moiety. Additionally, curcumin has multiple conjugated double bonds, enhancing its reactivity and interaction potential[9-12].

Despite the diverse pharmacological activities of curcumin (Table 1), its therapeutic application is significantly hindered by inherent limitations such as poor aqueous solubility, rapid metabolism, and limited systemic bioavailability. To overcome these challenges, a variety of delivery systems have been developed. These include nanoformulations, liposomal preparations, phospholipid complexes, and other novel drug delivery systems. Another important consideration in improving curcumin's bioavailability is its interaction with the gut microbiota, which plays a crucial role in its

Table 1 Pharmacological effects of curcumin

Pharmacological activity	Mechanisms/effects	Key points
Anti-inflammatory properties	Inhibition of NF-κB activation and suppression of inflammatory mediators; suppression of COX-2, LOX, and iNOS expression; modulation of pro-inflammatory cytokines (*e.g.*, TNF-α, IL-1β, IL-6); regulation of MAPK signaling pathways; inhibition of inflammatory transcription factors	Modulates gut microbiota
Antioxidant activities	Direct scavenging of free radicals; enhancement of cellular antioxidant defenses; upregulation of Nrf2 pathway; increase in antioxidant enzyme activities (SOD, CAT, GPx); metal ion chelation	Protects against oxidative stress-induced cellular damage
Anticancer properties	Cell cycle arrest and induction of apoptosis; Inhibition of cancer cell proliferation; modulation of microRNAs; suppression of angiogenesis; regulation of cancer stem cells; interference with signaling pathways (STAT3, Wnt/β-catenin, PI3K/Akt)	Gut microbiota interaction enhances effects
Immunomodulatory effects	Regulation of T cell differentiation and function; influence on B cell response; modulation of macrophage polarization; modification of dendritic cell function; alteration of natural killer cell activity	Significant impact on gut immunity
Neuroprotective activities	Protection of the blood-brain barrier; reduction of neuroinflammation; prevention of protein aggregation; enhancement of neuroplasticity; modulation of neurotransmitter systems	Gut-brain axis plays a crucial role
Cardiovascular protection	Improvement of endothelial function; reduction of atherosclerosis; modulation of lipid metabolism; prevention of cardiac hypertrophy; protection against ischemia-reperfusion injury	
Antidiabetic effects	Enhancement of insulin sensitivity; protection of β-cell function; regulation of glucose metabolism; reduction of advanced glycation end-products; amelioration of diabetic complications	Ameliorates diabetic complications
Hepatoprotective activities	Prevention of hepatic fibrosis; protection against drug-induced liver injury; reduction of hepatic steatosis; modulation of liver enzyme activities; enhancement of hepatic regeneration	
Antimicrobial properties	Broad-spectrum activity against bacterial, fungal, viral, and parasitic infections	Involves modulation of gut microbiota

TNF-α: Tumor necrosis factor-alpha; IL: Interleukin.

metabolism and overall efficacy. Recent scientific advancements have increasingly explored the relationship between curcumin and gut microbiota. Wang *et al*[13] investigated the modulatory effects of curcumin on gut microbiota as a potential therapeutic strategy[13]. Similarly, Liu *et al*[14] examined the bidirectional interaction between curcumin and gut microbiota[14], while Shen *et al*[15] emphasized the connection between curcumin, gut microbiota, and neuroprotection[15]. Further expanding on this field, Zhang *et al*[16] provided insights into how curcumin's modulation of gut microbiota may help ameliorate symptoms associated with Parkinson's disease (PD).

Despite these promising findings, there remains a need for further research to elucidate the mechanisms underlying curcumin's effects on gut microbiota and to explore its potential therapeutic applications in various disease contexts[2,3]. The personalized nature of the response to curcumin and turmeric underscores the importance of personalized medicine approaches in harnessing the therapeutic potential of natural compounds for microbiome modulation[7]. Understanding the complex interplay between curcumin and gut microbiota has substantial potential to enhance our comprehension of microbial-host interactions and facilitate the development of adjuvant therapies for an array of ailments. The aim of this study is to offer a thorough and comprehensive review of the influence exerted by curcumin on the gut microbiome, along with an exploration of its clinical applications.

CURCUMIN-AN OVERVIEW

Curcumin, a polyphenolic compound derived from the rhizome of *Curcuma longa*, commonly known as turmeric, has garnered significant attention in recent years due to its diverse pharmacological properties and potential clinical applications[17]. Curcumin is the primary bioactive constituent of turmeric and is extensively utilized in both culinary and medicinal contexts[17,18]. Despite its widespread use, curcumin exhibits poor systemic bioavailability, attributed to its low solubility and stability, which poses challenges for its therapeutic utilization[19]. Nevertheless, numerous studies have highlighted the remarkable biologic activities of curcumin, including its antioxidant, anti-inflammatory, and anticancer properties[17,20]. Additionally, curcumin has demonstrated promising effects in improving brain function, controlling obesity, and ameliorating diabetes[19].

These multifaceted pharmacological activities underscore the potential of curcumin as a therapeutic agent for various health conditions. In preclinical studies, curcumin has shown efficacy in inhibiting the proliferation of colon cancer cells and inducing apoptosis, suggesting its potential as an anticancer agent[17]. Furthermore, curcumin has been found to suppress mucosal expression of inflammatory mediators, highlighting its anti-inflammatory effects[17]. Notably, curcumin has been shown to modulate the gut microbiota, promoting the growth of beneficial bacteria such as butyrate-producing species, which may contribute to its anti-inflammatory and anticancer effects[20]. Additionally, curcumin's ability to ameliorate intestinal inflammation and modulate signaling pathways further enhances its therapeutic potential

[17].

Despite its challenges regarding bioavailability, ongoing research efforts are focused on developing novel curcumin formulations with enhanced bioavailability to maximize its therapeutic efficacy[19,20]. Moreover, studies have demonstrated the safety and tolerability of curcumin, making it an attractive candidate for clinical use[17]. Clinical trials investigating the effects of curcumin on various health outcomes, including metabolic disorders, neurodegenerative diseases, and cancer, are underway, highlighting its potential clinical applications[19].

ROLE OF GUT MICROBIOTA IN HEALTH AND DISEASE

The gut microbiome, comprising a diverse array of microorganisms, plays a crucial role in maintaining host health and homeostasis. Typically, the gut microbiome is dominated by several key phyla, including *Firmicutes, Bacteroidetes, Actinobacteria*, *Proteobacteria, Fusobacteria*, and *Verrucomicrobia*[21,22]. Among these, *Firmicutes* and *Bacteroidetes* are particularly abundant, with species such as *Lactobacillus, Bacillus*, *Clostridium, Enterococcus, Bacteroides*, and *Prevotella* commonly found[22]. Additionally, Actinobacteria, notably the Bifidobacterium genus, also contribute significantly to the gut microbiome composition. The stability of the gut microbiome is paramount, with a dynamic continuum of composition influenced by various factors such as age, diet, environment, and host genetics[21,23]. Throughout life, the gut microbiota composition undergoes changes, shaped by early microbial contact, genetic predisposition, dietary habits, and lifestyle factors[21,23].

The gut microbiome plays a critical role in numerous disease processes, impacting health outcomes through its influence on metabolism, immune responses, and physiological development. Research has identified associations between gut microbiota and a wide array of diseases, including hypercholesterolemia, respiratory allergies, anxiety, osteoarthritis, hypertension, celiac disease, inflammatory bowel disease (IBD), type 2 diabetes, hypertension, and colorectal cancer[24]. Studies have highlighted the therapeutic potential of manipulating the gut microbiota to treat diseases, with fecal microbiota transplantation emerging as a promising strategy for altering bacterial compositions and addressing conditions such as gastrointestinal disorders and metabolic diseases[25]. The gut microbiome's role in disease pathogenesis extends beyond gastrointestinal ailments, as evidenced by its involvement in allergic diseases, cancer, neurological disorders, and psychiatric illnesses[25]. Early microbial supplementation, probiotics, and specific microbial strains like *Lactobacillus johnsonii* and *Lactobacillus plantarum* may offer therapeutic benefits by promoting immune tolerance induction and restoring gut health[26]. Overall, the intricate interplay between the gut microbiome and disease processes underscores the importance of understanding and leveraging microbial contributions to develop novel approaches for disease prevention and management.

CURCUMIN AND THE GUT MICROBIOTA

Bacterial species involved: Curcumin supplementation has been associated with significant alterations in the composition and abundance of various bacterial species within the gut microbiota, with implications for health and disease. Several studies have highlighted the specific bacterial taxa affected by curcumin supplementation across different populations and health conditions[17,20,27]. Notably, *Escherichia-Shigella*, a genus encompassing pathogenic bacteria associated with gastrointestinal infections, decreased significantly following curcumin supplementation in patients with chronic kidney disease (CKD)[27]. Conversely, beneficial bacterial species such as *Lachnoclostridium* and *Lactobacillaceae spp*. showed significant increases in abundance after curcumin supplementation in CKD subjects, suggesting a potential role for curcumin in promoting gut microbial balance and diversity[27]. Moreover, curcumin intake has been shown to increase the abundance of butyrate-producing bacteria, such as *Clostridium* and *Bacteroides spp*., which are known for their anti-inflammatory and metabolic benefits[17]. A randomized controlled study found that curcumin supplementation led to changes in the abundance of *Clostridium*, *Collinsella*, and *Kluyvera*[7]. Furthermore, curcumin has been shown to reduce the relative abundance of potentially pathogenic bacteria such as *Blautia spp.* and *Ruminococcus spp*., which are associated with gut dysbiosis and inflammation[7]. In addition to promoting the growth of beneficial bacterial species, curcumin supplementation has been found to modulate the relative abundance of specific bacterial taxa associated with disease pathogenesis[28]. Curcumin has been found to increase butyrate production in the gut, which has important implications for gut health and immune function. Butyrate, a short-chain fatty acid produced by certain gut bacteria, serves as a crucial energy source for colonocytes and exhibits anti-inflammatory properties[8,17]. Table 2 shows the altered bacterial species in the gut due to curcumin.

MECHANISM OF ACTION OF CURCUMIN ON THE GUT MICROBIOME

Several studies elucidate the intricate interplay between curcumin and gut microbial composition, shedding light on its therapeutic potential in various health conditions[29,30]. One key mechanism by which curcumin influences the gut microbiome is through its ability to regulate microbial diversity and abundance. Xiao *et al*[30] revealed that curcumin supplementation can restore homeostasis in Th17/Treg responses within the gut, thereby modulating the composition of gut microbiota in mice with diabetic complications[30]. Additionally, curcumin has been shown to regulate the diversity and abundance of intestinal microbiota at various taxonomic levels, suggesting a broad-spectrum impact on microbial

Table 2 Altered bacterial species in the gut due to curcumin

Bacterial species altered	Ref.
Escherichia-Shigella, Lachnoclostridium, Lactobacillaceae spp.	[27]
Clostridium, Bacteroides, Parabacteroides, Collinsella, Kluyvera, Enterococcus spp., Blautia spp., Ruminococcus spp.	[7]
Butyrate-producing bacteria, Clostridium, Bacteroides spp., Beneficial gut microbiota	[17]
Blautia spp. MRG-PMF1	[20]
Lactobacilli, Clostridium perfringens, Anaerobic bacteria producing butyric acid	[18]
Akkermansia, Firmicutes/Bacteroidetes ratio	[6]

communities within the gut[30].

Findings from Burge *et al*[29] and Di Meo *et al*[28] highlight curcumin's ability to favor the growth of beneficial bacteria while reducing the abundance of pathogenic strains in the gut microbiome[29]. This modulation of microbial balance by curcumin is accompanied by a decrease in microbial richness and diversity, as well as the modulation of molecular pathways involved in intestinal inflammation[28]. For example, curcumin influences the intestinal barrier function by modulating tight junction proteins, thus protecting against inflammation-induced disruption of gut integrity. Mechanistically, curcumin attenuates lipopolysaccharide-induced inflammation by reducing the activation of p38 MAPK and myosin light chain kinase, as well as preventing the disruption of tight junction proteins[31]. Moreover, curcumin's interaction with gut microbiota indirectly influences neuroprotection through modulation of signaling pathways such as NF-kB and AP-1, which are involved in inflammatory responses within the gut[28]. The summarized mechanisms of action are presented in Table 3 and Figure 1.

EFFECT OF GUT MICROBIOME ON CURCUMIN

Conversely, emerging evidence suggests that the gut microbiome plays a crucial role in mediating the bioavailability, metabolism, and therapeutic effects of curcumin within the body. Pluta *et al*[32] and Augusti *et al*[33] underscore the impact of gut microbial composition on curcumin's pharmacokinetics and pharmacodynamics[32,33]. Gut microbiota influences curcumin bioavailability and transformation during digestion, with unique human phenolic metabotypes yielding different responses to curcumin[33]. Moreover, the metabolization of curcuminoids by human gut microbiota generates new colonic metabolites with potent pharmacological activities, suggesting a symbiotic relationship between curcumin and gut microbial communities[32].

The gut microbiome acts as a crucial determinant of curcumin's efficacy in various disease states. Zhang *et al*[34] elucidated how curcumin protects against cadmium-induced atherosclerosis by remodeling gut microbiota, restoring bacterial diversity, and reducing pathogenic loads[34]. The modulation of gut microbiota by curcumin contributes to its cardioprotective effects by reducing cadmium absorption and restoring microbial balance[34]. Additionally, the gut microbiota regulates curcumin's effects on microbial richness, diversity, and composition, further underscoring the bidirectional relationship between curcumin and gut microbial communities[35]. Moreover, curcumin enhances response to cytarabine therapy in acute myeloid leukemia by regulating gut microbiome composition, highlighting the therapeutic potential of targeting gut microbiota in conjunction with curcumin-based interventions[36]. Overall, the gut microbiome exerts a profound influence on curcumin's pharmacokinetics, pharmacodynamics, and therapeutic efficacy, highlighting the importance of considering microbial factors in optimizing curcumin-based interventions for various health conditions.

HEALTH IMPLICATIONS

Neurologic diseases: Curcumin exhibits promising therapeutic potential in various neurologic disorders, including Alzheimer's disease (AD), PD, multiple sclerosis (MS), ischemic brain injury, and anxiety (Figure 2). In AD models, curcumin demonstrates neuroprotective effects by mitigating memory impairment and metabolic dysfunction. Moreover, it modulates synaptic plasticity and metabolic pathways, potentially ameliorating AD-related symptoms. Additionally, curcumin enriches beneficial gut microbiota, thereby influencing cognitive functions indirectly[32,37]. In PD, curcumin improves motor deficits and neuroinflammation through modulation of the gut microbiota-metabolite axis. Furthermore, it provides neuroprotective effects and ameliorates motor deficits in PD models[38]. In MS, the curcumin derivative CMG alters gut microbiota composition, suppressing experimental autoimmune encephalomyelitis severity. This suppression correlates with changes in specific bacterial species abundance in feces and ileal contents[39]. In ischemic brain injury, curcumin reduces infarct volume, brain edema, and blood-brain barrier permeability while inhibiting tau protein hyperphosphorylation and disintegrating its fibers. Moreover, it improves cognitive deficits and neurological outcomes post-ischemia[32]. Curcumin treatment demonstrated significant improvements in brain connectivity and social behavior in mice, alongside alterations in gut microbiota composition[40]. In anxiety disorders, curcumin alleviates anxiety-like

Table 3 Mechanisms of action of curcumin

Mechanism of action	Ref.
Regulation of Th17/Treg balance	[30]
Modulation of microbial diversity and abundance	[30]
Improvement of gut microbiota composition	[30]
Influence on immune modulation	[29,50]
Restoration of gut flora balance	[17,29,34,35,50]
Enhancement of cytarabine response in acute myeloid leukemia	[36]
Indirect influence on neuroprotection through modulation of signaling pathways	[28,32]
Modulation of intestinal barrier function	[31]
Biotransformation by gut microbiota	[20,33,35]

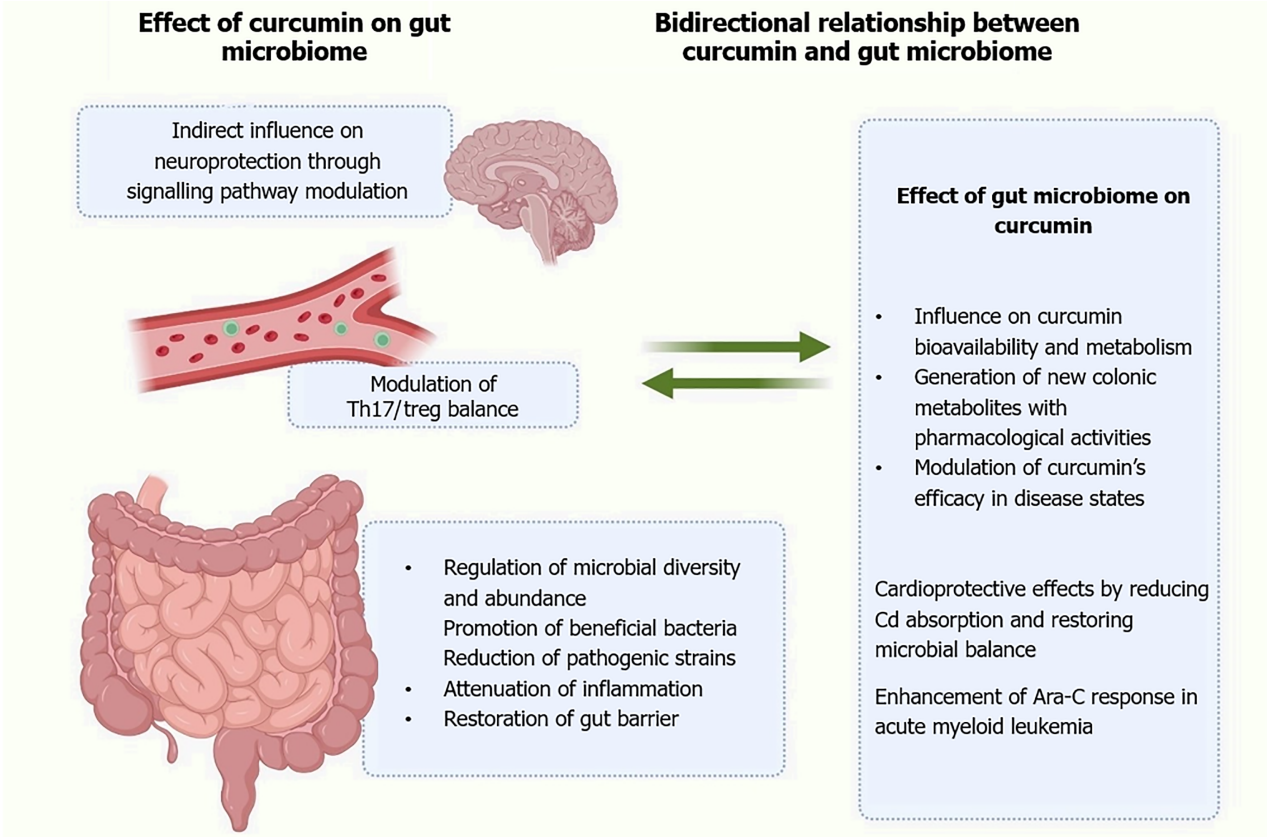

Figure 1 Effect of curcumin on the gut microbiome.

behaviors by modulating the microbiota-gut-brain axis and increasing phosphatidylcholine levels in the prefrontal cortex. Additionally, it influences lipid metabolism and gut microbiota composition to relieve anxiety symptoms[41]. Notably, curcumin's effects on working memory are independent of insulin and linked to body fatness in pre-diabetic individuals, suggesting its potential in cognitive enhancement[42]. Collectively, curcumin exerts its neuroprotective effects through various mechanisms, including scavenging free radicals, modulating synaptic plasticity, regulating neuroinflammation, and altering gut microbiota composition[28]. These multifaceted actions make curcumin a promising candidate for therapeutic intervention in neurologic diseases. Further research exploring curcumin's mechanisms of action and clinical efficacy is warranted to fully harness its therapeutic benefits in neurologic diseases.

GASTROINTESTINAL DISEASES

Numerous studies have demonstrated that curcumin supplementation can exert beneficial effects on gastrointestinal system health by modulating the composition and diversity of the gut microbiota. For instance, Xiao *et al*[30] found that

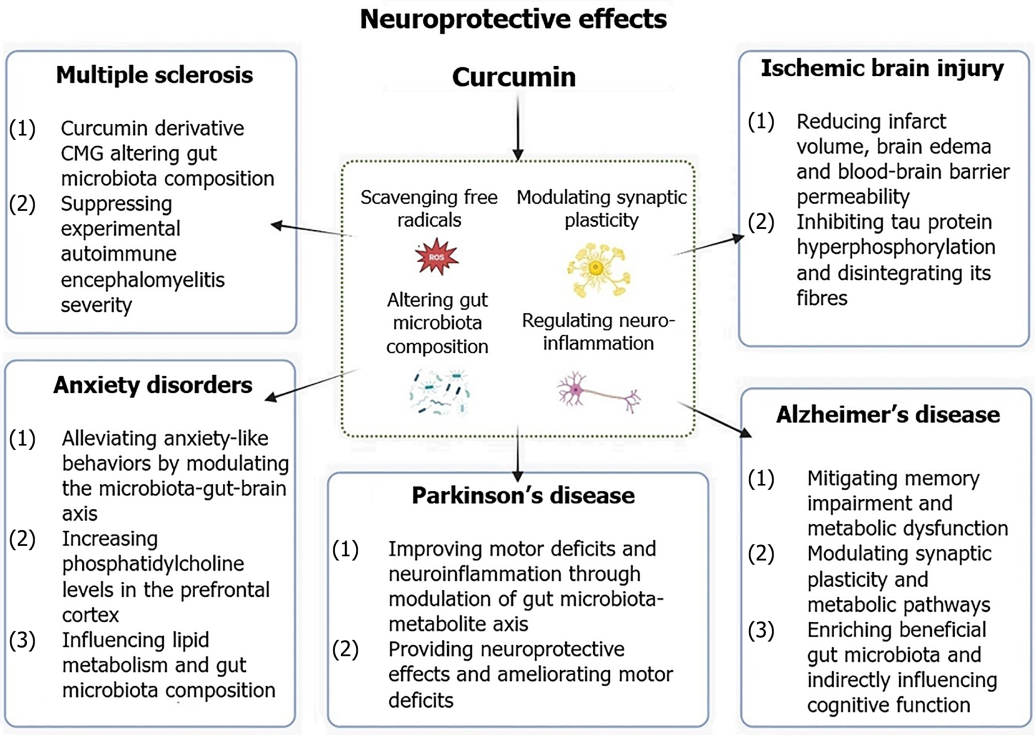

Figure 2 Neuroprotective effects of curcumin.

curcumin improved colitis in diabetic mice by regulating the balance of Th17/Treg cells and restoring intestinal microbiota composition[30]. Similarly, Burge *et al*[29] noted that curcumin supplementation can shift gut microbiota composition towards a profile enriched in short-chain fatty acid-producing bacteria, thereby promoting intestinal mucosal protection and mitigating inflammation associated with intestinal diseases[29]. Lopresti *et al*[43] found that curcumin extract was found to reduce gastrointestinal symptoms in adults. Despite not showing significant effects on the intestinal microbiota, the study observed a reduction in gastrointestinal symptoms following curcumin supplementation [43]. Curcumin's influence on the gut microbiome extends to diseases such as colorectal cancer. Farhana *et al*[44] demonstrated that a combination of curcumin and tocotrienol-rich fraction altered microbial diversity in colorectal cancer cells, suggesting a potential therapeutic synergy in inhibiting colon cancer cell growth[44]. Gan *et al*[45] reported that curcumin and resveratrol, when supplemented in the diet, alleviated intestinal inflammation and regulated gut microbiota composition in piglets, highlighting their potential as dietary interventions for improving gastrointestinal health[45]. In addition to its direct effects on gut microbiota, curcumin also exerts beneficial effects on the gastrointestinal system by enhancing intestinal barrier function. It does so by modulating tight junction proteins, which play a crucial role in maintaining the integrity of the intestinal barrier. By attenuating inflammation and enhancing barrier integrity, curcumin may help protect against gastrointestinal diseases characterized by intestinal barrier dysfunction, such as IBD and leaky gut syndrome[31]. Through its ability to modulate gut microbial composition, attenuate inflammation, and enhance intestinal barrier function, curcumin holds promise as a natural therapeutic agent for promoting gastrointestinal system health and potentially ameliorating a range of gastrointestinal disorders (Table 4). The mechanisms of action of the gut microbiome in gastrointestinal disorders are shown in Table 5.

METABOLIC DYSFUNCTION

Curcumin has garnered significant attention due to its potential therapeutic effects on metabolic dysfunction, particularly in relation to glucose regulation, insulin sensitivity, and diabetes management. Several studies have demonstrated that curcumin supplementation can lead to favorable alterations in gut microbiota composition. For instance, in a study by Hong *et al*[46], curcumin was found to increase the abundance of beneficial bacterial taxa such as *Lachnoclostridium* and *Lactobacillaceae*, while decreasing the levels of potentially harmful bacteria like *Escherichia-Shigella* in CKD patients[46]. Similarly, Zhang *et al*[34] observed that curcumin restored gut microbiota diversity and decreased the abundance of *Lactobacillus*, while increasing levels of *Akkermansia*, thereby mitigating cadmium-induced atherosclerosis[34].

These changes in gut microbial composition induced by curcumin supplementation have been linked to improvements in metabolic parameters. Huang *et al*[47] found that curcumin supplementation improved gut microbiota dysbiosis in diabetic rats, leading to enhanced intestinal barrier function and reduced blood glucose levels[47]. Xiao *et al*[30] reported that curcumin improved diabetes complications by modulating the balance between Th17 and Treg cells in conjunction with regulating gut microbiota composition, underscoring the interplay between immune regulation, gut microbiota, and

Table 4 Implications of gut microbiome in gastrointestinal disorders

Gastrointestinal disorder	Curcumin's effects	Mechanisms of action	Clinical implications
Inflammatory bowel disease	Ulcerative colitis. Reduces disease activity index and endoscopic scores. Increases beneficial bacteria (*Lactobacillus, Bifidobacterium*). Decreases pro-inflammatory bacterial species	NF-κB pathway inhibition; Modulates Th17/Treg balance through microbiota alterations; Improves barrier function	Efficacious as adjunct therapy with mesalamine
	Crohn's disease. Reduces inflammatory markers (TNF-α, IL-1β, IL-6). Strengthens epithelial barrier integrity	Modifies intestinal microbiota composition. Influences bacterial metabolite production	Shows promise in maintaining remission
Colorectal cancer	Suppresses growth of pro-carcinogenic bacteria. Enhances production of beneficial metabolites	Alters microbial diversity in colorectal cancer microenvironment; modulates bacterial enzyme activities related to carcinogenesis	Synergistic effects with conventional chemotherapy
IBS	Reduces abdominal pain and bloating. Normalizes bowel habits	Modifies gut microbiota composition. Improves gut-brain axis signaling	Effects vary across IBS subtypes (IBS-D *vs* IBS-C)
Celiac disease	Reduces intestinal inflammation	Modifies intestinal permeability. Influences microbiota adaptation to gluten-free diet	Potential role in managing non-responsive celiac disease
Gastric Disorders	*Helicobacter pylori* infection. Modification of gastric microbiota	Direct antimicrobial effects. Enhancement of mucosal defense	Synergistic effects with standard triple therapy
	Gastric cancer. Influences *Helicobacter pylori*-associated dysbiosis. Affects cancer stem cell populations	Modulates inflammatory responses	Potential role in prevention and therapy
Small intestinal bacterial overgrowth	Reduces bacterial overgrowth	Modifies small intestinal microbiota composition. Improves intestinal motility	Alleviates small intestinal bacterial overgrowth-associated symptoms
Radiation-induced enteritis	Reduces oxidative stress	Preserves beneficial microbiota. Modulates inflammatory response	Maintains intestinal barrier function
Drug-induced gastrointestinal injury	Non-steroidal anti-inflammatory drugs-induced damage. Maintains microbial homeostasis	Protects against mucosal injury; Reduces oxidative stress	Enhances mucosal recovery
	Chemotherapy-induced mucositis. Preserves microbiota diversity. Reduces inflammatory damage	Supports mucosal healing	Improves treatment tolerance

IBS: Irritable bowel syndrome; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin.

metabolic health[30]. The influence of curcumin on gut microbiota appears to extend beyond direct modulation of microbial populations to impact metabolic pathways. As highlighted by Shen and Ji, polyphenols like curcumin may exert therapeutic effects on metabolic diseases by regulating the gut microbiota[48]. By promoting a microbial profile associated with improved metabolic outcomes, curcumin holds promise as a potential therapeutic agent for addressing metabolic disorders through microbiota-targeted interventions.

MISCELLANEOUS

Cai *et al*[49] investigated curcumin's role in alleviating psoriasis-like inflammation by modulating gut microbiota composition, revealing a correlation between curcumin-induced gut microbiota changes and reductions in psoriasis-related inflammatory factors[49]. Augusti *et al*[33] explored the immunomodulatory properties of curcumin, highlighting its ability to combat inflammatory storms, such as those observed in coronavirus disease 2019. Importantly, curcumin's modulation of the gut microbiota was implicated in influencing disease outcomes, suggesting a potential mechanism by which curcumin exerts its immunomodulatory effects[33]. Liu *et al*[36] investigated curcumin's role in enhancing the response to cytarabine chemotherapy in AML, revealing that curcumin-mediated alterations in the gut microbiota sensitized the response to cytarabine treatment[36].

Collectively, these studies underscore the intricate relationship between curcumin, the gut microbiome, and disease modulation. By influencing gut microbiota composition and function, curcumin holds promise as a therapeutic agent for a wide range of diseases, including neurological disorders, inflammatory conditions, infectious diseases, and cancer. Further research elucidating the mechanisms underlying curcumin-gut microbiome interactions will be crucial for harnessing the full therapeutic potential of this natural compound in disease management and prevention.

Table 5 Mechanism of action of gut microbiome in gastrointestinal disorders

Mechanisms of action	Description	Implications
Direct effects on gut microbiota	Selective pressure on bacterial populations: Curcumin selectively inhibits harmful bacteria while promoting the growth of beneficial microbes	Helps restore a balanced gut microbiome
	Modification of Bacterial Metabolism: Alters metabolic pathways of gut bacteria, affecting their growth and activity	May reduce production of harmful bacterial metabolites
	Influence on bacterial adhesion and biofilm formation: Disrupts bacterial adhesion to gut mucosa and inhibits biofilm formation	Reduces infection risk and persistence of pathogens
	Effects on bacterial virulence factors: Curcumin can suppress the expression of bacterial virulence factors	Lowers pathogenicity of harmful bacterial strains
Host-microbiota interactions	Modulation of immune responses: Modulates gut-associated immune cells, reducing excessive inflammatory responses	Helps in managing inflammatory bowel conditions
	Enhancement of barrier function: Strengthens the intestinal epithelial barrier, preventing translocation of pathogens	Prevents gut permeability ("leaky gut")
	Regulation of mucus production: Promotes mucus secretion in the gut, aiding in the protection of the mucosal lining	Provides an additional layer of defense against pathogens
	Influence on enterocyte function: Enhances the function of enterocytes, the absorptive cells of the intestinal lining	Improves nutrient absorption and gut health
Metabolic effects	Alteration of short-chain fatty acid production: Modulates the production of short-chain fatty acids like butyrate.	Supports gut barrier integrity and reduces inflammation
	Modification of bile acid metabolism: affects the synthesis and transformation of bile acids, impacting digestion and gut health	May alter gut microbial composition and metabolism
	Influence on tryptophan metabolism: Modifies tryptophan metabolism, affecting serotonin production and gut-brain axis signaling	Potentially improves gut-brain communication and mood
	Effects on bacterial enzyme activities: Alters the activities of bacterial enzymes involved in various metabolic processes	Influences gut homeostasis and metabolic health

CHALLENGES AND FUTURE DIRECTIONS

Curcumin, despite its potential therapeutic benefits, faces numerous limitations and challenges that hinder its effectiveness in various disease contexts. One of the primary obstacles is its poor bioavailability, characterized by inadequate absorption and rapid metabolism[32,33,40]. This limitation impedes the attainment and maintenance of therapeutic concentrations of curcumin in the body, thereby limiting its clinical efficacy. Moreover, the bioavailability issues are compounded by challenges in achieving stable concentrations in target tissues[29,40]. These factors pose significant hurdles in realizing its therapeutic potential[28,39,41]. Furthermore, the lack of standardized formulations and inconsistent results from clinical trials contribute to the uncertainty surrounding curcumin's efficacy and safety[19,50]. Curcumin's safety profile is a concern, as evidenced by its cytotoxicity and potential DNA damage, particularly at high doses[42]. These limitations underscore the need for further research to overcome the challenges associated with curcumin's bioavailability, efficacy, and safety to fully harness its therapeutic potential.

Recent advances in understanding curcumin-gut microbiota interactions have opened new avenues for therapeutic applications while raising important questions for future research. Unlike previous reviews that focused on specific aspects of this relationship, our analysis reveals several critical areas requiring further investigation: (1) Temporal dynamics of microbiota changes; (2) Need for longitudinal studies examining the sustainability of curcumin-induced microbiota changes; (3) Investigation of optimal dosing schedules for maintaining beneficial microbiota alterations; (4) Population-specific responses; (5) Examination of genetic and environmental factors influencing individual responses to curcumin; (6) Development of predictive models for personalized curcumin interventions; (7) Novel delivery systems; (8) Investigation of microbiota-targeted delivery systems for enhanced curcumin efficacy; (9) Development of synbiotic formulations combining curcumin with specific probiotic strains; (10) Mechanistic studies; (11) Elucidation of direct *vs* indirect effects of curcumin on specific bacterial populations; (12) Investigation of bacterial metabolites mediating curcumin's therapeutic effects; (13) Clinical applications; (14) Design of microbiota-focused clinical trials for specific disease conditions; and (15) Development of biomarkers for monitoring curcumin-induced microbiota changes. These research directions represent important opportunities for advancing our understanding of curcumin-microbiota interactions and their therapeutic applications.

CONCLUSION

Curcumin, a polyphenolic compound derived from turmeric, exhibits multifaceted pharmacological properties, including

anti-inflammatory, antioxidant, and anticancer effects. Its ability to modulate gut microbiota composition and function further enhances its therapeutic potential. Through the regulation of microbial diversity and abundance, curcumin contributes to the maintenance of gut health and homeostasis, thereby exerting beneficial effects on various disease processes. Studies have demonstrated curcumin's efficacy in neurological disorders, gastrointestinal diseases, metabolic dysfunction, and beyond, with mechanisms involving immune modulation, neuroprotection, and inflammation regulation. However, challenges such as poor bioavailability, inconsistent formulations, and safety concerns warrant further investigation to optimize curcumin's therapeutic utility.

FOOTNOTES

Author contributions: Jeyaraman M and Jeyaraman N contributed to conceptualization; Ramasubramanian S contributed to acquiring clinical data and performing the data analysis; Balaji S and Ramasubramanian S contributed to manuscript writing; Jeyaraman M, Santos GS, da Fonseca LF and Lana JF helped in manuscript revision; Muthu S contributed to image acquisition; Jeyaraman M contributed to proofreading; Jeyaraman M and Lana JF contributed to administration. All authors have agreed to the final version to be published and agree to be accountable for all aspects of the work.

Conflict-of-interest statement: All authors declare no conflict of interest in publishing the manuscript.

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S-Editor: Liu H
L-Editor: Webster JR
P-Editor: Yu HG

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World Journal of Experimental Medicine

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World J Exp Med 2025 March 20; 15(1): 101771

DOI: 10.5493/wjem.v15.i1.101771

ISSN 2220-315x (online)

MINIREVIEWS

Bile acid therapy for primary biliary cholangitis: Pathogenetic validation

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Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification

Scientific Quality: Grade B

Novelty: Grade B

Creativity or Innovation: Grade B

Scientific Significance: Grade B

P-Reviewer: Li YC

Received: September 25, 2024

Revised: October 25, 2024

Accepted: November 7, 2024

Published online: March 20, 2025

Processing time: 91 Days and 10.3 Hours

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Abstract

Knowledge of the etiological and pathogenetic mechanisms of the development of any disease is essential for its treatment. Because the cause of primary biliary cholangitis (PBC), a chronic, slowly progressive cholestatic liver disease, is still unknown, treatment remains symptomatic. Knowledge of the physicochemical properties of various bile acids and the adaptive responses of cholangiocytes and hepatocytes to them has provided an important basis for the development of relatively effective drugs based on hydrophilic bile acids that can potentially slow the progression of the disease. Advances in the use of hydrophilic bile acids for the treatment of PBC are also associated with the discovery of pathogenetic mechanisms of the development of cholangiocyte damage and the appearance of the first signs of this disease. For 35 years, ursodeoxycholic acid (UDCA) has been the unique drug of choice for the treatment of patients with PBC. In recent years, the list of hydrophilic bile acids used to treat cholestatic liver diseases, including PBC, has expanded. In addition to UDCA, the use of obeticholic acid, tauroursodeoxycholic acid and norursodeoxycholic acid as drugs is discussed. The pathogenetic rationale for treatment of PBC with various bile acid drugs is discussed in this review. Emphasis is made on the mechanisms explaining the beneficial therapeutic effects and potential of each of the bile acid as a drug, based on the understanding of the pathogenesis of the initial stages of PBC.

Key Words: Primary biliary cholangitis; Treatment of primary biliary cholangitis with bile acids; Ursodeoxycholic acid; Obeticholic acid; Tauroursodeoxycholic acid; Norursodeoxycholic acid

Core Tip: The review is devoted to the issues of treatment of primary biliary cholangitis (PBC) with bile acid preparations. The mechanisms of beneficial action of ursodeoxycholic acid and its derivatives (tauroursodeoxycholic acid, obeticholic acid, norursodeoxycholic acid) are considered taking into account the pathogenetic mechanisms of PBC development known so far. The assumptions about the development of new therapeutic approaches in the treatment of PBC taking into account the discovery of the disruption of the mechanisms of bicarbonate formation by cholangiocytes in PBC were outlined. This may serve as a basis for the development of new targeting drugs aimed at local reduction of microRNA 506 activity or activation of anion exchanger 2 in cholangiocytes.

Citation: Reshetnyak VI, Maev IV. Bile acid therapy for primary biliary cholangitis: Pathogenetic validation. *World J Exp Med* 2025; 15(1): 101771
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/101771.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.101771

INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic cholestatic progressive liver disease belonging to the cholangiopathies[1]. In untreated patients there is a gradual progression of the disease with the development of damage to the cholangiocytes of the small bile ducts, leading to their proliferation, fibrosis and ductulopenia, accompanied by increasing cholestasis. The second step of the disease is the progression of cholestasis, which leads to the involvement of hepatocytes in the pathological process, their damage, the development of fibrosis and, finally, cholestatic cirrhosis and hepatic cell failure. The treatment of any disease depends on the discovery of the etiologic and pathogenetic mechanisms of its development, as well as the development of appropriate drugs. Because the etiology of PBC is still unknown, there are currently no etiotropic treatments with meaningful efficacy. Therefore, the treatment of patients with PBC is predominantly symptomatic, especially in the late stages of the disease. At the same time, certain successes in the pathogenesis of cholangiocyte and hepatocyte damage development in PBC have been achieved, that allowed to use hydrophilic bile acids for the treatment of this disease. The use of hydrophilic bile acids for the treatment of PBC is associated with significant progress in expanding our understanding of the physiology of the processes of bile formation and biliary excretion, as well as the role of bile acids in the injury and death of biliary epithelial cells (BECs, cholangiocytes) in PBC[2-4]. The new knowledge has helped to better delineate the pathophysiology of cholestasis and the adaptive responses of BECs and hepatocytes to the damaging effects of bile acids. Knowledge of the physicochemical properties of various bile acids and the adaptive responses of cholangiocytes and hepatocytes to them has served as an important basis for the development of relatively effective drugs based on hydrophilic bile acids that can potentially slow down the progression of the disease. The main principle of action of preparations containing hydrophilic bile acids consists in replacement and dilution of toxic (having strong detergent properties) primary bile acids by less toxic and more hydrophilic and easily excreted from the body. Bile acid therapy in PBC aims to slow disease progression, increase life expectancy and improve quality of life. In recent years, the list of hydrophilic bile acids used for the treatment of cholestatic liver diseases, including PBC, has been expanded, and in addition to ursodeoxycholic acid (UDCA), the use of obeticholic acid (OCA), tauroursodeoxycholic acid (TUDCA), and norursodeoxycholic acid (norUDCA) as drugs is being discussed. The review considers the mechanisms explaining the beneficial therapeutic effects and the potential of each of the bile acids as a drug based on the ideas about the pathogenesis of the initial stages of PBC[4]. UDCA was the first drug approved by the United States Food and Drug Administration for the treatment of PBC[5].

UDCA AS FIRST-LINE TREATMENT FOR PBC

In 1987, the German hepatologists Leuschner and Kurtz[6] reported beneficial effects of UDCA in patients with PBC, a disease previously known as primary biliary cirrhosis[7,8]. UDCA is the 7-beta epimer of primary chenodeoxycholic bile acid (CDCA), which has a hydroxy group on the 7-carbon atom at the beta position rather than the alpha position as in CDCA (Figure 1). It is these seemingly minor structural chemical differences that lead to significant pharmacotherapeutic differences between these two bile acids: UDCA is more hydrophilic and less hepatotoxic than CDCA. Studies by many scientists have provided the basis for the accumulation of evidence supporting a positive therapeutic effect, justifying the use of UDCA as a standard of care for the treatment of patients with PBC[8-14].

UDCA has been studied in numerous randomized, placebo-controlled trials in stage I-IV PBC with both positive and inconclusive results[11,15-18]. Clinical studies have shown that oral administration of UDCA at a dose of 13-15 mg/kg/day is well tolerated by patients and has a positive therapeutic effect in cholestatic liver diseases, including PBC[19]. Scientific publications indicate that UDCA improves biochemical markers of cholestasis (alkaline phosphatase, gamma-glutamyl transpeptidase), slows progression of PBC, and delays liver transplantation and death in most patients, with improved survival[20,21]. It has been shown that the efficacy of UDCA use depends on the stage of the disease: The earlier treatment is started (stage I and II), the more effective it is. Some authors believe that transplant-free survival in patients with early-stage PBC treated with UDCA was equivalent to that of age and sex-matched healthy controls[22-24]. The use of UDCA in PBC delays histologic progression of the disease and prolongs survival in patients without liver

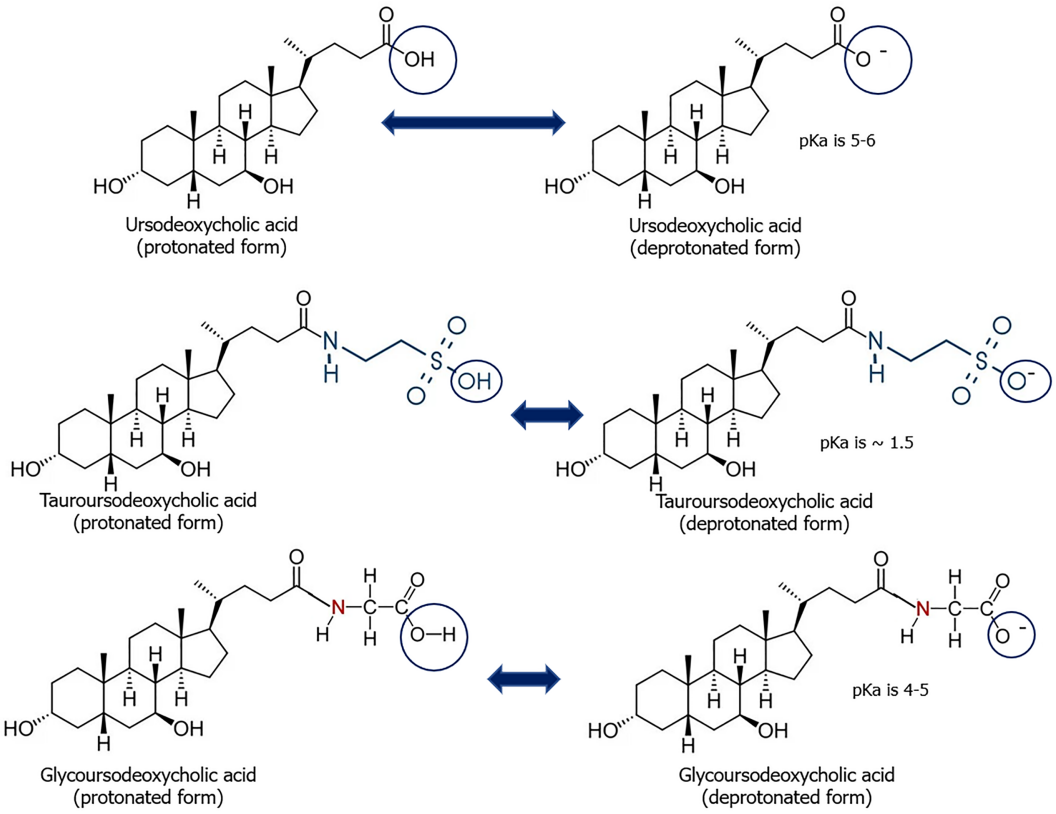

Figure 1 Protonated and deprotonated forms of ursodeoxycholic acid, tauroursodeoxycholic acid, and glycoursodeoxycholic acid.

transplantation, therefore this drug is recommended as first-line therapy for all patients with PBC[19,25]. Despite this, the efficacy of UDCA in PBC remains a matter of debate due to the lack of evidence of efficacy on hard endpoints (*e.g.*, survival or survival without liver transplantation), especially in patients who started UDCA drugs late in the disease[2]. The mechanism of the beneficial effect of UDCA is not fully understood. Clearly, it depends on both its physicochemical properties and its metabolism and enterohepatic circulation. In this regard, it is important to present the metabolism of UDCA in norm, on which factors it depends and how its metabolism changes in PBC.

METABOLISM OF UDCA AND FORMATION OF CONJUGATES WITH TAURINE AND GLYCINE

Human bile normally contains about 5% UDCA. When UDCA is administered orally to treat cholestatic liver diseases, its fraction in bile increases. After oral administration, most of UDCA is absorbed by passive diffusion in the small intestine and travels with the venous blood of the portal vein to the liver, where it is taken up by hepatocytes. The side chain of UDCA is conjugated to glycine or taurine (amidation) in the liver cell. Importantly, 75% of UDCA is conjugated to glycine and 25% to taurine, like the primary bile acids[26]. The UDCA conjugates carry out an enterohepatic circulation. UDCA conjugated to glycine or taurine enters the hepatic bile in a deprotonated state (Figure 1). At neutral [pondus hydrogenii (pH) = 7.4] or slightly alkaline physiological pH of hepatic bile, glycine and taurine conjugates of UDCA are deprotonated, which prevents them from entering cholangiocytes through the biliary bicarbonate "umbrella"[27,28]. The secretion of bicarbonate (HCO_3^-) by BEC to create the latter. Bicarbonate, which has buffering properties, maintains neutral or slightly alkaline pH of hepatic bile, creates a negative charge in the supraepithelial layer, and maintains bile acids in a deprotonated state[29]. Since negative charges repel each other, bile acids in ionized form do not approach the apical surface of cholangiocytes, preventing their entry into BECs. Such a condition has been termed biliary bicarbonate "umbrella". Therefore, in a normal healthy person, bile acids, including UDCA, enter the gallbladder and duodenum in ionized (deprotonated) form and conjugated with glycine or taurine. In the intestine, they take an active part in the emulsification and absorption of fats and fat-soluble vitamins thanks to their detergent properties.

RESPONSE OF UDCA CONJUGATES WITH GLYCINE AND TAURINE TO CHANGES IN THE PH OF THE HEPATIC BILE IN PBC

In recent decades, scientific evidence has emerged regarding the alterations in hepatic bile pH in PBC and its role in the development of cholangiopathy[27]. It has been shown that in PBC there is insufficient synthesis of HCO_3^- by cholan-

giocytes, which is accompanied by insufficient bicarbonate uptake into the bile ducts with simultaneous accumulation in BECs[27,30]. There is evidence that insufficient synthesis and entry of HCO_3^- into the bile ducts in PBC is due to decreased activity of inositol 1,4,5-trisphosphate receptor isoform 3 and chlorine/bicarbonate-anion exchanger 2 (AE2), caused by increased activity of microRNA 506 (miR-506) in cholangiocytes[30]. To date, the factors that trigger the increased expression of *miR-506* gene are still unknown.

As a result of insufficient bicarbonate intake into the bile ducts in PBC there is an acidification of the hepatic bile pH and alkalinization of pH inside small BECs, which leads to impaired metabolism of glycine (but not taurine) conjugates of bile acids, including UDCA. Underlying this impairment are differences in the physicochemical properties of glycine and taurine conjugates of bile acids, which determine their protonation and deprotonation states depending on the pH of the environment. The degree of protonation and deprotonation of bile acids depends on both the pH of the hepatic bile and on the dissociation constant (pKa) of the bile acids. The pKa values for unconjugated bile acids are 5-6[31,32]. Amidation reduces the pKa to 4-5 for glycine conjugates and 1-2 for taurine conjugates[31-33] (Figure 1). Acidification of hepatic bile pH in PBC allows unconjugated bile acids and their glycine conjugates to be easily protonated due to high pKa values[31, 32]. The low pKa values of the taurine conjugates of the bile acids indicate that they are stronger acids than the glycine conjugates. For this reason, taurine-conjugated bile acids will be present in a dissociated (deprotonated, ionized) form even at the highly acidic pH values of the hepatic bile. On the contrary, glycine conjugates of bile acids are weak acids and at the slightest change of bile pH in the acidic direction will quickly change to the protonated state. The latter allows them to easily overcome the biliary bicarbonate "umbrella" and penetrate into small BECs[32].

THE ROLE OF BICARBONATE AND HEPATIC BILE PH IN PROTECTING SMALL CHOLANGIOCYTES FROM BILE ACID INJURY

PBC is a chronic cholestatic progressive liver disease with destruction, apoptosis and necrosis of the epithelium of mainly small intralobular and septal bile ducts, with the development of ductulopenia and cholestasis, in the terminal stage of which liver cirrhosis develops[1]. Development of PBC already in asymptomatic stage of the disease is accompanied by damage to small cholangiocytes and development of ductulopenia leading to cholestasis. To date, there is no clear understanding of why the small cholangiocytes that line the intralobular, interlobular, and septal bile ducts are damaged in PBC. It is thought to occur due to an imbalance between aggression factors "bile acids" of the hepatic bile and the defense factors (biliary bicarbonate "umbrella") of the cholangiocytes[4].

Bile is an aggressive medium for cholangiocytes. The presence of bile acids in bile, which have strong detergent properties, can cause damage to the cell membranes of cholangiocytes. Hydrophobic bile acids are cytotoxic to many cell types[27]. Cholangiocytes are exposed to very high (millimolar) concentrations of hydrophobic bile acids. However, they show no evidence of cytotoxicity[34]. This resistance suggests the presence of mechanisms that protect cholangiocytes from the toxic effects of bile acids in the normal state. Known defense factors that enter the bile during its passage through the bile ducts include the production and secretion of mucin and HCO_3^- by cholangiocytes[35]. In physiological conditions the main function of BECs is biliary secretion of bicarbonate necessary for maintaining neutral or slightly alkaline pH of liver bile[29]. This pH maintains the bile acids in a deprotonated state. Bicarbonate is produced by cholangiocytes throughout the biliary tree.

Mucin glycoproteins are produced by the peribiliary glands (PBG)[36]. The latter are located in the wall of only large intrahepatic and extrahepatic bile ducts and are directly connected with their lumen. Mucin produced by PBG protects cholangiocytes from the damaging effects of bile acids only in large bile ducts[35]. The cholangiocytes of the large intra, and extrahepatic bile ducts have a double defense: The mucin produced by PBG and the bicarbonate. The intralobular, interlobular and septal bile ducts, which are affected in PBC, do not contain PBG, which is accompanied by the absence of mucin in these ducts[36]. As a result, only bicarbonate serves as a defense factor of small BECs at the level of intralobular, interlobular, and septal ducts.

EFFECT OF A DEFECTIVE BILIARY BICARBONATE "UMBRELLA" ON UDCA METABOLISM IN PBC

The primary mechanism responsible for the damage to small BECs and the development of cholestasis in PBC is the entry and accumulation of hydrophobic bile acids within cholangiocytes[4]. The mechanism of uncontrolled entry and accumulation of endogenous bile acids in small BECs is associated with a decrease in the protective role of bicarbonate in PBC. The hypothesis of a malfunctioning biliary bicarbonate "umbrella" is currently a topic of active debate[27,28,37]. This hypothesis is based on a number of clinical and experimental studies demonstrating a reduction in the synthesis of HCO_3^- by cholangiocytes, an insufficient influx it into the bile ducts, and a concurrent accumulation in BECs in PBC[27,30]. Insufficient production and supply of HCO_3^- in the lumen of the bile ducts results in the formation of a so-called defective biliary bicarbonate "umbrella". A shift in the pH of the intraductal "hepatic" bile occurs, moving towards a slightly acidic range, while the pH within the cholangiocytes increases, approaching a slightly alkaline range[30].

The acidification of hepatic bile pH and alkalinization of pH within small BECs in PBC results in the impaired metabolism of glycine (but not taurine) conjugates of primary bile acids and UDCA, which subsequently enter and accumulate in BECs. This results in the apoptosis of small cholangiocytes, the development of ductulopenia, and subsequent cholestasis and toxic "detergent" effects of bile acids on not only cholangiocytes but also hepatocytes as cholestasis progresses[4]. The presence of a mucin-containing glycocalyx layer on the apical surface of large cholan-

giocytes serves to protect them from the penetration and damaging effects of protonated conjugated and unconjugated bile acids. Therefore, they are not implicated in the pathogenesis of PBC development. The prolonged oral administration of UDCA preparations at a dose of 13-15 mg/kg/day has been demonstrated to result in a significant replacement of hydrophobic primary bile acids by less toxic and more hydrophilic UDCA. However, the ratio of glycine (75%) to taurine (25%) UDCA conjugates remains in favor of the former. This allows for the possibility of protonation and penetration of glycine (but not taurine) UDCA conjugates into small BECs through the defective biliary bicarbonate "umbrella" to the same extent as primary bile acids during the acidification of hepatic bile in patients with PBC[4,27]. The moderately alkaline pH within small BECs results in the deprotonation of bile acids. An accumulation of glycine conjugates of bile acids, including UDCA, is observed in cholangiocytes. This is a prerequisite for the cytotoxic (detergent) effects that they exert[27]. However, due to the hydrophilic properties of UDCA, its detergent (toxic, damaging) properties are less than those of primary bile acids, which results in a positive therapeutic effect.

Concurrently, taurine conjugates of UDCA remain in a deprotonated state and are unable to overcome the biliary bicarbonate "umbrella"[4]. Given that taurine conjugates of UDCA, which have low pKa, are in hepatic bile in a deprotonated state, it can be concluded that even at an acidic pH of hepatic bile in PBC, they will not penetrate inside BECs and will not have a damaging effect on cholangiocytes. However, the concentration of taurine conjugates of UDCA in hepatic bile is approximately four times lower than that of glycine conjugates. Consequently, to halt the progression of PBC more effectively, it is essential to enhance the supply of taurine conjugates of UDCA into hepatic bile. This can be achieved through the use of TUDCA, which will lead to the replacement of glycine conjugates of UDCA with taurine conjugates in hepatic bile. One possible avenue for future research is the alkalinization of hepatic bile. However, this is currently impossible due to the lack of appropriate drugs.

THE USE OF TUDCA AND ITS METABOLISM IN PBC

The aforementioned fundamental and clinical studies demonstrate that ionized (deprotonated, negatively charged) taurine conjugates of bile acids are unable to cross the biliary bicarbonate "umbrella" and penetrate into cholangiocytes [34,38-40]. These findings have suggested that TUDCA may have potential as a treatment for cholestatic liver diseases[41, 42]. Despite the fact that UDCA has become a recognized drug in the treatment of cholestatic liver disease a number of studies have been conducted with TUDCA, a natural component of human bile albeit in minute quantities. In patients with PBC, TUDCA was administered at doses of 500 mg, 1000 mg, and 1500 mg per day[43]. The analysis revealed no statistically significant clinical difference between the three doses[43]. The administration of TUDCA has been demonstrated to result in a notable improvement in serum parameter of hepatic enzymes associated with cholestasis and cytolysis. Additionally, favorable alterations in the composition of bile acids in bile have been observed. During drug administration, hepatic bile is enriched in TUDCA, indicating the replacement of primary bile acids. It is shown that a low dose (500 mg) of TUDCA was sufficient to achieve satisfactory enrichment of bile with taurine conjugates of UDCA and to improve biochemical indices[43]. Studies with similar results suggest that a daily dose of about 10 mg/kg body weight per day is appropriate[43,44]. TUDCA administration has also been demonstrated to contribute to the preservation of clinical and functional stability during the waiting period preceding terminal liver transplantation in patients with PBC[44]. Conversely, administration of unconjugated UDCA at the terminal stage of PBC does not exhibit such properties.

The mechanism by which TUDCA exerts a beneficial effect in patients with PBC is attributed to her low dissociation constant (pKa is 1.5-2). As a result, TUDCA exists in an ionized "deprotonated" state in acidified hepatic bile in PBC. The deprotonated state enables her incorporation into the enterohepatic circulation, thereby replacing the majority of glycine-conjugated primary bile acids with tauroursodeoxycholate. This results in a notable reduction in glycine conjugates of primary bile acids, accompanied by a considerable elevation in TUDCA within the hepatic bile. However, complete replacement of glycine conjugates of bile acids by taurine conjugates is not achievable, even with TUDCA administration. Nevertheless, a notable replacement of glycine conjugates of bile acids by taurine conjugates markedly reduces the influx and deleterious impact of the former on cholangiocytes, as well as in the advanced stage of cholestasis and on hepatocytes. Based on the pathogenetic mechanisms, TUDCA should contribute to a more significant suspension of the progression of PBC than when taking unconjugated UDCA. The deceleration of the rate of progression of PBC is depending on the quantity of substituted glycine conjugates of bile acids and the stage of the disease. The reduction in the glycine/taurine ratio observed in patients with PBC may be considered as a compensatory response of the body, aimed at maintaining bile acids in a deprotonated state[45-48]. Further multicenter long-term studies are required to ascertain the efficacy of TUDCA administration. The use of TUDCA in asymptomatic and early-stage disease is likely to be particularly efficacious. In case of positive response of patients to oral administration of UDCA, TUDCA and good tolerability of the drugs therapy should be continued for life.

ADMINISTRATION OF OCA IN PBC

While UDCA treatment has been demonstrated to yield favorable clinical outcomes in the majority of patients, approximately 30%-40% of patients with PBC do not respond adequately to therapy, resulting in an elevated risk of disease progression and significant complications[49]. Agonists of farnesoid X receptors (FXR) and peroxisome proliferator-activated receptors are considered as an efficacious treatment option for patients with cholestatic liver diseases who do not respond adequately to UDCA[25]. OCA is currently being investigated as a potential therapeutic option for patients

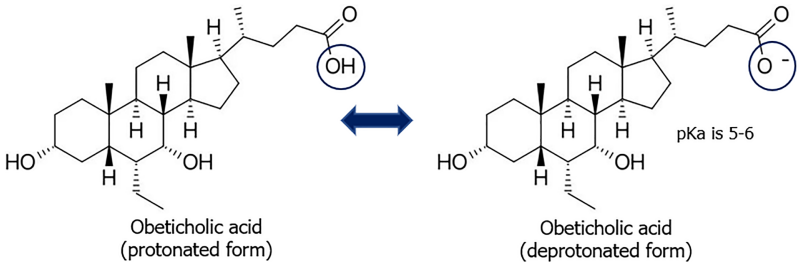

Figure 2 Formulation of the protonated and deprotonated form of obeticholic acid.

with PBC who have not responded to UDCA treatment[50]. In such instances, the treatment regimen is maintained through the concurrent administration of UDCA and OCA[49]. OCA has been approved for use as a second-line therapy for patients with PBC who have not responded adequately to UDCA, or as monotherapy in adults who are intolerant of UDCA[49,51,52]. The rationale for approval OCA was based on the reduction in alkaline phosphatase levels in patients with PBC, which is one of the biomarkers of PBC, and thus indicative of clinical improvement[52]. OCA is a semi-synthetic derivative of CDCA, also known as 6α-ethyl chenodeoxycholic acid, which has a strong affinity for the nuclear FXR (Figure 2)[53]. As a potent selective FXR agonist, OCA has been demonstrated to possess pronounced properties of suppressing bile acid synthesis in cholestatic liver diseases through the transcription of the *CYP7A1*[54].

Concurrently with the inhibition of bile acid synthesis, OCA *via* FXR induces the expression of the bile salt export pump protein, which is responsible for bile acid efflux from the hepatocyte[3,54]. As a result, hepatocytes are protected from the accumulation and toxic "detergent" effects of bile acids[55]. Good tolerability and significant improvement in liver biochemical parameters associated with cholestasis demonstrated in clinical trials of OCA in PBC[49,52]. Results of a randomized, double-blind, phase 3 study comparing OCA to placebo showed that approximately 50% of patients achieved a significant reduction in serum alkaline phosphatase levels, a marker that predicts disease progression in PBC [49]. Cutaneous pruritus was most common adverse event in PBC patients and OCA dose-dependent[51]. According to Li *et al*[51] skin itching was more frequent in the combination therapy group (61.3%) than in the monotherapy group (42.3%). In patients with PBC, OCA is recommended at a dose of 5 mg daily, which is more effective than doses of 10 mg ($P = 0.001$), 25 mg ($P = 0.06$), and 50 mg ($P = 0.04$) OCA, with the lowest risk of side effects[52].

METABOLISM OF OCA IN PBC PATIENTS AFTER ORAL ADMINISTRATION

In the small intestine, OCA is taken up by enterocytes and transported with the venous blood of the portal vein to hepatocytes, where it is conjugated with glycine (75%) and taurine (25%) in the same ratio as the primary bile acids. The mechanism of action of OCA is the inhibition of primary bile acid synthesis *via* FXR, accompanied by a decrease in their levels in hepatocytes and hepatic bile, and the substitution of primary bile acids by OCA. Induction of expression of bile acid transporter proteins on hepatocyte membrane leads to decrease of bile acid content in hepatocytes, which is very important for prevention of their damaging effect on membrane structures of liver cells. And on the other hand, against the background of OCA administration and increased intake of bile acids into hepatic bile, there is an increase of bile acid excretion into the systemic blood flow due to the presence of ductulopenia and intrahepatic cholestasis in PBC. And the more pronounced the cholestasis, the greater is the efflux of OCA into the general bloodstream and the greater is the probability of development of an undesirable side effect, skin itching. The entry of increased amounts of OCA into the general bloodstream leads to the involvement of the kidneys and skin in the process of her excretion from the body. Getting into the skin and having strong detergent (on lipid components of myelin sheath of nerve fibers) and irritating properties on nerve endings OCA causes intensification of skin itching, which has a dose-dependent effect[51,52].

In spite of decrease in accumulation and damaging effect of bile acids on hepatocytes, OCA administration does not reduce toxic, detergent effect on cholangiocytes, which is due to OCA metabolism. The predominant glycine conjugates of primary bile acids and OCA in acidified hepatic bile of PBC patients undergo protonation (Figure 2). This promotes overcoming of biliary bicarbonate "umbrella" by glycine conjugates of OCA and her entry into the cholangiocytes. There is an accumulation and damaging effect of glycine conjugates of primary bile acids and OCA on cholangiocytes, due to the change in pH of hepatic bile to acidic region and alkalinization of BECs cytosol in patients with PBC. OCA is not recommended in patients with advanced stage PBC[51,52]. Given the differing mechanisms of action of UDCA and OCA, combined therapy using both drugs are recommended for refractory PBC. The combined use of UDCA and OCA has been shown to result in a positive therapeutic effect due to the inhibition of primary bile acid synthesis by OCA and the replacement of toxic, hydrophobic primary bile acids with more hydrophilic, less toxic UDCA for cholangiocytes and hepatocytes[51]. Given the mechanisms of action of bile acid preparations as above described, it can be postulated that the combination of TUDCA and OCA will prove to be a more efficacious treatment. The use of OCA in combination with tauroursodeoxycholate may become a vital means of pharmacotherapy for patients with refractory PBC. The therapeutic efficacy of OCA can likely be enhanced by developing a novel drug formulation, specifically a taurine conjugate of OCA that is sulfated at the 3rd carbon atom of the cyclopentane-perhydrophenanthrene ring. It is anticipated that the latter will result in a reduction of the compound's toxic properties, an increase in her water solubility, and enhanced excretion from the body *via* urine[56,57].

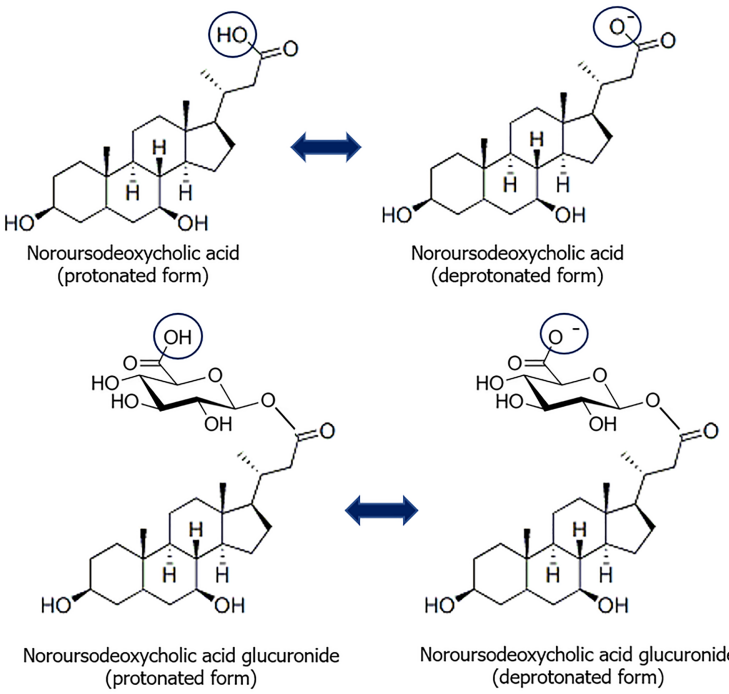

Figure 3 Formulas of norursodeoxycholic acid and its glucuronide in protonated and deprotonated forms.

THE USE OF NORUDCA IN CHOLESTATIC DISEASES

The administration of norUDCA has been described as an alternative to UDCA or for concomitant use with it for the treatment of a number of cholestatic liver and biliary tract diseases[58]. NorUDCA is a derivative of UDCA with a shortened side chain, in which one methyl group is absent. This structural modification confers relative resistance to the side-chain amidation (Figure 3)[59]. Nordihydroxy bile acids, such as norUDCA, are excreted into the bile partly unchanged and partly as glucuronide or sulfate conjugate[59,60]. In humans, the majority of norUDCA is metabolized *via* side-chain glucuronidation in hepatocytes, rather than through amidation with glycine or taurine[61]. The glucuronidation of the side chain, as opposed to amidation, endows norUDCA with distinctive physiological and pharmacological attributes. Instead of undergoing a complete enterohepatic circulation, it is capable of undergoing cholehepatic shunting (Figure 4)[61-63]. As a result of side chain glucuronidation, there is significant renal elimination of the glucuronide C-23 ester of norUDCA[61]. It seems probable that renal excretion occurs as a consequence of the efflux of norUDCA glucuronide from the hepatocyte to blood plasma *via* the basolateral membrane, with the involvement of a transporter belonging to the multiple drug resistance family (Figure 4)[64].

NorUDCA is considered as a bile acid with choleretic properties, what is associated with her cholehepatic shunting[61,65]. Based on animal studies, it has been shown that the physicochemical properties of norUDCA glucuronide promote its constant flow through the BECs of the bile ducts "cholehepatic circulation", which may be of therapeutic importance[61]. Due to its hydrophilic properties and glucuronidation of side-chain, norUDCA is considered a promising pharmacological agent for the treatment of a variety of cholestatic liver and biliary diseases. NorUDCA has been successfully tested clinically in patients with primary sclerosing cholangitis[58]. A double-blind, randomized, multicenter, placebo-controlled, comparative phase III study on oral administration of norUDCA at a dose of 1500 mg/day for the treatment of primary sclerosing cholangitis was conducted. NorUDCA administration resulted in a dose-dependent decrease in serum levels of alkaline phosphate and other liver enzymes after 12 weeks of treatment[66]. NorUDCA was effective both in patients who had previously taken UDCA (whether or not they responded to UDCA therapy) and in patients who had not previously taken UDCA[66,67]. According to the authors, the drug was well tolerated. The number of treatment-related adverse events was similar in all groups[58,66].

METABOLISM OF NORUDCA DURING ORAL ADMINISTRATION

The mechanism of action that mediates the beneficial effects of norUDCA continues to be the subject of ongoing research [68,69]. Beuers *et al*[70] suggest that it is likely that norUDCA passing through cholangiocytes stimulates bicarbonate secretion by BECs to maintain a protective biliary bicarbonate "umbrella". However, this statement is not supported by experimental studies[71]. Denk *et al*[71] showed that norUDCA administration has a choleretic effect only in normal isolated perfused rat liver and has no anticholestatic effect in an experimental model of induced cholestasis. But norUDCA taurine conjugate (TnorUDCA) was effective, although inferior to UDCA taurine conjugate[71]. Indirectly, these data indicate another mechanism of action of norUDCA. The physicochemical properties of norUDCA suggest the

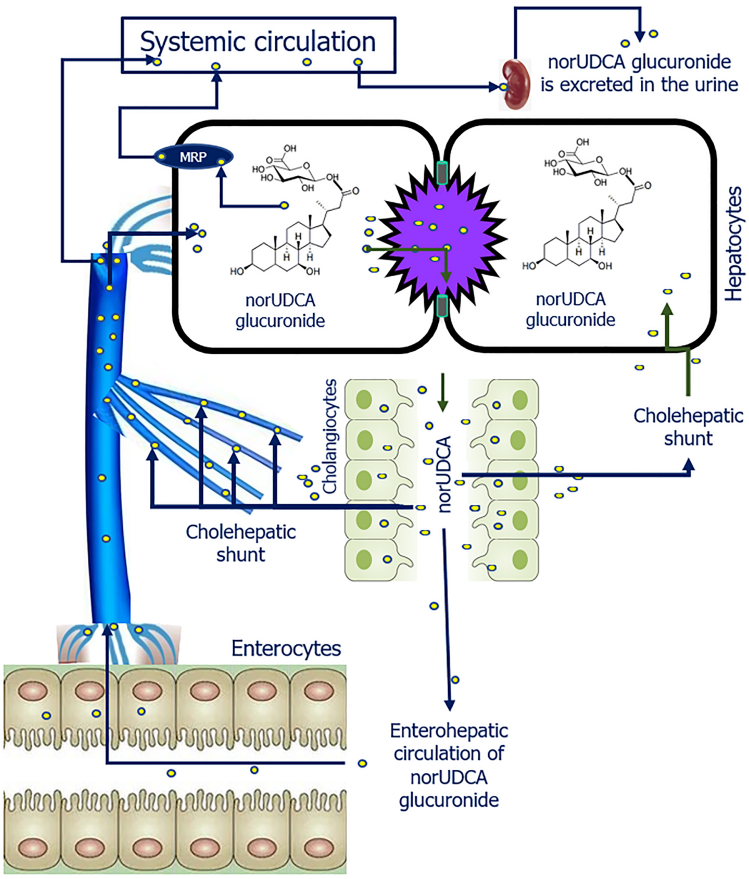

Figure 4 Cholehepatic bypass and metabolism of norursodeoxycholic acid glucuronide. norUDCA: Norursodeoxycholic acid glucuronide; MRP: Multidrug-resistance protein.

following metabolic mechanism. After being absorbed in the intestine, norUDCA is transported through the portal vein system to the liver, where it is taken up by the hepatocytes. In liver cells, the side chain of norUDCA is glucuronidated (glucuronides of norUDCA are formed), giving her the properties of a very weak acid. The latter, entering hepatic bile and having properties of very weak acid, is protonated and easily overcomes biliary bicarbonate "umbrella" enters cholangiocytes. At normal pH of the BECs cytosol, it is excreted into the peribiliary space and, once in the blood, is transported to hepatocytes and the systemic circulation (Figure 4). The portion of norUDCA glucuronides that enters the liver cells is included into the cholehepatic (and only partially into the enterohepatic) circulation and partially enters the systemic circulation by eflux[64]. There is a partial replacement of primary bile acids by the glucuronide norUDCA, which has a less toxic effect on hepatocytes and BECs due to the formation of esters with glucuronic acid. The portion of norUDCA glucuronides that enters the systemic circulation due to the presence of glucuronic acid in the molecule is easily excreted from the body by the kidneys[61]. This mechanism is most likely the basis for the beneficial effect of norUDCA when used in patients with primary sclerosing cholangitis[58].

However, in PBC, bicarbonate supply to the bile ducts is disturbed, hepatic bile acidification and pH alkalinization within the cholangiocytes occur. Glucuronides of norUDCA are very easily protonated (due to acidification of hepatic bile pH) and overcoming the biliary bicarbonate "umbrella" enter cholangiocytes. Since inside BECs at PBC there is alkalinization of cytosol, glucuronides of norUDCA can be deprotonated and their exit to peribiliary space will be worsened. This will lead to retention and accumulation of norUDCA glucuronides in cholangiocytes, with subsequent damaging effect, although to a lesser extent than primary bile acids due to their hydrophilicity and glucuronidation of the side chain. Probably, this mechanism can explain the absence of anticholestatic effect in the experimental model of induced cholestasis[71]. Denk *et al*[71] showed that conjugation of norUDCA with taurine is necessary to achieve the anticholestatic effect. Taurine conjugates of norUDCA are a strong acid. And they will not be protonated in hepatic bile, will not be in the BECs, and will not be part of the cholehepatic shunt. Meanwhile, TnorUDCA will be part of the enterohepatic circulation and will be a substitute for primary bile acids. This may explain the Denk *et al*[71] efficacy result of TnorUDCA similar to the taurine conjugate of UDCA. It may be possible to increase the therapeutic efficacy of TnorUDCA by sulfation or glucuronidation of the third carbon atom of the cyclopentane-perhydrophenanthrene ring. The latter should reduce her toxic properties, increase her water solubility and her excretion from the body with urine[56,57,61].

CONCLUSION

Treatment of PBC remains a challenge, as the cause causing this chronic, slowly progressive cholestatic disease has not been identified. Advances in the use of hydrophilic bile acids for the treatment of PBC are associated with progress in the study of the physicochemical properties of bile acids and the disclosure of the pathogenetic mechanisms of cholangiocyte damage development and appearance of the first signs of this disease. The use of bile acid preparations (UDCA, TUDCA, OCA, norUDCA) in the treatment of PBC has resulted in slowing the progression of the disease and improving the quality of life in these patients. Unfortunately, the treatment of PBC with bile acid preparations is not associated with a complete cure of the disease. The revelation of the mechanisms underlying the positive therapeutic effect of these drugs, described in this review, demonstrates the limited efficacy of bile acid drugs.

Therefore, there is an urgent need for the development of new, more effective drugs and treatment methods for this cholestatic disease. At the same time, new drugs should take into account the data on the mechanism of development of initial signs of PBC, on metabolism of various forms and conjugates of hydrophilic bile acids used for treatment of this disease, and also new targets revealed by deeper study of the pathophysiology of the disease[4,72]. New drugs based on UDCA and her derivatives should contain in their structure taurine in the side chain, as well as glucuronic acid or sulfogroup at the 3-carbon atom of the cyclopentane-perhydrophenanthrene ring in order to increase the efficacy, improve urinary excretion and reduce the number of side effects. The taurine conjugates will maintain the deprotonated form of the bile acids in the acidified hepatic bile of PBC patients, and the sulfogroup or glucuronic acid will reduce toxicities and increase aqueous solubility and renal excretion. At the same time, intestinal absorption of such drugs will be reduced, which will need to be taken into account when selecting the dosage of the drug. Such drugs are designed to better stop the symptoms and further inhibit PBC from progressing. The development of such drugs and the conduct of experimental and multicenter clinical trials can be expected in the near future. After 35 years of using UDCA as a unique drug of choice for patients with PBC, a number of targets have been identified based on a deeper understanding of the pathophysiology of the disease. These include the discovery of impaired mechanisms of bicarbonate formation by cholangiocytes in PBC, through decreased activity of inositol-1,4,5-trisphosphate receptor isoform 3 and chlorine/bicarbonate AE2, caused by increased miR-506 activity. This may provide a rationale for the future development of new drugs aimed at locally reducing miR-506 activity or activating the AE2 in cholangiocytes. It is likely to be one of the new therapeutic approaches in the treatment PBC or will complement existing methods that use hydrophilic bile acids.

FOOTNOTES

Author contributions: Reshetnyak VI and Maev IV have contributed to the study conception and design, literature review and analysis, drafting, critical revision and editing, and final approval of the final version; they contributed equally to this article, they are the co-first authors of this manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Bai Y
L-Editor: A
P-Editor: Yu HG

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World Journal of Experimental Medicine

Submit a Manuscript: https://www.f6publishing.com

World J Exp Med 2025 March 20; 15(1): 99516

DOI: 10.5493/wjem.v15.i1.99516

ISSN 2220-315x (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Prevalence of *RUNX1* gene alterations in *de novo* adult acute myeloid leukemia

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Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification
Scientific Quality: Grade B
Novelty: Grade B
Creativity or Innovation: Grade B
Scientific Significance: Grade B

P-Reviewer: Deng J

Received: July 24, 2024
Revised: September 17, 2024
Accepted: October 22, 2024
Published online: March 20, 2025
Processing time: 154 Days and 17.3 Hours

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Abstract

BACKGROUND

Acute myeloid leukemia (AML) is a complicated disease with uncontrolled hematopoietic precursor proliferation induced by various genetic alterations. Runt-related transcription factor-1 (*RUNX1*) is commonly disrupted by chromosomal translocations in hematological malignancies.

AIM

To characterize *RUNX1* gene rearrangements and copy number variations in newly diagnosed adult AML patients, with an emphasis on the impact of clinical and laboratory features on the outcome.

METHODS

Fluorescence in situ hybridization was used to test *RUNX1* gene alterations in 77 newly diagnosed adult AML cases. *NPM1*, *FLT3/ITD*, *FLT3/TKD*, and *KIT* mutations were tested by PCR. Prognostic clinical and laboratory findings were studied in relation to *RUNX1* alterations.

RESULTS

RUNX1 abnormalities were detected by fluorescence in situ hybridization in 41.6% of patients: 20.8% had translocations, 22.1% had amplification, and 5.2%

had deletion. Translocations prevailed in AML-M2 (P = 0.019) with a positive expression of myeloperoxidase (P = 0.031), whereas deletions dominated in M4 and M5 subtypes (P = 0.008) with a positive association with CD64 expression (P = 0.05). The modal chromosomal number was higher in cases having amplifications (P = 0.007) and lower in those with deletions (P = 0.008). *RUNX1* abnormalities were associated with complex karyotypes (P < 0.001) and were mutually exclusive of *NPM1* mutations. After 44 months of follow-up, *RUNX1* abnormalities affected neither patients' response to treatment nor overall survival.

CONCLUSION

RUNX1 abnormalities were mutually exclusive of *NPM1* mutations. *RUNX1* abnormalities affected neither patients' response to treatment nor overall survival.

Key Words: Acute myeloid leukemia; Deletion; Disease-free survival; Fluorescence *in-situ* hybridization; Karyotyping; *RUNX1*

Core Tip: In the current study, we characterized the runt-related transcription factor-1 (*RUNX1*) gene rearrangements and copy number variations in patients with newly diagnosed adult acute myeloid leukemia with an emphasis on the impact of clinical and laboratory features on the outcome. *RUNX1* abnormalities were mutually exclusive of *NPM1* mutations. *RUNX1* abnormalities affected neither patients' response to treatment nor overall survival.

Citation: Abd El-Ghany HM, El Ashry MS, Abdellateif MS, Rabea A, Sultan N, Abd El Dayem OY. Prevalence of *RUNX1* gene alterations in *de novo* adult acute myeloid leukemia. *World J Exp Med* 2025; 15(1): 99516
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/99516.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.99516

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous hematologic cancer characterized by the clonal growth of myeloid blasts in the blood, bone marrow (BM), and/or other tissues. It is the most prevalent type of acute leukemia in adults[1]. Over the past few decades, the discovery of recurrent structurally balanced and unbalanced chromosomal abnormalities has significantly influenced the clinical management of patients with AML. These chromosomal abnormalities are the most significant prognostic markers where they can define specific clinicopathologic entities of the disease. The current recommendations of the European Leukemia Network for genetic testing in AML are primarily focused on risk stratification in order to identify an effective therapeutic strategy[2].

Runt-related transcription factor 1 (RUNX1) is the founding member of the mammalian core-binding transcription factor family, which also includes RUNX2, RUNX3, and their non-DNA-binding cofactor CBF[3]. The significance of the transcription factor *RUNX1* in the t (8; 21) translocation in AML attracted initial interest. Since its discovery, RUNX1 has been found to play significant roles not only in leukemia but also in the development of the normal hematopoietic system [3]. Additionally, *RUNX1* has been associated with epithelial tissue development and carcinogenesis[4]. The *RUNX1* gene occupies approximately 261 kb on the long arm of chromosome 21. It controls the expression of genes involved in hematopoietic differentiation, ribosome synthesis, cell cycle regulation, p53, and transforming growth factor signaling pathways *via* interacting with various proteins through its domains[5].

Four forms of acquired *RUNX1* genetic abnormalities have been identified in AML: (1) Translocations involving *RUNX1* that result in fusion genes; (2) Molecular mutations; (3) *RUNX1* amplifications; and (4) Partial or total deletions of the *RUNX1* gene. It has been observed that *RUNX1* deletions and gains were most common in patients with unfavorable cytogenetics and that the prognosis differed dramatically, being best for individuals with *RUNX1* translocations and worst for those with deletions[6].

There have been reports of the *RUNX1* gene fusing with over 40 partner genes encoding structurally diverse proteins. Some RUNX1-fusions are frequent in AML, and their partner genes are known to be implicated in recurrent translocations, including *RUNX1 RUNX1T1*/t (8; 21) (q22; q22), t (3; 21) (q26.2; q22), t (1; 21) (p36; q22), and t (16; 21) (q24; q22). These translocations have been investigated extensively, and their potential prognostic influence is uncertain. Others have been documented in only a small number of trials, and their potential prognostic influence is uncertain[7].

Genetic testing of patients with newly diagnosed AML with *RUNX1* fluorescence in situ hybridization (FISH) probe provides the opportunity to identify more instances with minor rearrangements or new partner genes of the *RUNX1* locus. This will improve our understanding of the prognosis for these instances and may ultimately aid in the establishment of a very successful therapeutic treatment plan[6] that offers tailored therapy options for a large number of patients and future opportunities to prevent the development of AML[8].

This study aimed to determine the prevalence of *RUNX1* gene changes in patients with newly diagnosed AML and their influence on clinical outcomes. Various prognostic markers and other clinical and laboratory results were examined in relation to the expression of *RUNX1* genetic variations.

MATERIALS AND METHODS

Patients

Among the 263 adult patients who were diagnosed with AML between January 2018 and July 2019, 77 adult patients with *de novo* AML were included in this study. All patients were presented to the Outpatient Clinic of the Medical Oncology Department of the National Cancer Institute in Cairo, Egypt. Patients with acute promyelocytic leukemia were omitted from the study since their therapy and prognosis differ significantly from other patients with AML. Additionally, those with a history of hematologic disorders were excluded. The diagnosis of AML was based on morphological assessment of peripheral blood (PB) and BM smears, cytochemistry, immunophenotyping by flow cytometry, conventional cytogenetics, and molecular studies according to French-American-British (FAB) and World Health Organization parameters[9].

All patients underwent standard induction chemotherapy with the 3 + 7 treatment protocol (doxorubicin as a 3-day brief infusion and cytarabine 100 mg/m^2 as a 7-day continuous infusion). Depending on their risk assessment, patients who achieved complete remission (CR) were offered consolidation with high-dose cytarabine and human leukocyte antigen matching, followed by allogeneic BM transplantation. Refractory cases were given a high-dose cytarabine-based regimen for re-induction.

Clinical endpoints

Response to induction therapy was evaluated between days 14 and 28 post-induction. Response was categorized as CR, partial remission, stable disease, relapsed disease, or refractory disease. CR was defined as BM blasts 5%, absence of blasts with Auer rods, lack of extramedullary illness, neutrophil count > 1.0 × 10^9/L, platelet count > 100 × 10^9/L, and independence from red cell transfusions[10]. Patients who attained CR were divided into two groups termed normal recovery or delayed recovery based on whether they achieved CR before or after day 35, respectively[11]. Treatment failures were due to either disease resistance or relapse. The resistant disease was defined as the inability to achieve CR after completion of the initial treatment, with evidence of residual leukemia by PB and/or BM examination. Relapse was defined as BM blasts ≥ 5%, recurrence of blasts in PB, or the development of extramedullary illness.

Disease-free survival (DFS) was only defined for patients who attained a CR. It was calculated from the date of CR until the date of relapse or death, regardless of cause, censoring patients who were still alive at the time of the last follow-up. Overall survival (OS) was estimated from the date of protocol inclusion to the date of death or last follow-up/ measured from the date of diagnosis to the date of death or last follow-up.

Cytogenetic analysis

Pretreatment diagnostic conventional karyotyping was applied to BM samples employing G-banded metaphase cells from unstimulated 24-h cultures following the standard techniques. Using the IKAROS imaging system, at least 20 metaphases were analyzed in the majority of cases (Metasystems, Altlussheim, Germany). Through using International System for Human Cytogenetic Nomenclature, the karyotypes were interpreted (ISCN 2016)[12]. FISH was performed according to the manufacturer's instructions using locus-specific probes XL *RUNX1* dual-color Break-Apart probe (MetaSystems) to detect *RUNX1* rearrangements, deletions, and amplifications, which together represented total *RUNX1* abnormalities.

A minimum of 10 metaphases and 200 interphase nuclei were studied using a fluorescent microscope (AxioImager.Z1 mot; Carl Zeiss Ltd., Hertshire, United Kingdom) with the proper filter settings. The ISIS imaging system was utilized for image capture and processing (Metasystems).

Molecular detection of fusion gene transcripts and mutational analysis

Total RNA was extracted from BM or PB samples using Qiagen RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse transcribed using a high-capacity complementary DNA reverse transcription Kit (Applied Biosystems, Waltham, MA, United States) for the identification of fusion transcripts t (9; 22) (q34; q11), t (8; 21) (q22; q22), and inv (16) (p13q22) in accordance with the BIOMED-1 guidelines[13]. Mutation analysis of four other significant molecular marker genes, *NPM1*, *FLT3/ITD*, *FLT3/TKD*, and *KIT*, was carried out using genomic DNA-PCR as directed by the manufacturer.

Immunophenotype analysis

In all cases, blast cells in bone marrow aspiration samples were immunophenotyped using an EPICS XL Coulter Flow Cytometer (Beckman Coulter, Hialeah, FL, United States). A large panel of myeloid markers [myeloperoxidase (MPO), CD13, CD33, CD117, CD14, and CD15], lymphoid markers (CD10, CD19, CD22, CD79a, CD20, Cyto IgM, Kappa and Lambda for B lymphoid series, and CD3, CD2, 4, 8, 7, and 5 for T lymphoid series), and the stem cell marker CD34, as well as CD56 and human leukocyte antigen-DR, were used to confirm the diagnosis of AML.

Statistical analysis

Using version 22 of the statistical software program SPSS, data were analyzed (IBM, Armonk, NY, United States). According to the relevant normality test, quantitative data were described as mean ± standard deviation or median and interquartile ranges or as numbers and percentages for qualitative data. The relationship between *RUNX-1* anomaly and patient clinical characteristics was evaluated using the χ^2 test and/or Fisher exact test, where applicable. Numerical variables from two groups were compared using the Mann-Whitney test. The Kaplan-Meier test was utilized for survival analysis, whilst the log-rank test was utilized to compare survival curves. All tests were run with an alpha level of 0.05 and a confidence interval of 95%.

Compliance with ethical standards

Every patient gave a written informed consent. The study was conducted in accordance with the Helsinki Declaration of 2011 and was approved by the internal review board of the National Cancer Institute and the Faculty of Medicine Research Ethics Committee at Cairo University (Code: MS-38-2020).

RESULTS

Patient characteristics

The median age of the 77 Egyptian patients with *de novo* AML was 42 (range of 18 to 82) years. Males represented 61% (47/77) of patients. Thirty-eight patients (49.4%) were AML-M2, while 22 patients (28.6%) were AML-M4. Based on genetic findings, the patients with AML were classified according to the European LeukemiaNet genetic risk classification into 18 patients (23.4%) with low risk, 42 patients (54.5%) with intermediate risk, and 17 patients (22.1%) with high-risk stratification. By molecular screening, recurrent translocations were identified in 15/77 cases (19.5%); 7 cases (9.1%) with t (8; 21) (q22; q22), 6 cases (7.8%) with inv (16) (p13q22), and 2 cases (2.6%) with t (9; 22) (q34; q11.2). Forty cases (51.9%) achieved CR, while 36 cases (46.8%) died before day 28. The detailed demographic, clinical, and laboratory characteristics of our patients are illustrated in Table 1.

Cytogenetics analysis

Excluding 8 patients who failed to show mitosis, 22 out of 69 patients (31.9%) had normal karyotyping, while 9/69 patients (13.0%) had a complex karyotype including 4 cases with recurrent translocations. The median of the modal chromosome number (MCN) was 45.8 (range: 32-53); 10 patients (14.5%) had hypodiploidy, while 12 cases (17.4%) had a hyperdiploid karyotype (MCN > 46) including 4 cases with concurrent recurrent translocations, 2 cases with inv (16) (p13q22), 1 case with t (8; 21) (q22; q22), and 1 case with t (9; 22) (q34; q11.2).

RUNX1 aberrations in de novo AML

By FISH, *RUNX1* gene abnormalities were found in 32/77 patients (41.6%): 16 patients (20.8%) showed *RUNX1* rearrangements; 17 patients (22.1%) had *RUNX1* amplifications; and 4 patients (5.2%) had *RUNX1* deletion encompassing the whole *RUNX1* gene (Figure 1A).

Out of 16 cases with *RUNX1* translocations, 7 cases (43.8%) had t (8; 21) (q22; q22), 2 cases (12.5%) had t (1; 21) (Figure 1B-D), 1 case (6.3%) had t (16; 21), and 6 cases (37.5%) had unidentified partner chromosome. One or more copies of chromosome 21 in a hyperdiploid karyotype were gained in 4/17 cases (23.5%), while 10/ 17 cases (58.8%) showed *RUNX1* duplications including 4 cases with concurrent *RUNX1* translocations and 1 case with isochromosome 21. Three cases (17.6%) failed to show mitosis. Therefore, the differentiation could not be done.

Two out of four cases (50%) had -21; 1 case with a hypodiploid karyotype and another case with concurrent inv16 in a complex karyotype. Deletion of *RUNX1* was found in 2/4 cases (50%) in a diploid karyotype including 1 case with concurrent *RUNX1* translocations. The clinical characteristics of patients with and without different *RUNX1* aberrations at diagnosis are presented in Table 1.

Correlation of RUNX1 aberrations with clinical features and hematological findings

Although no statistically significant correlation between *RUNX1* abnormalities and hepatomegaly, lymphadenopathy, or splenomegaly was found (P = 0.434, 0.808, and 0.404, respectively), patients with *RUNX1* amplification presented with splenomegaly (P = 0.009) and 52.9% of them had lymphadenopathy (P = 0.076).

Hypercellular BM was more frequent in patients with *RUNX1* abnormalities (68.8%) and translocations (62.5%) than normocellular and hypocellular marrow (P = 0.042 and 0.09, respectively).

In *RUNX1* translocation positive cases, AML-M2 (43.8%) was the most frequent FAB subtype, and M0 and M7 tended to be more frequent among them than in *RUNX1* translocations negative cases (75% *vs* 25% and 100% *vs* 0%, respectively; P = 0.019). In addition, *RUNX1* translocations were positively associated with MPO expression (P = 0.031). Regarding *RUNX1* deletion, all cases were of the myeloid with monocytic phenotype (FAB-M4 and M5) (P = 0.008) and were positively associated with the expression of CD64 (P = 0.050). Otherwise, there was no significant difference between *RUNX1* positive and negative cases in different clinical characteristics. The comparison of clinical characteristics of patients with and without *RUNX1* gene alterations is shown in (Table 2 and Table 3).

Association of RUNX1 aberrations with cytogenetic and molecular abnormalities

There was a highly statistically significant relation between *RUNX1* copy number variations and MCN where positive cases to *RUNX1* amplification tended to have higher MCN, while *RUNX1* deletion cases tended to have lower MCN (P = 0.007 and 0.008, respectively). Cases positive to *RUNX1* abnormalities, translocations, and amplifications tended to have complex karyotypes compared to *RUNX1*-negative cases (P = 0.000, 0.001, and 0.001, respectively). There was no statistically significant correlation between all types of *RUNX1* abnormalities and other different cytogenetic abnormalities (as -2, -3, -7, -11, -13, -22, +8, +13, +17), inv16, and t (9; 22) (P = 0.623, 0.670, and 0.806, respectively).

To investigate the interaction of gene mutations in the pathogenesis of adult AML, screening of mutational status of four other genes was performed. Among the 32 patients with *RUNX1* abnormalities, 4 cases showed additional molecular abnormalities including 2 cases with *RUNX1* deletion, of which one had an *FLT3-ITD* mutation and the other case had concomitant *FLT3/TKD* and *NPM1* mutations, 1 case with t (8; 21) and *c-KIT* mutation, and 1 case with *RUNX1* ampli-

Table 1 Clinical features of the assessed patients with acute myeloid leukemia

Parameter	Frequency	Percent	Median (IQR)
Sex			
Male	47	61	
Female	30	39	
Age in years			42 (18-82)
< 50	52	67.5	
≥ 50	25	32.5	
TLC as × 10^9/L			20 (1-377)
Hb in g/dL			8.2 ± 2.39
Platelets as × 10^9/L			32 (1-658)
PB blast as %			53 (0-63)
BM blast as %			69 (20-97)
MCN			45.8 ± 2.62
< 46	10	14.5	
46	47	68.1	
> 46	12	17.4	
BM cellularity			
Hypocellular	3	3.9	
Normocellular	11	14.3	
Hypercellular	63	81.8	
Hepatomegaly			
Absent	57	74	
Present	20	26	
Splenomegaly			
Absent	60	77.9	
Present	17	22.1	
Lymphadenopathy			
Absent	52	67.5	
Present	25	32.5	
FAB classification			
M0	4	5.2	
M1	10	13	
M2	38	49.4	
M4	22	28.6	
M5a	2	2.6	
M7	1	1.3	
t (8; 21)			
Absent	70	90.9	
Present	7	9.1	
inv16			
Absent	71	92.2	
Present	6	7.8	

t (9; 22)			
Absent	75	97.4	
Present	2	2.6	
Genetic risk			
High	17	22.1	
Intermediate	42	54.5	
Low	18	23.4	
FLT3-ITD			
Wild	69	89.6	
Mutant	8	10.4	
FLT3-TKD			
Wild	75	97.4	
Mutant	2	2.6	
C-KIT			
Wild	76	98.7	
Mutant	1	1.3	
NPM			
Wild	63	81.8	
Mutant	14	18.2	
BM blast on day 15			0.03 (0-1)
BM cellularity on day 15			
Hypocellular	41	68.3	
Normocellular	11	18.3	
Hypercellular	8	13.3	
BM blast on day 28			0.03 (0-1)
BM cellularity on day 28			
Hypocellular	6	14.6	
Normocellular	21	51.2	
Hypercellular	14	34.1	
CR			
Negative	37	48.1	
Positive	40	51.9	
Delayed CR			
Negative	70	90.9	
Positive	7	9.1	
Resistance			
Negative	67	87	
Positive	10	13	
Relapse			
Negative	63	81.8	
Positive	14	18.2	
Death			
Negative	20	26	

Positive	57	74
Early death		
Negative	41	53.2
Positive	36	46.8

BM: Bone marrow; CR: Complete remission; FAB: French-American-British; Hb: Hemoglobin; IQR: Interquartile range; MCN: Modal chromosomal number; PB: Peripheral blood; TLC: Total leucocytic count.

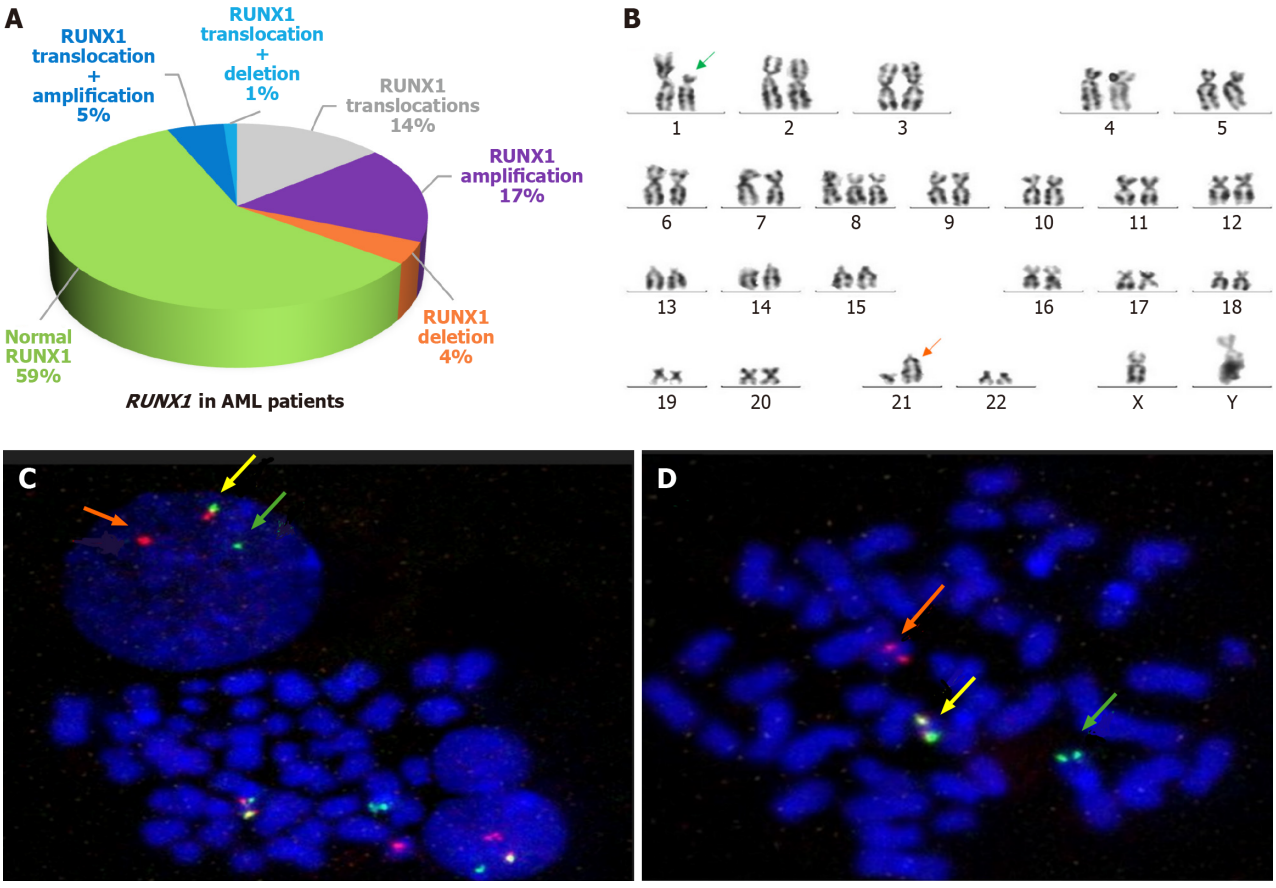

Figure 1 Runt-related transcription factor-1 in patients with acute myeloid leukemia. A: Runt-related transcription factor-1 (*RUNX1*) gene alterations in acute myeloid leukemia (AML) cases; B: G-banded karyotype of a case of t (1; 21). 48, XY, +X, t (1; 21) (p36; q22), +8; C: Interphase fluorescence in situ hybridization using *RUNX1* break apart probe showing a split of *RUNX1* signal; D: Metaphase fluorescence in situ hybridization using a *RUNX1* break apart probe showing a split of the *RUNX1* signal. The magnification factor is × 63.

fication with *NPM1* mutation.

FLT3-ITD mutations tended to be more prevalent in *RUNX1*-negative cases compared to positive cases. Out of 8 patients with AML with *FLT3-ITD* mutations, 7 patients (87.5%) were negative for *RUNX1* abnormalities, while only 1 patient was positive for *RUNX1* abnormalities. However, this relation was statistically insignificant (P = 0.078). Similarly, *NPM1* mutations were rarely seen in *RUNX1* abnormalities and translocations (P = 0.034 and 0.034, respectively). Otherwise, there were no significant differences between *RUNX1* positive and negative cases regarding other cytogenetic and molecular abnormalities, as shown in Table 2 and Table 3 (P > 0.05).

RUNX1 translocations, amplifications, and deletions were more frequent in the intermediate risk group (43.8%, 64.7%, and 50.0%) and the high-risk group (31.3%, 29.4%, and 50.0%) than in the low risk group (25.0%, 5.9%, and 0%), but the relationship was not statistically significant (P = 0.542, 0.150, and 0.288, respectively).

Impact of RUNX1 aberrations on response to treatment and clinical outcome

The response to treatment at day 28 of starting chemotherapy revealed that 36 of 77 (46.8%) cases died before day 28, and 33 cases (42.9%) achieved CR at day 15, 7 cases (9.1%) achieved delayed CR, 10 cases (13%) were resistant to treatment, and eventually 57 cases (74%) died.

Fourteen cases (18.2%) relapsed after achieving CR, 9/14 cases (64.2%) were positive to *RUNX1* abnormalities. One patient had t (9; 22) (q34; q11.2), and another patient had inv(16). The detailed karyotype and analysis of the outcome of

Table 2 Association between all runt-related transcription factor-1 abnormalities and runt-related transcription factor-1 translocations with the clinicopathological features of the patients with acute myeloid leukemia

Parameter		*RUNX1* abnormalities		*P* value	*RUNX1* translocation		*P* value
		Negative	Positive		Negative	Positive	
Age in years, median (IQR)		43.0 (20-69)	35.0 (18-78)	0.472	42.5 (20-78)	30.5 (18-70)	0.156
TLC as × 10^9/L, median (IQR)		44.7 (1-377)	20.8 (2-191)	0.620	34.8 (1-377)	20.1 (2-107)	0.187
Hb in g/dL, mean ± SD		8.38 ± 2.30	7.93 ± 2.50	0.421	8.15 ± 2.20	8.37 ± 3.10	0.748
Platelets as × 10^9/L, median (IQR)		24 (12-266)	30 (13-185)	0.549	26 (12-226)	27 (13-185)	0.390
PB blast as %, median (IQR)		60.0 (0-99)	54.0 (5-92)	0.426	53.0 (0-99)	68.5 (33-92)	0.950
BM blast as %, median (IQR)		66 (30-97)	62 (20-90)	0.598	66 (20-97)	70 (36-90)	0.711
MCN, mean ± SD		45.90 ± 0.67	45.80 ± 4.10	0.852	46.00 ± 2.50	45.30 ± 3.10	0.362
Sex, *n* (%)	Male	28 (62.2)	19 (59.4)	0.817	40 (65.6)	7 (43.8)	0.151
	Female	17 (37.8)	13 (40.6)		21 (34.4)	9 (56.3)	
BM cellularity, *n* (%)	Hypocellular	1 (2.2	2 (6.3)	0.042	3 (4.9)	0 (0.0)	0.009
	Normocellular	3 (6.7)	8 (25.0)		5 (8.2)	6 (37.5)	
	Hypercellular	41 (91.1)	22 (68.8)		53 (86.9)	10 (62.5)	
Hepatomegaly, *n* (%)	Absent	35 (77.8)	22 (68.8)	0.434	45 (73.8)	12 (75.0)	0.920
	Present	10 (22.2)	10 (31.3)		16 (26.2)	4 (25.0)	
Splenomegaly, *n* (%)	Absent	37 (82.2)	23 (71.9)	0.404	47 (77.0)	13 (81.3)	0.718
	Present	8 (17.8)	9 (28.1)		14 (23.0)	3 (18.8)	
Lymphadenopathy, *n* (%)	Absent	31 (68.9)	21 (65.6)	0.808	41 (67.2)	11 (68.8)	0.907
	Present	14 (31.1)	11 (34.4)		20 (32.8)	5 (31.3)	
MPO, *n* (%)	Negative	3 (6.7)	5 (15.6)	0.265	4 (6.6)	4 (25.0)	0.031
	Positive	42 (93.3)	27 (84.4)		57 (93.4)	12 (75.0)	
CD34, *n* (%)	Negative	26 (57.8)	16 (50.0)	0.643	35 (57.4)	7 (43.8)	0.330
	Positive	19 (42.2)	16 (50.0)		26 (42.6)	9 (56.3)	
CD64, *n* (%)	Negative	35 (77.8)	22 (68.8)	0.434	45 (73.8)	12 (75.0)	0.920
	Positive	10 (22.2)	10 (31.3)		16 (26.2)	4 (25.0)	
CD14, *n* (%)	Negative	39 (86.7)	29 (90.6)	0.728	53 (86.9)	15 (93.8)	0.447
	Positive	6 (13.3)	3 (9.4)		8 (13.1)	1 (6.3)	
FAB, *n* (%)	M0	1 (2.2)	3 (9.4)	0.241	1 (1.6)	3 (18.8)	0.019
	M1	5 (11.1)	5 (15.6)		9 (14.8)	1 (6.3)	
	M2	27 (60.0)	11 (34.4)		31 (50.8)	7 (43.8)	
	M4	11 (24.4)	11 (34.4)		19 (31.1)	3 (18.8)	
	M5	1 (2.2)	1 (3.1)		1 (1.6)	1 (6.3)	
	M7	0 (0.0)	1 (3.1)		0 (0.0)	1 (6.3)	
Complex, *n* (%)	Negative	40 (100.0)	20 (69.0)	< 0.001	50 (94.3)	10 (62.5)	0.001
	Positive	0 (0.0)	9 (31.0)		3 (5.7)	6 (37.5)	
t (8; 21), *n* (%)	Negative	45 (100.0)	25 (78.1)	0.001	61 (100.0)	9 (56.3)	< 0.001
	Positive	0 (0.0)	7 (21.9)		0 (0.0)	7 (43.8)	
inv16, *n* (%)	Negative	41 (91.1)	30 (93.8)	0.670	55 (90.2)	16 (100.0)	0.191
	Positive	4 (8.9)	2 (6.3)		6 (9.8)	0 (0.0)	
t (9; 22), *n* (%)	Negative	44 (97.8)	31 (96.9)	0.806	59 (96.7)	16 (100.0)	0.463

	Positive	1 (2.2)	1 (3.1)		2 (3.3)	0 (0.0)	
Cytogenetic risk, *n* (%)	High	8 (17.8)	9 (28.1)	0.310	12 (19.7)	5 (31.3)	0.542
	Intermediate	24 (53.3)	18 (56.3)		35 (57.4)	7 (43.8)	
	Low	13 (28.9)	5 (15.6)		14 (23.0)	4 (25.0)	
FLT3-ITD, *n* (%)	Wildtype	38 (84.4)	31 (96.9)	0.078	53 (86.9)	16 (100.0)	0.126
	Mutant	7 (15.6)	1 (3.1)		8 (13.1)	0 (0.0)	
C-KIT, *n* (%)	Wildtype	45 (100.0)	31 (96.9)	0.416	61 (100.0)	15 (93.8)	0.208
	Mutant	0 (0.0)	1 (3.1)		0 (0.0)	1 (6.3)	
NPM, *n* (%)	Wildtype	33 (73.3)	30 (93.8)	0.034	47 (77.0)	16 (100.0)	0.034
	Mutant	12 (26.7)	2 (6.3)		14 (23.0)	0 (0.0)	
BM blast on day 28, *n* (%)		0.04 (0-1)	0.02 (0-1)	0.082	0.03 (0-1)	0.04 (0-1)	0.789
BM cellularity on day 28, *n* (%)	Hypocellular	4 (19.0)	2 (10.0)	0.507	5 (16.7)	1 (6.3)	0.831
	Normocellular	9 (42.9)	12 (60.0)		15 (50.0)	6 (54.5)	
	Hypercellular	8 (38.1)	6 (30.0)		10 (33.3)	4 (36.4)	
CR, *n* (%)	Negative	23 (51.1)	14 (43.8)	0.644	30 (49.2)	7 (43.8)	0.699
	Positive	22 (48.9)	18 (56.3)		31 (50.8)	9 (56.3)	
Delayed CR, *n* (%)	Negative	43 (95.6)	27 (84.4)	0.093	57 (93.4)	13 (81.3)	0.131
	Positive	2 (4.4)	5 (15.6)		4 (6.6)	3 (18.8)	
Relapse, *n* (%)	Negative	40 (88.9)	23 (71.9)	0.075	52 (85.2)	11 (68.8)	0.128
	Positive	5 (11.1)	9 (28.1)		9 (14.8)	5 (31.3)	
Death, *n* (%)	Negative	9 (20.0)	11 (34.4)	0.192	15 (24.6)	5 (31.3)	0.589
	Positive	36 (80.0)	21 (65.6)		46 (75.4)	11 (68.8)	
Early death, *n* (%)	Negative	21 (46.7)	20 (62.5)	0.247	30 (49.2)	11 (68.8)	0.163
	Positive	24 (53.3)	12 (37.5)		31 (50.8)	5 (31.3)	

BM: Bone marrow; CR: Complete remission; FAB: French-American-British; Hb: Hemoglobin; IQR: Interquartile range; PB: Peripheral blood; MCN: Modal chromosomal number; MPO: Myeloperoxidase; *RUNX1*: Runt-related transcription factor-1; SD: Standard deviation; TLC: Total leucocytic count.

those patients are summarized in Supplementary Table 1.

Although positive cases to *RUNX1* abnormalities tended to have delayed CR and relapse compared to negative cases (71.4% *vs* 28.6% $P = 0.093$ and 64.3% *vs* 35.7% $P = 0.075$, respectively), the relationship was not statistically significant. The statistical analysis showed the absence of a relationship between the achievement of CR and *RUNX1* abnormalities ($P = 0.644$), *RUNX1* translocation ($P = 0.699$), *RUNX1* amplification ($P = 0.926$), and *RUNX1* deletion ($P = 0.616$), as shown in Table 2 and Table 3.

After a follow-up period of 44.2 months, the present study showed that there was no significant difference between positive and negative *RUNX1* aberration cases regarding the OS (Figure 2). Patients with *RUNX1* deletion had significantly poorer DFS than those without *RUNX1* deletion (mean: 3.03 months *vs* 27.20 months, respectively; $P < 0.001$). No other significant differences were observed between any other type of *RUNX1* alterations and negative cases regarding DFS (Figure 3).

DISCUSSION

The role of *RUNX1* mutations in cytogenetically normal AML had been identified. However, the prognostic impact of *RUNX1* translocations other than t (8; 21) (q22; q22), *RUNX1* deletions, and amplifications are still unknown. As a result, addressing such interactions is critical for further risk classification and eventually the development of a successful therapeutic plan.

In this study, FISH was used to screen for *RUNX1* gene alterations in 77 newly diagnosed adult patients with *de novo* AML, and the results were compared to clinical characteristics and prognosis. *RUNX1* abnormalities were divided into four categories: (1) *RUNX1* translocations; (2) *RUNX1* amplifications; (3) *RUNX1* deletions; and (4) *RUNX1* abnormalities, which encompass all three types.

Table 3 Association between runt-related transcription factor-1 amplifications and runt-related transcription factor-1 deletion with clinicopathological features of acute myeloid leukemia patients

Parameter		*RUNX1* amplification		*P* value	*RUNX1* deletion		*P* value
		Negative	Positive		Negative	Positive	
Age in years, median (IQR)		42.0 (18-70)	36.0 (20-78)	0.694	41.5 (18-78)	21.5 (21-22)	0.175
TLC as × 10^9/L, median (IQR)		44.7 (1-377)	19.2 (2-91)	0.873	29.1 (1-377)	105.3 (19-191)	0.630
Hb in g/dL, mean ± SD		8.33 ± 2.50	7.70 ± 1.70	0.359	8.20 ± 2.40	8.10 ± 1.50	0.950
Platelets as × 10^9/L, median (IQR)		24 (12-226)	45 (21-185)	0.484	26 (12-226)	24 (18-30)	0.663
PB blast (%), median (IQR)		60 (0-99)	38 (5-72)	0.515	59 (0-99)	47 (40-54)	0.232
BM blast (%), median (IQR)		66 (30-97)	60 (20-90)	0.35	65.5 (20-97)	74.5 (71-78)	0.613
MCN, mean ± SD		45.4 ± 2.5	47.5 ± 2.4	0.007	46 ± 2.1	42.5 ± 7	0.008
Sex, *n* (%)	Male	34 (56.7)	13 (76.5)	0.168	45 (61.6)	2 (50.0)	0.641
	Female	26 (43.3)	4 (23.5)		28 (38.4)	2 (50.0)	
BM cellularity, *n* (%)	Hypocellular	1 (1.7)	2 (11.8)	0.137	3 (4.1)	0 (0.0)	0.626
	Normocellular	8 (13.3)	3 (17.6)		11 (15.1)	0 (0.0)	
	Hypercellular	51 (85.0)	12 (70.6)		59 (80.8)	4 (100.0)	
Hepatomegaly, *n* (%)	Absent	47 (78.3)	10 (58.8)	0.125	53 (72.6)	4 (100.0)	0.568
	Present	13 (21.7)	7 (41.2)		20 (27.4)	0 (0.0)	
Splenomegaly, *n* (%)	Absent	51 (85.0)	9 (52.9)	0.009	56 (76.7)	4 (100.0)	0.57
	Present	9 (15.0)	8 (47.1)		17 (23.3)	0 (0.0)	
Lymphadenopathy, *n* (%)	Absent	44 (73.3)	8 (47.1)	0.076	49 (67.1)	3 (75.0)	0.743
	Present	16 (26.7)	9 (52.9)		24 (32.9)	1 (25.0)	
MPO, *n* (%)	Negative	6 (10.0)	2 (11.8)	0.833	8 (11.0)	0 (0.0)	1
	Positive	54 (90.0)	15 (88.2)		65 (89.0)	4 (100.0)	
CD34, *n* (%)	Negative	34 (56.7)	8 (47.1)	0.584	38 (52.1)	4 (100.0)	0.121
	Positive	26 (43.3)	9 (52.9)		35 (47.9)	0 (0.0)	
CD64, *n* (%)	Negative	45 (75.0)	12 (70.6)	0.758	56 (76.7)	1 (25.0)	0.052
	Positive	15 (25.0)	5 (29.4)		17 (23.3)	3 (75.0)	
CD14, *n* (%)	Negative	51 (85.0)	17 (100.0)	0.194	66 (90.4)	2 (50.0)	0.065
	Positive	9 (15.0)	0 (0)		7 (9.6)	2 (50.0)	
FAB, *n* (%)	M0	2 (3.3)	2 (11.8)	0.368	4 (5.5)	0 (0.0)	0.014
	M1	6 (10.0)	4 (23.5)		10 (13.7)	0 (0.0)	
	M2	32 (53.3)	6 (35.3)		38 (52.1)	0 (0.0)	
	M4	17 (28.3)	5 (29.4)		19 (26.0)	3 (75.0)	
	M5	2 (3.3)	0 (0)		1 (1.4)	1 (25.0)	
	M7	1 (1.7)	0 (0)		1 (1.4)	0 (0.0)	
Complex, *n* (%)	Negative	52 (94.5)	8 (47.1)	0.001	57 (87.7)	3 (75.0)	0.436
	Positive	3 (5.5)	6 (35.3)		8 (12.3)	1 (25.0)	
t (8; 21), *n* (%)	Negative	54 (90.0)	16 (94.1)	0.602	66 (90.4)	4 (100.0)	0.516
	Positive	6 (10.0)	1 (5.9)		7 (9.6)	0 (0.0)	
inv16, *n* (%)	Negative	55 (91.7)	16 (94.1)	0.739	68 (93.2)	3 (75.0)	0.282
	Positive	5 (8.3)	1 (5.9)		5 (6.8)	1 (25.0)	
t (9; 22), *n* (%)	Negative	59 (98.3)	16 (94.1)	0.395	71 (97.3)	4 (100.0)	0.737

	Positive	1 (1.7)	1 (5.9)		2 (2.7)	0 (0.0)	
Cytogenetic risk, *n* (%)	High	12 (20.0)	5 (29.4)	0.15	15 (20.5)	2 (50.0)	0.288
	Intermediate	31 (51.7)	11 (64.7)		40 (54.8)	2 (50.0)	
	Low	17 (28.3)	1 (5.9)		18 (24.7)	0 (0.0)	
FLT3-ITD, *n* (%)	Wildtype	52 (86.7)	17 (100.0)	0.188	66 (90.4)	3 (75.0)	0.361
	Mutant	8 (13.3)	0 (0)		7 (9.6)	1 (25.0)	
C-KIT, *n* (%)	Wildtype	59 (98.3)	17 (100.0)	0.592	72 (98.6)	4 (100.0)	0.814
	Mutant	1 (1.7)	0 (0)		1 (1.4)	0 (0.0)	
NPM, *n* (%)	Wildtype	47 (78.3)	16 (94.1)	0.173	60 (82.2)	3 (75.0)	0.717
	Mutant	13 (21.7)	1 (5.9)		13 (17.8)	1 (25.0)	
BM cellularity on day 28, *n* (%)	Hypocellular	6 (20.0)	0 (0)	0.152	5 (12.8)	1 (25.0)	0.22
	Normocellular	13 (43.3)	8 (47.1)		21 (53.8)	0 (0.0)	
	Hypercellular	11 (36.7)	3 (25.0)		13 (33.3)	1 (25.0)	
CR, *n* (%)	Negative	29 (48.3)	8 (47.1)	0.926	36 (49.3)	1 (25.0)	0.616
	Positive	31 (51.7)	9 (52.9)		37 (50.7)	3 (75.0)	
Delayed CR, *n* (%)	Negative	56 (93.30	14	0.177	66 (90.4)	4 (100.0)	0.516
	Positive	4 (6.7)	3 (25.0)		7 (9.6)	0 (0.0)	
Relapse, *n* (%)	Negative	50 (83.3)	13	0.496	61 (83.6)	2 (50.0)	0.149
	Positive	10 (16.7)	4		12 (16.4)	2 (50.0)	
Death, *n* (%)	Negative	13 (21.7)	7	0.125	19 (26.0)	1 (25.0)	0.964
	Positive	47 (78.3)	10		54 (74.0)	3 (75.0)	
Early death, *n* (%)	Negative	30 (50.0)	11	0.41	39 (53.4)	2 (50.0)	0.894
	Positive	30 (50.0)	6		34 (46.6)	2 (50.0)	

BM: Bone marrow; CR: Complete remission; FAB: French-American-British; Hb: Hemoglobin; IQR: Interquartile range; MCN: Modal chromosomal number; PB: Peripheral blood; MPO: Myeloperoxidase; *RUNX1*: Runt-related transcription factor-1; SD: Standard deviation; TLC: Total leucocytic count.

In agreement with Haferlach *et al*[6], total *RUNX1* abnormalities were detected in 41.6% of cases of which *RUNX1* amplification was the most common alteration (22.1%) followed by *RUNX1* translocations (20.8%) then RUNX1 deletion (5.2%). Baldus *et al*[14] investigated 12 patients with AML with complicated karyotypes and chromosome 21 anomalies and showed that amplification of two chromosome 21 areas was frequently seen in AML with complex karyotypes using comparative genomic hybridization, supporting the notion that gain of chromosome 21 material appears to be a nonrandom event implicated in AML. Baldus *et al*[14] reasoned that this might be related to the function of a specific gene or set of genes.

The clinical and genetic characteristics of patients with and without *RUNX1* alterations were compared. In agreement with Yamato *et al*[15], there were no statistically significant differences in any form of *RUNX1* abnormalities with respect to age, sex, total leucocytic count, hemoglobin level, platelet count, PB count, or BM blast cell counts at presentation. Tang *et al*[16] discovered that male patients had a higher rate of *RUNX1* alterations than female patients, while Haferlach *et al*[17] reported that patients with *RUNX1* deletion were considerably older than those with two *RUNX1* copies and had a lower WBC count. On the other hand, Said *et al*[18] used reverse transcription-quantitative PCR to explore the role of *RUNX1* gene expression in Egyptian patients with AML and found that male patients had significantly higher *RUNX1* expression. This discrepancy can be attributed to the difference in the technique used, a difference in the sample size, and variation in inclusion criteria between the two studies, in spite of conducting both studies on the same race.

The current data showed that splenomegaly is common in patients with *RUNX1* amplification, and the majority of them had lymphadenopathy. Hypercellular marrow was also more frequent than normocellular and hypocellular marrow in *RUNX1*-abnormalities and translocations. No published research had associated *RUNX1*-abnormalities with hepatomegaly, splenomegaly, lymphadenopathy, or BM cellularity at the time of diagnosis, to our knowledge.

Of interest, 43.8% of patients with *RUNX1* translocation were FAB AML-M2 and were associated with MPO expression. Also, M0 and M7 were more prevalent in *RUNX1* translocation positive cases than in negative instances. All patients with *RUNX1* deletion had a myeloid with monocytic phenotype (FAB-M4 and M5) and were favorably related with CD64 expression. These results matched those of Haferlach *et al*[6], who found that 45.2% of *RUNX1* translocation cases were FAB type M1 and M2. However, in contrast to our findings, they found that in cases of *RUNX1* deletion, M0 was the most common AML subtype. This discrepancy can be attributed to the racial and sample size variations. While

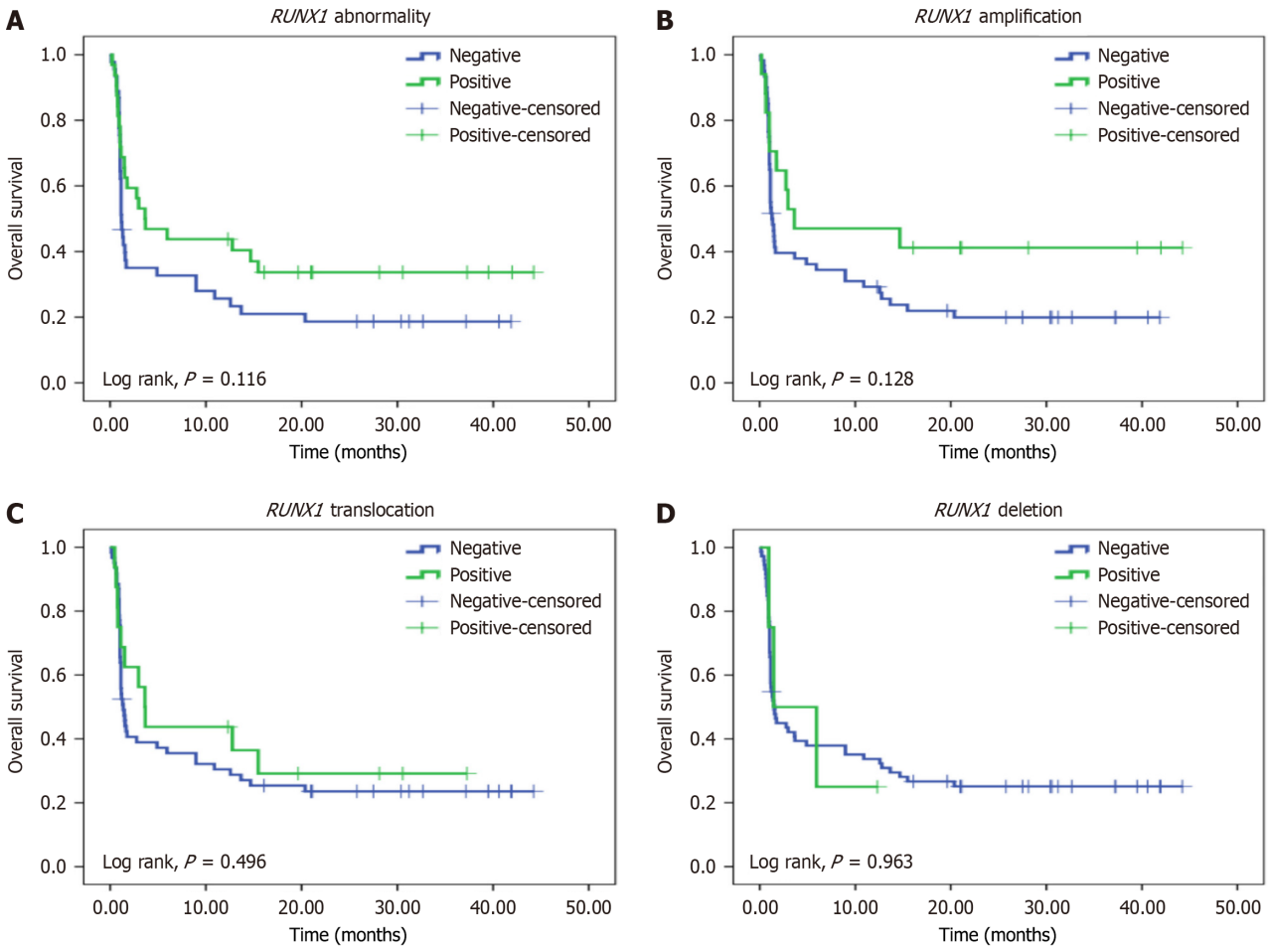

Figure 2 Kaplan-Meier survival curves. A: Runt-related transcription factor-1 (*RUNX1*) abnormality; B: *RUNX1* amplification; C: *RUNX1* translocation; D: *RUNX1* deletion on overall survival rates in acute myeloid leukemia patients.

Said *et al*[18] found that out of 42% of patients with AML classified as M2, translocation t (8; 21) was found in only 6.6% of the cases. The researchers also discovered no link between FAB subtypes and *RUNX1* expression.

In keeping with earlier studies, translocations, amplifications, and deletions of *RUNX1* were more common in the intermediate and high-risk groups, although not statistically significant.

In addition, as previously reported[17,19,20], positive cases of *RUNX1* amplification have a higher MCN, whereas *RUNX1* deletion cases have a lower MCN. This could be explained by the fact that chromosomal gain is nonrandom in patients with AML with hyperdiploid karyotypes, and chromosome 21 is one of the most frequently gained chromosomes in these circumstances.

The present data showed that *RUNX1* abnormalities, translocations, and amplifications are associated with more complex karyotypes than *RUNX1*-negative instances, which supports prior research[6,14,17]. It was concluded that chromosome 21 amplifications are common in people with complicated karyotypes.

In terms of other molecular mutations, 8 cases tested positive for the *FLT3-ITD* mutation. *RUNX1* translocations and amplifications were all negative, but *RUNX1* deletion was positive in 1 patient (12.5%). These relationships, however, did not exhibit statistical significance. According to Said *et al*[18], patients with higher *RUNX1* expression were more likely to have *FLT3-ITD* mutation than patients with lower *RUNX1* expression. This suggested that *RUNX1* could operate as an oncogene that causes leukemogenesis and as a surrogate marker for other mutations, particularly *FLT3-ITD*, when expressed at high levels.

Furthermore, *NPM1* mutations were mutually exclusive of *RUNX1* abnormalities and *RUNX1* translocations. This suggests that *RUNX1* mutation shares a similar genetic pathway role with *NPM1* mutations in leukemia development. This was supported by the study of Zuo *et al*[21], who stated that *NPM1* mutant interacts with *PU.1/CEB-PA/RUNX1* transcription factor complexes to block myeloid differentiation. Additionally, *NPM1* mutations were found at a lower frequency in *RUNX1* copy number variation-positive patients than in negative cases, but the difference was not statistically significant. These *RUNX1* mutations have genetic characteristics that are similar to those previously described in patients with AML[6,15,17].

There was no statistically significant association between any form of *RUNX1* modification and the achievement of CR or OS in terms of clinical outcome. Although the median DFS differed significantly amongst the three types of *RUNX1* changes (9.5, 25.7, and 1.5 months, respectively), *RUNX1* amplifications had the best prognosis. Only those with *RUNX1* deletion exhibited a considerably worse outcome than cases without the mutation. Consistently, previous research has

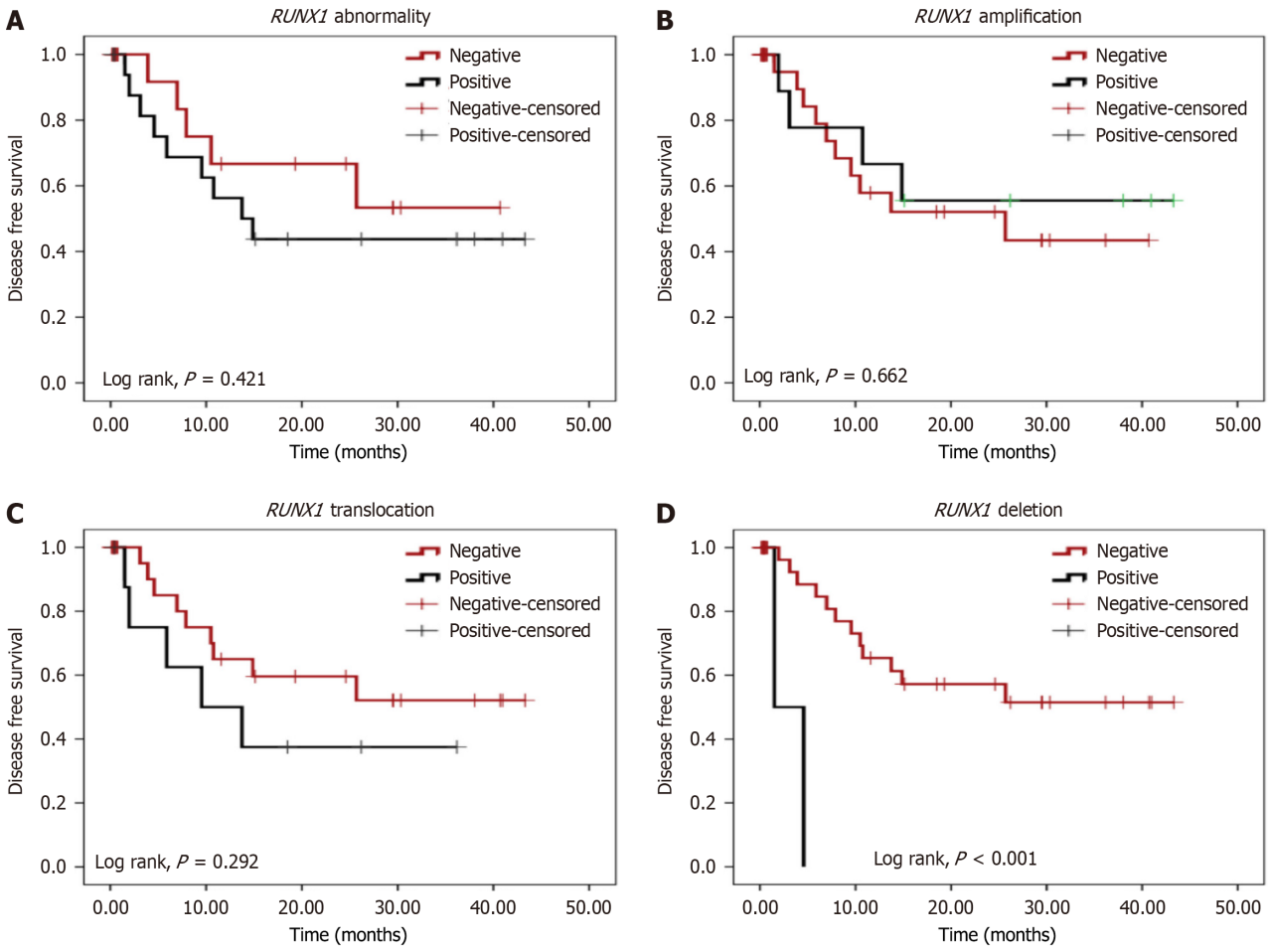

Figure 3 Kaplan-Meier survival curves. A: Runt-related transcription factor-1 (*RUNX1*) abnormality; B: *RUNX1* amplification; C: *RUNX1* translocation; D: *RUNX1* deletion on disease-free survival of patients with adult acute myeloid leukemia.

found that OS varies dramatically between the different forms of *RUNX1* alterations, with *RUNX1* deletions having the worst outcome[6].

On the contrary, other studies[6,22] found that the outcome differed considerably across *RUNX1* alterations and was better in individuals with *RUNX1* translocations. Discrepancies in the results could be related to the fact that most research looked at *RUNX1* mutations in combination with cytogenetic abnormalities, whereas just a few looked at the impact of *RUNX1* translocations, amplifications, and deletions in AML cases from different risk groups. Furthermore, it is thought that the different sorts of techniques used in the studies are a key source of heterogeneity. Said *et al*[18] found no significant impact of *RUNX1* expression on OS or DFS rates, but Chen *et al*[23] found that *RUNX1* mutation was linked to a lower risk-free survival rate.

Nine out of seventeen (53%) *RUNX1* amplification positive cases had *RUNX1* duplications. Four cases (29.4%) had *RUNX1* translocations at the same time, and five cases (29.4%) obtained one or more copies of chromosome 21, four of which were in a hyperdiploid karyotype. All of these are favorable prognostic markers that may help patients with positive *RUNX1* amplification live longer. This could explain the disparity in clinical outcomes between our data and that of others.

Furthermore, when inv16 and t (9; 22) cases with favorable prognosis were excluded from statistical analysis, the mean DFS in positive *RUNX1* deletion cases was 3.033 months compared to 27.231 months in negative *RUNX1* deletion cases. This suggests that *RUNX1* deletion has the worst prognosis, even if other strong prognostic markers like inv16 and t (9; 22) are present.

CONCLUSION

Our data presented a pilot study for *RUNX1* gene alterations in a cohort of patients with *de novo* AML. *RUNX1* abnormalities were detected in 41.6% of patients. *RUNX1* translocations occurred predominantly in FAB M2, M0, and M7 while *RUNX1* deletions were of myeloid with monocytic phenotype (FAB-M4 and M5). Cases positive for *RUNX1* abnormalities, translocations, and amplifications tended to have complex karyotypes. *RUNX1* abnormalities were mutually exclusive of *NPM1* mutations. *RUNX1* deletion was an independent adverse parameter for DFS. Further trials with larger numbers of *RUNX1* abnormal cases are warranted to further highlight the prognostic features and the

predictive significance of this abnormality.

ACKNOWLEDGEMENTS

The authors would like to thank all the patients who were included in this study.

FOOTNOTES

Author contributions: Abd El-Ghany HM and Abd El Dayem OY supervised the work and revised the paper; Rabea A managed and performed a follow-up of the patients; Abdellateif MS shared in the molecular work and analyzed the data; Sultan N performed the cytogenetics work and collected the data; El Ashry MS supervised the cytogenetic work and wrote the manuscript.

Institutional review board statement: The study was conducted following the Helsinki Declaration of 2011 and was approved by the internal review board of the National Cancer Institute and the Faculty of Medicine Research Ethics Committee at Cairo University (Code: MS-38-2020).

Informed consent statement: Every patient gave written informed consent.

Conflict-of-interest statement: The authors declare no competing interests.

Data sharing statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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S-Editor: Lin C
L-Editor: Filipodia
P-Editor: Zheng XM

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World Journal of Experimental Medicine

Submit a Manuscript: https://www.f6publishing.com

World J Exp Med 2025 March 20; 15(1): 96988

DOI: 10.5493/wjem.v15.i1.96988

ISSN 2220-315x (online)

ORIGINAL ARTICLE

Observational Study

Haematology results, inflammatory haematological ratios, and inflammatory indices in cervical cancer: How is the difference between cancer stage?

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Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification

Scientific Quality: Grade B
Novelty: Grade B
Creativity or Innovation: Grade B
Scientific Significance: Grade B

P-Reviewer: Rusman RD

Received: May 20, 2024
Revised: October 22, 2024
Accepted: November 1, 2024
Published online: March 20, 2025
Processing time: 219 Days and 16.2 Hours

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Abstract

BACKGROUND

Cervical cancer is a prevalent form of cancer affecting women worldwide and it is the second most common cancer among women in Indonesia, accounting for 8.5% of all cancer-related deaths. Cervical cancer progression can be evaluated through laboratory tests to detect anaemia, an increased platelet count, and elevated inflammatory markers, therefore, effective laboratory examination is crucial for early detection and treatment of cervical cancer.

AIM

To evaluate the association between laboratory findings (haematology, haemato-

logy index, and inflammatory index) and the clinical stage of cervical cancer.

METHODS

This cross-sectional study analyzed adult cervical cancer patients' data from medical records and laboratory results including sociodemographic status, histopathological finding, clinical stage, and complete haematology examination. Numerical data was analyzed by the one-way ANOVA (normal data distribution), while the Kruskal-Wallis test was used for non-parametric data (abnormal distribution), followed by appropriate post-hoc analysis. The categorical data was analyzed by the Chi-square or Fisher Exact tests. The significance level was established at a P value < 0.05.

RESULTS

This study involved the data of 208 adult cervical cancer patients and found no association between age, marital history, parity history, hormonal contraceptive use and cervical cancer stages. There were significant differences in the clinical laboratory test results based on the clinical stage of cervical cancer, including haemoglobin levels (P < 0.001), leucocytes (P < 0.001), neutrophils (P < 0.001), monocytes (P = 0.002), lymphocytes (P = 0.006), platelets (P < 0.001), neutrophil-lymphocyte ratio/NLR (P < 0.001), lymphocyte-monocyte ratio/LMR (P < 0.001), and platelet-lymphocyte ratio/PLR (P < 0.001). There were also significant differences in the systemic inflammatory index (SII) and systematic inflammatory response index (SIRI) between stage III + IV cervical cancer and stage II (SII P < 0.001; SIRI P = 0.001) and stage I (SII P < 0.001; SIRI P = 0.016), associated with the shifts in previously mentioned complete haematological values with cancer advancement.

CONCLUSION

The haematological parameters, inflammatory haematological ratios, and inflammatory indices exhibited significant differences between cervical cancer stages, therefore these tests can be utilized to evaluate cervical cancer progression.

Key Words: Cervical cancer; Haematology; Haematology index; Inflammation; Malignancy

Core Tip: The current investigation of 208 adult cervical patients found that hematologic parameters such as leucocyte, neutrophil, monocyte, and platelet counts vary significantly depending on the cervical cancer clinical stage. There were significant changes in inflammatory haematological ratios (neutrophil-lymphocyte ratio/NLR, platelet-lymphocyte ratio/PLR, and lymphocyte-monocyte ratio/LMR) and inflammatory indices (systemic immune-inflammation index/SII and systemic inflammation response index/SIRI), particularly between patients with stage III + IV and those with stage II and I cervical cancer. The analysis revealed that the cervical cancer clinical stage is highly related to the hematologic parameters.

Citation: Liana P, Syahbiran HG, Sari NP, Rahadiyanto KY, Nurwany R, Nurhidayat W, Umar TP. Haematology results, inflammatory haematological ratios, and inflammatory indices in cervical cancer: How is the difference between cancer stage? *World J Exp Med* 2025; 15(1): 96988

URL: https://www.wjgnet.com/2220-315x/full/v15/i1/96988.htm

DOI: https://dx.doi.org/10.5493/wjem.v15.i1.96988

INTRODUCTION

Cervical cancer is characterized by the uncontrolled growth of aberrant cells in the cervix uteri[1]. This cancer is the fourth most prevalent form of cancer affecting women worldwide[2] and is the second most prevalent cancer in Indonesian women with 36964 new cervical cancer cases and 20708 fatalities accounting for 8.5% of all cancer-related deaths[3]. Sexual contact is the primary factor contributing to disease transmission by exposure to the human papillomavirus (HPV). HPV is the primary oncogenic virus in women, responsible for around 90% of cervical cancer cases when there is a persistent high-risk HPV infection including HPV type 16 and 18[4].

Cervical cancer is frequently diagnosed at an advanced stage due to the lack of detection methods but is one of the most treatable forms of cancer if identified within the pre-cancerous stage[5]. A meta-analysis of 53,233 participants demonstrated that the incidence of late-stage cervical cancer patient presentation accounted for 60.66% of all cases worldwide, with Africa (62.60%) and Asia (69.30%) having higher rates than the global average[6]. This is primarily due to the individual's educational attainment, economic circumstance, geographical location, and pre-referral diagnosis by primary healthcare professionals[6,7]. This would undoubtedly exacerbate the death rate of cervical cancer, particularly in low-income nations, therefore, early identification is crucial in this case.

Various laboratory tests can be used to evaluate cervical cancer progression[8]. For instance, advanced cancer can cause chronic anaemia due to excessive cytokines directly and indirectly suppressing erythropoiesis[9]. Tumours also release cytokines that stimulate the formation of megakaryocytes and thrombopoiesis, resulting in an elevated platelet count[10]. Chronic inflammation also promotes the advancement of tumours and makes them more resistant to treatment. Therefore, cancer progression is linked to various inflammatory pathways, such as nuclear factor kappa B, Janus kinase/signal transducers and activators of transcription, toll-like receptor, and several proinflammatory cytokines [*e.g.*, interleukin (IL), interferon, and tumour necrosis factor][11]. Thus, there may be alterations in the leucocyte count and the com-position of leucocytes including neutrophils, lymphocytes, and monocytes[12]. For example, there is a correlation bet-ween the neutrophil-lymphocyte ratio (NLR) and cancer severity with the NLR tending to increase as the cancer progresses[13]. NLR is linked to increased cytokines [IL-1, IL-6, IL-7, IL-8, IL-12, IL-17, granulocyte colony-stimulating factor (G-CSF), and monocyte chemoattractant protein-1] that boost the activity of tumour macrophages[14]. Furthermore, reduced lymphocytes during advanced cancer may impact immune surveillance, causing diminished CD4+ T cells and an altered CD4+/CD8+ ratio related to rapid tumour growth and lymph node infiltration which are associated with platelet-lymphocyte ratio (PLR) elevation[15]. Moreover, there is evidence of elevated levels of inflammatory markers, such as the systemic inflammation index (SII) and systemic inflammatory response index (SIRI) in some cancers, including lung, pancreatic, and breast cancers[16–18]. However, SIRI prognostic significance has not been widely examined in cervical cancer, although nomogram creation utilizing SIRI, International Federation of Gynecology and Obstetrics (FIGO) stage, and lymphovascular invasion could better predict cervical cancer prognosis than FIGO stage alone because it may indicate the dynamic tumour burden and immune response status in patients[19].

Therefore, this study aimed to evaluate the association between laboratory findings (haematology, inflammatory haematological ratios, and inflammatory indices) and the clinical stage of cervical cancer to facilitate early diagnosis of cervical cancer and ultimately enhance the prognosis of people living with cancer by utilizing readily available biomarkers.

MATERIALS AND METHODS

This cross-sectional study collected data of cervical cancer patients from the Medical Records and Central Laboratory Installation of Dr Mohammad Hoesin General Hospital in Palembang, Indonesia (a tertiary-level facility). Cervical cancer patients aged ≥ 18 years and diagnosed from August 2022 to July 2023 were recruited using the total sampling technique considering a minimum sample size of 207, with an enrolment ratio of two, $\alpha = 0.05$, and $\beta = 80\%$. The study was approved by the medical and health research ethics committee of the Faculty of Medicine, Universitas Sriwijaya (Protocol No. 301-2023).

The data included information on the patient's sociodemographic status (such as age, marital status, parity, and hormonal contraceptive use), histopathological examinations, clinical stage of cervical cancer, and a comprehensive haematological examination (haemoglobin, leucocyte, neutrophil, monocyte, lymphocyte, and platelet counts). Additionally, haematological indices such as the NLR, PLR, and lymphocyte-monocyte ratio (LMR), as well as inflammatory indices, namely the SIRI and SII were also recorded[20].

The clinical stages of cervical cancer were classified according to the FIGO 2018 staging classification of cervical cancer, categorizing the disease into four main stages: I, II, III, and IV[21]. The participants were categorized into three distinct stages: Stage I, stage II, and stage III + IV due to the limited number of samples available in Stage IV. The NLR was determined by dividing the number of neutrophils by the number of lymphocytes, while the PLR was calculated by dividing the numbers of platelets and lymphocytes. LMR was established by dividing the number of lymphocytes by the number of monocytes. SIRI was determined by multiplying the monocyte count by the neutrophil count and dividing it by the lymphocyte count. Meanwhile, the SII was obtained by multiplying the platelet count by the neutrophil count and then dividing it by the lymphocyte count. The haematological analysis was conducted using a Sysmex XN-1000 machine.

The statistical analysis was conducted using IBM SPSS software version 26.0 (Armonk, NY: IBM Corp), with a significance level set at P value < 0.05. The data normality was assessed using the Kolmogorov-Smirnov test on a sample size of more than 50 participants. A univariate analysis was conducted to examine the frequency distribution of each variable. Normally distributed data was analyzed by one-way analysis of variance (ANOVA) and the Kruskal-Wallis test was applied to non-normally distributed data, followed by the appropriate post hoc test, either Tukey (homogenous sample), Games-Howell (non-homogenous sample) or Bonferroni correction test (non-parametric data). The χ^2 or Fisher Exact tests were employed for categorical data.

RESULTS

This analysis involved 208 cervical cancer patients with an average age of 48.5 years who were categorized into three groups: Stage I ($n = 25$), stage II ($n = 51$), and stage III + IV ($n = 132$). Most patients (98.5%) were married with multiparity and grand multiparity as the most common parity status and five patients had a prior record of hormonal contraception. Based on histopathological analysis, the most common type of cervical cancer was squamous cell carcinoma (68.6%), followed by adenocarcinoma, mixed types, and other types. There is no statistically significant association between age and the clinical stage of cervical cancer. Furthermore, there is no statistically significant association between marital status ($P = 0.41$), parity history ($P = 0.34$), and history of hormonal contraceptive usage ($P = 0.39$) and the clinical stage of cervical cancer. Similar findings apply to parity history, hormonal contraception use, and histological classification.

The hematologic results presented in Table 1 showed that several haematologic markers including haemoglobin ($P < 0.001$), leucocyte counts ($P < 0.001$) and platelet counts ($P < 0.001$) exhibited statistically significant variations depending on the clinical stage. Leucocyte counts, including neutrophils ($P < 0.001$), monocytes ($P = 0.002$), and lymphocytes ($P = 0.006$) exhibited comparable results. The progressive increase in leucocytes, platelets, neutrophils, and monocytes illustrated the association between cervical cancer stage and haematologic results. Concurrently, haemoglobin levels exhibited a progressive decline with the increasing severity of cancer stages and varied significantly between each stage ($P < 0.001$). The leucocyte counts were significantly different between stage III + IV and stage II ($P = 0.042$) and stage I ($P < 0.001$). Neutrophils exhibited a notable disparity between stage III + IV and stage II ($P = 0.006$) as well as stage I ($P < 0.001$). However, only significant differences between stage III + IV and stage II were observed for monocytes ($P = 0.002$) and lymphocytes ($P = 0.005$).

Furthermore, analysis of the haematological indices NLR ($P < 0.001$), PLR ($P < 0.001$), and LMR ($P < 0.001$) revealed notable variations depending on the clinical stage. The NLR and PLR results were comparable, with notable differences in the levels of these indicators between patients with stage III + IV and stage II (NLR $P < 0.001$; PLR $P = 0.001$) and stage I (NLR $P < 0.001$; PLR $P < 0.001$). However, LMR was only different between stage I and stage II ($P = 0.004$) and stage III + IV ($P = 0.019$), suggesting that monocytes are not significantly elevated between these clinical stages. These changes are attributed to lower lymphocyte counts and elevated neutrophil and platelet counts along with disease progression.

There were significant differences in all inflammatory indexes based on the severity of cervical cancer ($P < 0.001$). Post-hoc analysis revealed significant differences in SII values between patients with stage III + IV and those with stage II ($P < 0.001$) and stage I ($P < 0.001$). Similarly, the SIRI was significantly different between stage III + IV and stage II ($P = 0.001$) and stage I ($P = 0.016$), demonstrating increased inflammation in patients with a more advanced cancer stage.

DISCUSSION

The present investigation evaluated the association between laboratory findings (haematology, haematology index, and inflammatory index) and the clinical stage of cervical cancer using data from 208 patients with an average age of 48.5 ± 10.25 years which is consistent with the global average age of cervical cancer diagnosis (53 years, range: 45 to 68 years)[22]. Moreover, the lack of an association between parity history and hormonal contraceptive use with cancer stage is consistent with findings from several earlier studies[23-26].

There were significant differences in the laboratory findings among the various stages of cervical cancer. The haemoglobin levels decreased with cancer progression in line with the study of Kunos *et al*[27] which demonstrated a significant association ($P = 0.01$) between pre-therapy haemoglobin levels and the cervical cancer stage[27]. Another investigation conducted in Tianjin, China also demonstrated a notable association between the presence of anaemia and the cervical cancer stage ($P = 0.002$)[28]. The causes of anaemia in cervical cancer are multifaceted. It can arise from a haemorrhage associated with the vulnerability of the newly formed blood vessels or from cytokine activation which inhibits the generation of erythropoietin, hindering the body's ability to use iron and decreasing the formation of erythroid precursors[9,29,30].

Non-haematopoietic cancers, including cervical cancers frequently exhibit leukemoid response, characterized by leucocytosis caused by factors external to the bone marrow[31]. This study demonstrated a notable disparity in leucocyte counts among the different cancer stages with the most substantial increase observed in stages III and IV. Prior studies have also demonstrated that individuals with advanced cervical cancer exhibited more leucocyte abnormalities. Moreover, the increased neutrophil and monocyte counts, and decreased lymphocytes observed in the present study correlated with the progressive nature of cervical cancer. Previously, neutrophilia was demonstrated to be the most reliable indication of tumour cell invasiveness, which is directly linked to the malignancy severity[32]. The precise mechanism of neutrophilia within the tumour remains uncertain but it involves many cytokines including G-CSF, IL-1, and IL-6 produced by the tumour[14,33]. The present study observed a notable disparity in lymphocyte levels in advanced clinical stages (III-IV), in line with a previous study that reported an initial rise in lymphocytes during stages I and II, followed by a sharp decline at stage IV (a decrease of 22.8% compared to healthy individuals)[8]. The decreased lymphocytes are due to a decline in the immune system's capacity to combat and eradicate tumour cells, promoting its development[34]. The suppressive effect of neutrophils on lymphocytes is also an indicator of weakened immune system [35]. There was considerable variation in monocytes dependent on the cancer stage in the present study, contradictory to prior studies that found no association between monocytosis and different disease stages. Nevertheless, elevated monocytes are a negative prognostic indicator in patients with cervical cancer[36]. Monocytosis itself may be associated with several confounding factors including smoking and drinking history, as well as liver metastasis[37].

Elevation of platelet count in this study is consistent with an advanced cancer stage. Indeed, individuals with thrombocytosis were more commonly diagnosed with advanced stages (IIB-IVB) of cervical cancer[38]. Elevated platelet counts can be initiated by the secretion of cytokines including vascular endothelial growth factor and transforming growth factor-beta[39] as platelets function as a storage site that triggers the release of growth factors, which in turn stimulate the formation of new blood vessels, tumour proliferation, invasiveness, and growth[40].

As cervical cancer progresses, lymphocyte counts drop, resulting in higher NLR and PLR readings and lower LMR, as evidenced by the notable disparity in NLR[41,42] and PLR[43] across different cervical cancer clinical stages. A meta-analysis found a negative correlation between increased PLR and the prognosis of stage I and II cervical cancer patients (HR = 1.61; 95%CI: 1.21-2.15; $P = 0.001$) as well as stage I and IV patients (HR = 1.47; 95%CI: 1.19-1.81; $P < 0.001$)[43]. Prabawa *et al*[41] demonstrated a notable disparity in PLR levels ($P = 0.001$) between the initial and later phases of cervical cancer[41]. Elevated NLR and PLR are indicative of impaired lymphocyte function since reduced lymphocyte

Table 1 Sample characteristics, *n* (%)/mean ± SD/ median (minimum-maximum)

Variables	Total (*n* = 208)	Stage I (*n* = 25)	Stage II (*n* = 51)	Stage III + IV (*n* = 132)	*P* value
Demographics					
Age (years) (*n* = 208)	48.5 ± 10.25	44.8 ± 9.08	49.52 ± 10.80	48.80 ± 10.15	0.14[a]
> 40 tahun	41 (19.6)	18 (72.00)	40 (78.4)	109 (82.6)	0.44[c]
18-40 tahun	167 (79.9)	7 (28.00)	11 (21.60)	23 (17.4)	
Marital status (*n* = 203)					0.41[c]
Married	200 (98.5)	24 (100)	51 (100)	125 (97.7)	
Not married	3 (1.5)	0	0	3 (2.3)	
Parity history (*n* = 199)					0.34[c]
Multiparity and grand multiparity	175 (87.90)	23 (95.8)	42 (84.00)	110 (88.00)	
Nulliparity dan primiparity	24 (12.10)	1 (4.2)	8 (16.00)	15 (12.00)	
Hormonal contraception use (*n* = 173)					0.39[c]
Yes	5 (2.90)	1 (4.3)	0	4 (3.8)	
No	168 (97.10)	22 (95.7)	46 (100)	168 (97.10)	
Histolopathological classification					
Histopathological findings (*n* = 175)					0.33[c]
Squamous cell carcinoma	120 (68.60)	13 (59.10)	28 (63.60)	79 (72.5)	
Adenocarcinoma, mixed, and other	55 (31.40)	9 (40.9)	16 (36.4)	30 (27.5)	
Laboratory examinations					
Haemoglobin (g/L) (*n* = 204)	102.5 ± 24.7	124.6 ± 11.4	109.3 ± 21.2	95.4 ± 24.6	< 0.001[a]
Leucocyte (× 10^9/L) (*n* = 204)	9.85 (4.05-29.60)	7.47 (5.15-13.56)	8.84 (4.32-22.34)	11.08 (4.05-29.60)	< 0.001[b]
Neutrophil (× 10^9/L) (*n* = 186)	6.50 (1.52-25.16)	4.78 (2.66-8.90)	5.69 (3.14-17.20)	7.43 (1.52-25.16)	< 0.001[b]
Monocyte (× 10^9/L) (*n* = 186)	0.65 (0.18-2.10)	0.55 (0.33-1.22)	0.53 (0.18-1.55)	0.69 (0.27-2.10)	0.002[b]
Lymphocyte (× 10^9/L) (*n* = 186)	2.19 ± 2.15	2.18 ± 0.56	2.47 ± 0.72	2.09 ± 0.76	0.006[a]
Thrombocyte (× 10^9/L) (Neutrophil (× 10^9/L) (*n* = 186)	375.5 (99-1143)	307.00 (99.00-446.00)	364.00 (131.00-1143.00)	397.00 (147.00-791.00)	< 0.001[b]
Neutrophil lymphocyte ratio (*n* = 186)	3.13 (0.60-31.33)	2.37 (0.96-4.22)	2.31 (1.02-7.55)	3.78 (0.60-31.33)	< 0.001[b]
Lymphocyte monocyte ratio (*n* = 186)	3.29 (0.67-13.50)	3.77 ± 0.94	4.21 (1.86-13.50)	3.00 (0.67-8.25)	< 0.001[b]
Platelet lymphocyte ratio (*n* = 186)	176.01 (63.82-779.08)	141.03 (77.67-232.27)	138.96 (70.91-561.12)	200.49 (63.82-779.08)	< 0.001[b]
Systematic inflammation index (*n* = 186)	1195.47 (160.20-12282.67)	719.00 ± 309.59	1159.36 (308.71-5715.00)	1609.14 (160.20-12282.67)	< 0.001[b]
Systematic inflammatory response index (*n* = 186)	1.99 (0.25-16.54)	1.51 ± 0.80	1.17 (0.40-6.88)	2.96 (0.25-16.54)	< 0.001[b]

[a]One-Way Anova.
[b]Kruskal Wallis.
[c]χ^2 tests.

count leads to diminished immune system efficacy in combating tumour cells, facilitating tumour progression[44]. Furthermore, both NLR and PLR had a substantial capability to predict patients with tumour stages IIB and above as well as lymph node metastasis[8]. The levels of these indicators rise in patients with more advanced or aggressive illness, as seen by a growth in tumour size, nodal stage, and number of metastatic lesions[15,43,45,46]. The study demonstrated a decline in LMR during the advanced stages of cervical cancer in line with a previous study which reported a correlation between LMR and tumour stage (P = 0.012), as well as parametrial involvement (P = 0.022) and adjuvant therapy (P < 0.001)[47]. A low LMR is significantly correlated with specific clinicopathological parameters that are suggestive of a poor prognosis and aggressive illness[48].

In the current study, the inflammatory indicators, namely SII and SIRI, exhibited substantial differences among different clinical stages. Essentially, increased inflammatory markers were observed as a protective reaction of the body against internal or external damage, such as the development of tumours[11]. A prior investigation reported that elevated SII and SIRI are significantly linked to the likelihood of recurrence in individuals with early-stage cervical cancer. However, only a high SII relates to mortality[20]. SIRI strongly correlates with inflammatory haematological ratios, including NLR, PLR, and MLR, in matched and unmatched datasets ($P < 0.001$)[19]. Additionally, SII can differentiate the prognosis of patients in various FIGO stages, providing a valuable complement to the FIGO stage and increasing the sensitivity of screening for high-risk individuals to establish the most suitable personalised treatment[49]. Furthermore, a nomogram incorporating SIRI, FIGO stage, and lymphovascular invasion gave a better prognostic value with a c-index of 0.8, significantly higher than the FIGO stage alone ($P < 0.001$). Also, an increase in SIRI by > 75% at eight weeks after resection surgery was a risk factor for death and these patients had the worst prognosis (hazard ratio = 3.30, 95%CI: 2.08–5.25, $P < 0.001$)[19]. The inhibition of lymphocytes and T cell responses, along with elevated neutrophils, can contribute to tumour advancement, angiogenesis, and metastasis[35,50], thereby creating an inflammatory milieu. Before therapy, alterations in neutrophils and lymphocytes can indicate the extent of systemic inflammation or stress[51].

This study has several limitations including insufficient medical record data for several cervical cancer patients. The problem involves a lack of laboratory and sociodemographic data including hormonal contraceptives, which is seldom communicated in the study population. In addition, the assessment of patient outcomes, such as survival, tumour regression, or recurrence, was not conducted per this study's cross-sectional design. Furthermore, a confounding analysis was not possible due to limited data availability.

CONCLUSION

There were notable variations in the haematological parameters (haemoglobin and leucocyte, platelet, neutrophil, monocyte, and lymphocyte counts), inflammatory haematological ratios (NLR, PLR, and LMR), and inflammatory indices (SII and SIRI) across the different clinical stages of cervical cancer. Subsequent investigations should evaluate all blood-related measures and indicators, along with supplementary inflammatory markers to evaluate treatment effectiveness. Furthermore, these indicators could potentially be used to determine prognosis.

FOOTNOTES

Author contributions: Liana P and Sari NP conceptualized the study; Liana P and Syahbiran HG performed the research; Liana P, Sari NP, Rahadiyanto KY, Nurwany R, and Umar TP determined the methodology; Liana P, Sari NP, Rahadiyanto KY, Nurwany R, Nurhidayat W, and Umar TP conducted data validation and analysis; Liana P, Syahbiran HG, Sari NP, and Umar TP wrote the manuscript; Liana P and Umar TP revised the manuscript for important intellectual content; and all authors have read and approved the final version of the manuscript.

Institutional review board statement: The study has received approval from the medical and health research ethics committees of the Faculty of Medicine, Universitas Sriwijaya, under Protocol No. 301-2023.

Informed consent statement: No identifiable human data were used for this study.

Conflict-of-interest statement: All the authors report having no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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S-Editor: Liu H
L-Editor: A
P-Editor: Zhao YQ

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World Journal of Experimental Medicine

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World J Exp Med 2025 March 20; 15(1): 97460

DOI: 10.5493/wjem.v15.i1.97460

ISSN 2220-315x (online)

ORIGINAL ARTICLE

Prospective Study

Diagnostic utility of microRNA profiles in cavitatory and non-cavitatory pulmonary tuberculosis: Research protocol

Swathy Moorthy, Emmanuel Bhaskar, Shivakumar Singh, Santhi Silambanan

Specialty type: Medicine, research and experimental

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification

Scientific Quality: Grade D, Grade D

Novelty: Grade C, Grade C

Creativity or Innovation: Grade C, Grade C

Scientific Significance: Grade C, Grade C

P-Reviewer: Jaramillo-Rangel G

Received: May 30, 2024

Revised: October 31, 2024

Accepted: December 9, 2024

Published online: March 20, 2025

Processing time: 209 Days and 10.6 Hours

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Abstract

BACKGROUND

Tuberculosis (TB) is a common infection causing huge morbidity and mortality to mankind. The analytical methods used in diagnosing TB are not sensitive in paucibacillary infections and also require trained technical personnel. MicroRNAs are stable in serum and other body fluids, and hold great potential in the diagnosis of TB.

AIM

To analyze the dysregulated microRNA profiles among patients with cavitatory and non-cavitatory pulmonary TB.

METHODS

The prospective study will be conducted in a tertiary care center in India. Adult patients with newly diagnosed pulmonary TB will be included. There will be two groups: Patients with sputum positive pulmonary TB with cavity and without cavity (group1), and apparently healthy individuals (group 2). The participants will undergo sputum examination, Xpert *Mycobacterium TB* complex/resistance to rifampin (*Mtb*/RIF) assay, chest X-ray, and blood investigations and serum microRNA detection. Ethics approval has been obtained. Written informed consent will be obtained. Appropriate statistical analyses will be used.

RESULTS

MicroRNAs will be correlated with sputum positivity, Xpert *Mtb*/RIF assay, radiological involvement, inflammatory markers, and course of the disease among

cases and controls.

CONCLUSION
MicroRNAs could serve as potential diagnostic biomarkers in diagnostically challenging TB patients.

Key Words: Imaging; Inflammatory marker; MicroRNA; Molecular diagnosis; Pulmonary tuberculosis; CBNAAT

Core Tip: Tuberculosis (TB) is a multisystem infectious disease. The route of entry of *Mycobacterium tuberculosis* is *via* the respiratory system, hence the commonest presentation is lung TB. It has various presentations from subtle lesions to cavitation in the lung. If not treated in time, it spreads to various organs which can increase morbidity and mortality. Current diagnostic tools lack sensitivity and are time-consuming. Identification of the microRNA profiles in TB could help in devising point-of-care testing which may be used at bed side or physician consulting rooms.

Citation: Moorthy S, Bhaskar E, Singh S, Silambanan S. Diagnostic utility of microRNA profiles in cavitatory and non-cavitatory pulmonary tuberculosis: Research protocol. *World J Exp Med* 2025; 15(1): 97460
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/97460.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.97460

INTRODUCTION

Tuberculosis (TB) is a leading cause of disability, ranking thirteenth among the various causes which lead to mortality[1]. In countries where TB is common, the prevalence is 3.54%, while in other countries the prevalence is 1.43%[2]. In 2020, many deaths were due to TB, which could either be due to under-diagnosis or inappropriate management[3]. In 2016, 1.3 million deaths were due to TB alone and 0.37 million deaths were observed among human immunodeficiency virus-TB co-infection[4]. In 2021, more than 10 million individuals were found to be infected with *Mycobacterium tuberculosis* (*Mtb*), and the number of deaths due to TB was around two million[1]. According to the meta-analysis by Placeres *et al*[2], the prevalence of latent TB is 51.61% and 40.24% in high- and low-burden countries, respectively.

The BRICS countries, including India, China, South Africa, Russia and Brazil, are considered to be high TB-burden regions. Among these countries, India and China are affected more than other countries due to the large population. Due to the lack of awareness about the early stage of the disease, there is an increasing spread among the community. In spite of implementation of extensive measures for an early diagnosis and treatment, there is a continued gap between the clinical manifestation of the disease and treatment[5]. The United Nations Sustainable Development Goals has decided to control TB epidemic by the year 2030[1]. India marks the year 2022 as a milestone for the TB surveillance by the TB Elimination Program. It recorded a 13% increase in case notifications compared to the year 2021[6]. The clinical presentation and diagnosis of TB are complex due to the increase in the aging population, along with the increase in the prevalence of drug-resistant strains of *Mtb*[4].

Pulmonary TB

Mtb generally enters the human body through the lungs and starts replicating inside the macrophages, thus forming granulomas. The bacteria have the ability to evade the immune system in the lungs, and continue to multiply in the macrophages. The bacilli in the lungs have three fates: In 70%-80% of individuals, the infection is latent and is unable to infect others, which is called latent TB infection; in 10%-20% of individuals, caseous granuloma opens up disseminating infection through the breath of the infected person to other members in the family as well as neighborhood; and in the rest 10%, the bacteria spread beyond the lungs to establish extrapulmonary TB[7,8]. Most TB related deaths can be prevented with early diagnosis and appropriate treatment. However, the limited availability of a reliable diagnostic tool poses a major obstacle in the control of this epidemic. The currently available standard methods of TB diagnosis rely majorly on the adequacy of bacterial load. Owing to this limitation, emphasis has been laid on the need for the development of diagnostic tests which could be biomarkers of host responses. These tools can be used for diagnosis of the disease and monitoring the treatment outcomes[9,10].

Diagnosis of pulmonary TB

According to the World Health Organization 2013, pulmonary TB refers to the confirmation of TB based on the evidences from either a clinical or lab diagnosis of TB that involves the lower respiratory tract. In a person with latent inactive TB, the bacteria may be present in the body without causing disease[11]. Routinely done chest X-ray is not sensitive or specific, and shows a normal picture in spite of the disease presence. Chest computerized tomography can be sensitive in detecting microscopic or undefined lesions, which cannot be detected by chest X-ray. Further confirmation is made possible by detecting the bacilli in the sputum specimens. However, not all patients with active TB will be able to cough out enough sputum for laboratory analysis[11].

Sputum in diagnosis of pulmonary TB

For the diagnosis of TB, two sputum specimens have to be examined by microscopy. Microscopic examination of sputum smear is quick, easy, and cost-effective for detecting TB. However, at least 5000-10000 bacilli/mL of sputum must be present and the report is obtained within a day. Examination of Ziehl-Neelsen staining of sputum smears by light microscopy is widely used. Based on the number of bacilli in the smears, they are graded and thus the infectivity of the person. However, smears are not fool-proof in that patients with acid fast bacilli smears which are negative have been shown to have cultures positive for acid-fast-bacilli[11]. The laboratory test which is used for substantiating TB is culture. Also, it is mandatory to perform drug-sensitivity testing and genetic makeup of the organism to improve diagnostic accuracy and management. *Mtb* is grown on solid media, which can be the Lowenstein-Jensen slope or broth media. Liquid media are better, since the results will be available within two weeks[11]. In the recent times, diagnosis is made possible in early TB infection, with the advent of molecular assays such as nucleic acid amplification (NAA) test. A positive NAA test is considered to be diagnostic, especially for those who have an increased risk for the disease. Hence, public health TB programs at the community level, have the access to NAA testing for quicker diagnosis of TB. NAA testing has high positive predictive value and has the ability to rapidly identify the presence of *bacilli* in most smear-negative but culture-positive specimens[11].

Other investigations

The tuberculin skin test and interferon-gamma release assays are the immunology-based assays. But, the limitation with these assays is that they fail to detect the infection in the early stages. Since immune response takes at least eight weeks to get established, the test becomes positive only after this period[12].

MicroRNAs in pulmonary TB

There is a growing interest in identifying relevant microRNAs in the blood of patients using a PCR-based assay[13-15]. This could further enable identification of latent and active TB, as well as extrapulmonary TB. Circulating microRNAs play regulatory roles in various metabolic pathways and serve as ideal markers to detect *Mtb*[16]. MicroRNAs are 18-25-nucleotide-long non-coding RNAs and are stable in the body fluids[17-20]. MicroRNAs are considered to be ideal biomarkers, since they are easily accessible in the peripheral circulation, and have high specificity, sensitivity, and stability. MicroRNAs as biomarkers of disease, have been demonstrated in many malignancies and common infectious diseases[21-23]. During the disease process, certain microRNAs get up-regulated while few get down-regulated compared to healthy individuals. MicroRNAs such as miR-146, -31, and -150 are down-regulated while miR-16, -20, -21, -29, -30, -99, -155, -193, -223, -299, -365, -486, and let-7 family are upregulated in various stages of pathogenesis of the disease[8]. There are limited studies on the dysregulated microRNAs among the specific clinical subtypes of TB.

Several studies have been conducted over the last decade, for categorizing microRNAs as biomarkers of TB. Since TB at the latent stage is very inconclusive, these markers should be able to differentiate latent disease form the active one[24-26]. Pro-apoptotic microRNAs are found to be down-regulated in TB. Few microRNAs are activated by the toll-like receptor (TLR) pathway, which regulates inflammation by targeting interleukin-1α. MicroRNAs, by negatively regulating the insulin-like growth factor (IGF) pathway, target cell differentiation. In the macrophages, the IGF pathway activates lipopolysaccharide induced nuclear factor kappa B with release of inflammatory mediators[8]. The observed link between dysegulated microRNAs and active TB paves the way for better understanding of the pathogenic mechanisms[27].

TB is ranked the second leading cause of mortality among all the infectious diseases. This could be due to inadequacy in performance of the existing biomarkers to differentiate the varied presentations of pulmonary TB. MicroRNAs could serve as ideal diagnostic biomarkers of pulmonary TB. MicroRNAs could differentiate cavitatory from non-cavitatory pulmonary TB so that targeted therapy can be initiated according to the type and extent of the disease. MicroRNAs being very stable and can be implemented as point-of-care testing in diagnostically challenging groups of TB patients. These tests can be effectively utilized by the clinicians in the outpatient department and in the patient wards, and can also be used by community health workers in the society. Thus, this may offer hope on the eradication of TB in a planned and strategic way.

Therefore, circulating microRNAs could be a promising diagnostic tool which shall address the different aspects of the disease. It has been well established that the cavitatory TB has higher prevalence rates of multi-drug-resistant TB, higher relapse rates, and more complications in the long run. Hence, identifying the microRNAs specific for the group, would help in prognosticating the patients.

MATERIALS AND METHODS

Study design

This is a proof-of-concept study, so only a convenient sample has been chosen. MicroRNAs will be altered in all the TB infected patients. So, the expected percentage of microRNA positivity among the cases is 100%. This sample size is calculated based on the assumption that the expected percentage of microRNA positivity among the patients affected with TB is 100%. Studies of microRNAs in TB are available. But they have not been associated with the type and extent of lesions in the lungs. The existing diagnostic tools have a sensitivity and specificity both up to 90%. The microRNA profiles could be better than the existing diagnostic tests, and they have the potential to offer a more than 90% sensitivity as well as specificity. However, no reproducible data is available in the published literature on the difference in proportion of upregulation between cases and non-cases, as it is highly variable across the published studies. So,

computing sample size based on this might not be feasible. The protocol aims to study the upregulated and downregulated microRNAs and their association with the clinical subtypes of pulmonary TB.

Primary objectives: (1) To generate microRNA profiles specific to clinical subtype of tuberculosis (*i.e.*, cavitatory and non-cavitatory variants); (2) To correlate the microRNA profiles with sputum positivity in pulmonary TB patients; (3) To compare microRNA profiles with Xpert *Mtb*/resistance to rifampin (RIF) assay, inflammatory markers, and imaging techniques in patients with pulmonary TB; and (4) To compare the clinical course of the disease at the end of three months and six months with the baseline microRNA patterns in respective sub-groups of pulmonary TB.

Null hypothesis: MicroRNA profiles shall not be significantly altered to diagnose cavitatory and non-cavitatory TB.

Alternate hypothesis: Altered profiles of microRNAs could assist in the diagnosis of cavitatory and non-cavitatory tuberculosis.

With regard to sample size in convenient sampling, there are studies saying that a minimum of 30 could be the adequate sample size. Some studies say that 20 is adequate or 10 could be adequate, and there is no adequate information with regard to this sample size. However, the most important anticipated drawback could be bias. But the chances for bias in this study could be less since we are trying to identify profiles (set of upregulated and downregulated) of microRNAs specific to cavitatory and non-cavitatory TB. Moreover, we have stringent inclusion and exclusion criteria for inclusion of study participants.

We have considered geographical proximity, availability at a given time, or availability of financial support. Probably this study could facilitate us in conducting an in-depth study in future.

Inclusion criteria

Clinical, radiological, smear and culture proved new cases to whom treatment has not been initiated.

Group 1: This group will be composed of sputum-positive (acid-fast staining or CBNAAT) pulmonary TB patients. Sputum-positive (acid-fast staining or CBNAAT) pulmonary TB patients will be further classified as cavitatory (n = 20) or non-cavitatory type (n = 20) by chest X-ray or CT scanning (n = 20).

Group 2: This group will consist of age- and gender-matched persons who are free of acute illness and with no history of pre-existing chronic medical illness (n = 13).

Exclusion criteria

The exclusion criteria will be: (1) Age > 60 years and < 18 years; (2) Current smoking or alcoholism; (3) Presence of any prior chronic medical illness (diabetes mellitus, hypertension, liver disease, renal disease, endocrine disease, cerebro- and cardiovascular diseases, autoimmune disorders, haematological disorders other than iron deficiency anaemia, and recently cured cancer or active cancer); (4) Pregnancy; (5) Drug-resistant TB; (6) TB patients with human immunodeficiency virus co-infection; (7) Previously treated with anti-tuberculosis therapy, and other active lung infections like community acquired pneumonia; (8) and On drugs such as corticosteroids, anti-inflammatory drugs, anticonvulsants, and anticancer drugs.

The policies have changed in India. Most of the TB patients are being managed by the smaller government district hospitals. Hence, the number of TB patients approaching tertiary care hospitals like our institution is a slightly lesser compared to the scenario which existed few years back. Hence, the time taken to include participants also could take longer.

Ethics statement

The proposed study will be conducted at Sri Ramachandra Institute of Higher Education and Research, Chennai, India. The Institutional Ethics Committee (IEC) has approved the study (IEC number IEC/21/JUN/163/43). The outcome variables will be performed at baseline, and the patients will be followed as per the standard of care. The study is registered with Indian Council of Medical Research, India, CTRI/2023/08/056740 (https://ctri.nic.in/Clinicaltrials/Login.php).

Investigations to be done

All the patients will be subjected to analysis of sputum smear and culture, Xpert *Mtb*/RIF assay, chest X-ray, complete blood count, interleukin 6 (IL-6) and matrix metalloproteinase-1 (MMP-1), and serum microRNAs. Statistical analyses will be done. A p-value less than 0.05 will be considered statistically significant. The Xpert *Mtb*/RIF assay is a test that simultaneously detects *Mtb* complex and RIF. There is a chronic inflammatory state associated with TB which could be reflected by analysis of IL-6. One of the enzymes involved in cavity formation is MMP-1, measurement of which could help in identifying early cases of cavitatory TB. MMP-1 levels can be associated with microRNA profiles.

Isolation and analysis of microRNAs from serum samples

Five milliliter of venous blood is collected into a sterile vacutainer. Samples are centrifuged at 3000 rpm for 10 minutes, and the supernatant serum is aliquoted and stored immediately at -80°C until analysis. Total RNA from serum samples is then isolated using TRIzol and further purified using a RNeasy minikit according to the manufacturer's instructions. The concentration and quality of RNA are measured with a Nanodrop spectrophotometer and checked by gel electrophoresis. After RNA isolation from the samples, microRNA labelling and hybridization and microanalysis of the RNA (equal

amounts of RNA from 5 participants of each respective group is pooled for profiling) are done according to the standard guidelines.

Real-time PCR analysis

To confirm that the pattern of specific differentially expressed microRNAs, a validation study using independent samples is performed. Reverse transcription-PCR is performed to confirm the array results. Each sample is normalized on the basis of an appropriate endogenous control. The experiment is conducted in triplicate. Statistically significant occurrence is used to evaluate the diagnostic effect of the candidate microRNAs.

PureFast® microRNA mini spin purification kit (containing Carrier RNA, Lysis buffer, Wash Buffer-1, Wash Buffer-2, and Spin columns with collection tube and elution buffer) and microRNA real-time kit are procured from HELINI Biomolecules, Chennai, India.

cDNA synthesis protocol

cDNA Synthesis Detection Mix contains cDNA, microRNA-cDNA primer, RT enzyme, and purified microR. PCR vials are centrifuged briefly before placing into the thermal cycler. cDNA synthesis thermal profile takes place in two steps. qPCR Detection Mix contains probe PCR master mix, microR PP mix, PCR grade water, and cDNA.

Real-time PCR thermal profile

After 45 cycles, relative gene expression analysis is automatically done by the qPCR machine software and results are interpreted. Quantification of gene expression of interest is accomplished by measuring the fractional cycle number at which the amount of expression reaches a fixed threshold (Ct), which is directly related to the amount of product. The PCR cycle at which fluorescence measured by the instrument reaches a threshold value is called threshold cycle (Ct), which is set at a point that is above the background signal. The threshold cycle is inversely proportional to the log of the initial copy number. The amplification plot with amplification cycles *vs* fluorescence units is shown in Figure 1.

The dysregulated microRNAs in pulmonary TB patients are compared with those in healthy controls. Further the microRNAs will be correlated with sputum positivity, Xpert *Mtb*/RIF assay, radiological involvement, inflammatory markers, and the course of the disease.

Statistical analysis

Categorical variables will be analyzed by the χ^2 or Fischer's exact test. Continuous variables will be analyzed by one-way analysis of variance or the Kruskal-Wallis test. *Post-hoc* analysis using the least significant difference test will be used to analyze the results of the statistical comparisons. A p-value less than 0.05 will be considered statistically significant. Statistical analyses will be done with SPSS version 16.

RESULTS

During the progression of the disease, specific microRNAs will be selectively upregulated or downregulated. This phenomenon varies depending on the clinical presentation of the disease and whether it manifests as pulmonary TB with cavity formation or without cavity. This distinct modulation of microRNA expression acts as a pivotal determinant, offering unique signatures that can effectively indicate the pathogenic stage of the disease.

The inclusion criteria for patients participating in the study necessitate their identification *via* sputum assays, the Xpert *Mtb*/RIF test, and confirmation of radiological lung involvement. All patients will undergo comprehensive blood tests to evaluate parameters such as complete blood count and inflammatory markers. Subsequently, the identified microRNAs will be correlated with sputum test results, the Xpert *Mtb*/RIF findings, the extent of radiographic lung infiltration, and the levels of inflammatory markers.

Throughout the disease trajectory, all the patients will be consistently administered the standard treatment regimen appropriate for their diagnosis. The ongoing clinical progression of the disease will be closely observed and documented, juxtaposed with detailed laboratory assessments that monitor the microRNA profiles and the disease.

DISCUSSION

Biological indicators called microRNAs can be utilized to differentiate between various TB infection stages or therapeutic responsiveness. By attaching themselves to mRNA in the cytoplasm of the cell, they regulate the expression of genes. MicroRNAs are being explored as potential biomarkers for TB because they are sensitive, specific, and accessible. MicroRNA signals that can differentiate between individuals with active TB and healthy controls or those with latent TB have been found in a number of investigations. MicroRNAs are crucial in pathogen-host interactions, according to new research. Since they have been consistently and frequently found in the blood, circulating microRNAs have the potential to be used as molecular markers for a variety of physiological and pathological disorders. With a respectable level of sensitivity and specificity, upregulated miR-29a may distinguish TB patients from healthy controls. These circulating microRNAs are projected to influence a number of considerably enriched pathways, the majority of which are implicated in the regulation of the cytoskeleton, acute-phase response, and inflammatory response[28].

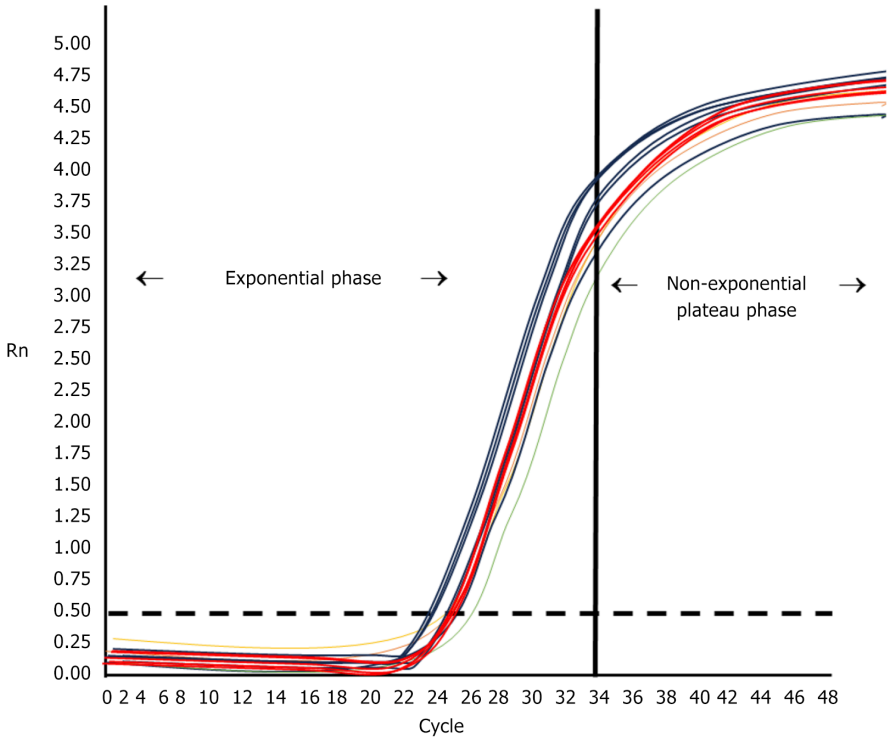

Figure 1 Amplification plot (Rn *vs* Cycle) of a microRNA.

It may be possible to distinguish between tuberculoma with and without decay using serum miR-155, miR-191, and miR-223. Serum levels of miR-26a, miR-191, miR-222, and miR-320 distinguish between fibrotic cavitary TB (FCT) and tuberculoma with degradation. Patients with FCT and those with tuberculoma without decay have different levels of serum expression of miR-26a, miR-155, miR-191, miR-222, and miR-223. As a result, the degree and direction of expression of the set of microRNAs might be used to characterize different TB course variations with varying levels of destruction and inflammatory process severity. Due to their ability to simultaneously regulate several genes, microRNAs are being investigated as potential treatment targets for TB. For instance, miR-155 can support the survival of *Mtb*-specific T lymphocytes while also providing protection against mycobacterial infection[29].

Altered microRNAs have shown promise as potential diagnostic biomarkers in the complex and challenging presentations of TB patients. These specialized RNA molecules could play a crucial role in identifying TB cases that are difficult to diagnose accurately through traditional methods. Additionally, microRNAs could not only aid in diagnosing TB but also serve as prognostic markers that are closely linked with the clinical outcomes and various laboratory investigations related to the disease. Moreover, the involvement of microRNAs in TB cases opens up exciting possibilities for personalized medicine approaches where treatment strategies could be tailored based on the unique microRNA profiles of individual patients. By understanding the intricate relationship between these altered microRNAs and disease progression, healthcare providers may be able to make more informed decisions regarding treatment plans and predict patient responses to specific interventions.

CONCLUSION

MicroRNA signatures offer a window into the pathophysiology of TB, shedding light on the molecular mechanisms underlying the disease's manifestation and progression. Through further research and validation studies, these microRNAs could potentially revolutionize TB management by providing clinicians with valuable tools to improve diagnostic accuracy, predict treatment outcomes, and monitor disease progression in real time. In conclusion, the emerging role of altered microRNAs as potential diagnostic and prognostic markers in TB patients represents a significant step forward in combating this infectious disease. By harnessing the power of these tiny but influential molecules, healthcare professionals can strive towards more effective and personalized care strategies for individuals affected by TB.

ACKNOWLEDGEMENTS

The authors thank Sri Ramachandra Institute of Higher Education and Research for providing the necessary infrastructure for conducting this research.

FOOTNOTES

Author contributions: Moorthy S, Bhaskar E, Singh S, and Silambanan S designed the research study; Moorthy S, Bhaskar E, and Santhi S performed the research; Moorthy S and Santhi S contributed new reagents and analytic tools; Moorthy S, Bhaskar E, Singh S, and Silambanan S analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

Institutional review board statement: The Institutional Ethics Committee has approved the study (IEC number IEC/21/JUN/163/43).

Clinical trial registration statement: The study is registered with Indian Council of Medical Research, India, CTRI/2023/08/056740 (https://ctri.nic.in/Clinicaltrials/Login.php).

Informed consent statement: Written informed consent will be obtained from all participants.

Conflict-of-interest statement: The authors declare that there were no conflicts of interest.

Data sharing statement: All the required data are provided in the article itself.

CONSORT 2010 statement: The authors have read the CONSORT 2010 statement, and the manuscript was prepared and revised according to the CONSORT 2010 statement.

Country of origin: India

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S-Editor: Liu H
L-Editor: Wang TQ
P-Editor: Zhang XD

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World Journal of Experimental Medicine

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World J Exp Med 2025 March 20; 15(1): 94022

DOI: 10.5493/wjem.v15.i1.94022

ISSN 2220-315x (online)

ORIGINAL ARTICLE

Basic Study

SARS-CoV-2 proteins show great binding affinity to resin composite monomers and polymerized chains

Pedro Henrique Sette-de-Souza, Moan Jéfter Fernandes Costa, Boniek Castillo Dutra Borges

Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification

Scientific Quality: Grade B
Novelty: Grade B
Creativity or Innovation: Grade B
Scientific Significance: Grade B

P-Reviewer: Arumugam EAP

Received: March 10, 2024
Revised: October 3, 2024
Accepted: October 30, 2024
Published online: March 20, 2025
Processing time: 291 Days and 0.7 Hours

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Abstract

BACKGROUND

Due to saliva and salivary glands are reservoir to severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2), aerosols and saliva droplets are primary sources of cross-infection and are responsible for the high human–human transmission of SARS-CoV-2. However, there is no evidence about how SARS-CoV-2 interacts with oral structures, particularly resin composites.

AIM

To evaluate the interaction of SARS-CoV-2 proteins with monomers present in resin composites using in silico analysis.

METHODS

Four SARS-CoV-2 proteins [*i.e.* main protease, 3C-like protease, papain-like protease (PLpro), and glycoprotein spike] were selected along with salivary amylase as the positive control, and their binding affinity with bisphenol-A glycol

dimethacrylate, bisphenol-A ethoxylated dimethacrylate, triethylene glycol dimethacrylate, and urethane dimethacrylate was evaluated. Molecular docking was performed using AutoDock Vina and visualised in Chimera UCSF 1.14. The best ligand–protein model was identified based on the binding energy (ΔG–kcal/moL).

RESULTS

Values for the binding energies ranged from -3.6 kcal/moL to -7.3 kcal/moL. The 3-monomer chain had the lowest binding energy (*i.e*. highest affinity) to PLpro and the glycoprotein spike. Non-polymerised monomers and polymerised chains interacted with SARS-CoV-2 proteins *via* hydrogen bonds and hydrophobic interactions. Those findings suggest an interaction between SARS-CoV-2 proteins and resin composites.

CONCLUSION

SARS-CoV-2 proteins show affinity to non-polymerised and polymerised resin composite chains.

Key Words: Composite resins; COVID-19; SARS-CoV-2; Dental restorations; Molecular docking simulation; Dentistry

Core Tip: The severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) may interact with monomers of resin composites; triethylene glycol dimethacrylate has the smallest affinity with SARS-CoV-2 among monomers; bisphenol-A glycol dimethacrylate and bisphenol-A ethoxylated dimethacrylate show a remarkable affinity mainly with papain-like protease.

Citation: Sette-de-Souza PH, Fernandes Costa MJ, Dutra Borges BC. SARS-CoV-2 proteins show great binding affinity to resin composite monomers and polymerized chains. *World J Exp Med* 2025; 15(1): 94022
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/94022.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.94022

INTRODUCTION

Saliva and salivary glands are a significant reservoir for severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) [1]. Aerosols and saliva droplets are primary sources of cross-infection and are responsible for the high human–human transmission of SARS-CoV-2[2,3]. Once saliva wets oral tissues, tooth structures, and dental restoratives present in the oral cavity, SARS-CoV-2 can bind to them, thereby increasing the permanence of microorganisms in the mouth.

Among dental restoratives, resin composites are widely used to restore decayed teeth[4] due to their aesthetic properties and capacity to preserve healthy tooth tissues. Such materials contain organic monomers such as bisphenol A glycol dimethacrylate [bisphenol-A glycol dimethacrylate (Bis-GMA)], bisphenol A ethoxylated dimethacrylate [bisphenol-A ethoxylated dimethacrylate (Bis-EMA)], triethylene glycol dimethacrylate (TEGDMA), and urethane dimethacrylate (UDMA), along with inorganic filler particles[5]. Those monomers present chemical components such as hydroxyl, oxygen, and nitrogen that affect intermolecular interactions with substrates[6,7]. It has been demonstrated that, aside from taking shelter on dental biofilms, SARS-CoV-2 can interact with oral tissues and tooth structures[8,9]. However, it remains unclear whether SARS-CoV-2 proteins interact with resin composites.

Knowing the sites within the mouth that can harbour SARS-CoV-2 is essential for understanding its spread once saliva is not the only oral harbour for viruses[9]. However, the mechanism by which SARS-CoV-2 colonises dental biofilm remains unclear. At the same time, the acquired pellicle (AP) may form on any exposed surface, including dental materials, through the selective adsorption of proteins[10]. Thus, SARS-CoV-2 proteins may interact with dental materials and collaborate in the formation of AP.

Given the above, in silico analyses play a remarkable role in investigations involving cellular and molecular processes [11,12]. The molecular docking method, which entails searching for probable interactions between microorganisms' proteins and substrates, has been used worldwide as the first step to understanding probable interactions with SARS-CoV-2[13]. That computational approach is an essential tool due to the urgent need to better understand SARS-CoV-2's effects on human health.

Against that background, in our study we evaluated the possible interaction of SARS-CoV-2 proteins with monomers and polymers present in resin composite in silico. The null hypothesis tested was that an interaction between the SARS-CoV-2 proteins and the monomers and other proteins would not occur.

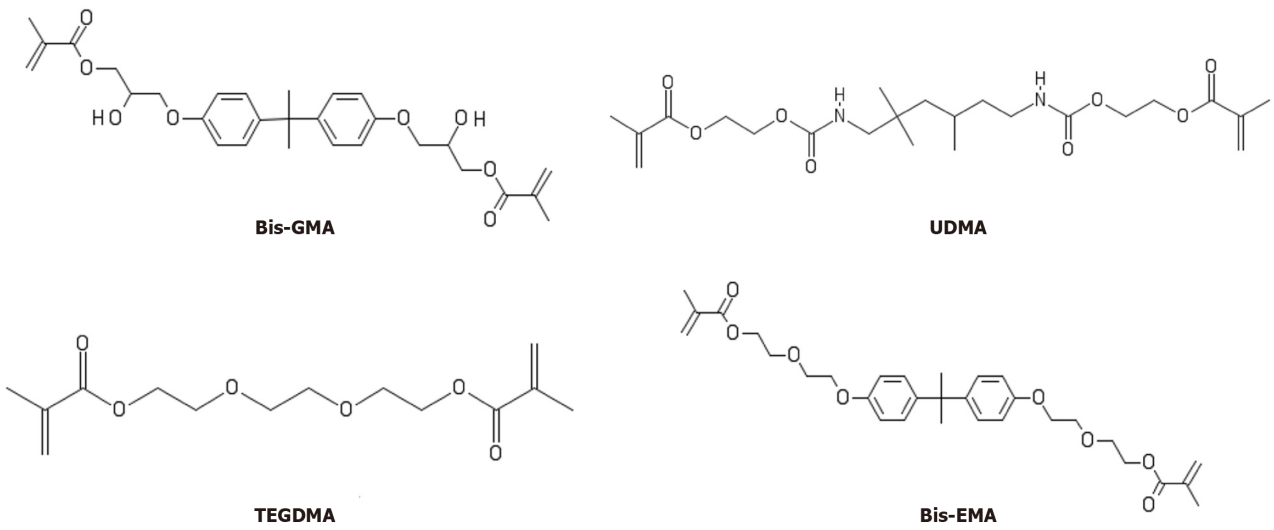

Figure 1 Chemical structure of the tested monomers: Bisphenol-A glycol dimethacrylate, bisphenol-A ethoxylated dimethacrylate, triethylene glycol dimethacrylate, and urethane dimethacrylate. Bis-EMA: Bisphenol-A ethoxylated dimethacrylate; Bis-GMA: Bisphenol-A glycol dimethacrylate; UDMA: Urethane dimethacrylate; TEGDMA: Triethylene glycol dimethacrylate.

MATERIALS AND METHODS

Protein selection and structure preparation

SARS-CoV-2 has some proteins involved in biological processes related to coronaviruses[14]. Thus, to simulate a whole new coronavirus, four different SARS-CoV-2 protein groups-the main protease (Mpro) (PDB: 6LU7), 3C-like protease (3CLpro) (PDB: 6M2N), papain-like protease (PLpro) (PDB: 6W9C), and glycoprotein spike (PDB: 6VYB)-were selected in light of previous studies[15–17]. For a positive control, we used salivary amylase (PDB: 3BLP) because it is involved in AP formation on multiple surfaces[10].

The crystal structures of SARS-CoV-2 proteins were obtained from the GenBank National Center for Biotechnology Information (RRID: SCR_002760). The AutoDock (RRID: SCR_012746) was used to delete duplicated chains, heteroatoms, and water molecules, as well as add polar hydrogens atoms and the charge of all atoms in the protein structure. Gasteiger charges were computed, and the structure was saved as a PDBQT file for the docking studies.

Ligand selection and structure preparation

The monomers Bis-GMA ($C_{29}H_{36}O_8$, PubChem CID: 15284), TEGDMA ($C_{14}H_{22}O_6$, PubChem CID: 7979), UDMA ($C_{23}H_{38}N_2O_8$, PubChem CID: 170472), and Bis-EMA ($C_{31}H_{40}O_8$, PubChem CID: 92523) were used in this study. Their chemical structures appear in Figure 1.

After retrieving SMILE codes from the National Center for Biotechnology Information's chemical structure library (RRID: SCR_004284), we constructed multiple chains through monomer combination using PubChem Draw (RRID: SCR_021249). We also linked the individual chains to simulate the natural polymerised resin composite. Next, we simulated various polymerised chains linking the monomer methacrylate regions during polymerisation, after which we transformed the new SMILE code in a PDB file in Chimera UCSF 1.14 (RRID: SCR_004097).

The rotatable bonds of the ligands were defined using AutoDock, and the structures were saved as PDBQT files for use in the docking studies.

Docking procedure

The Autogrid algorithm created the three-dimensional grids to generate the grid parameter files (RRID: SCR_015982). Each grid map was set to the centre of chain A, docking parameters were set according to the protein (Table 1), and all analyses were performed with a/an exhaustiveness value of 8.

Molecular docking was performed using AutoDock Vina (RRID: SCR_011958), and the best ligand–protein model was identified based on the binding energy (ΔG–kcal/moL)[18].

Docking visualisation

The results obtained through the docking procedure were visualised in Chimera UCSF 1.14 (RRID: SCR_004097). The two-dimensional interactions of the complex protein–ligand structure, including hydrogen bonds and bond lengths, were analysed in LigPlot+ (RRID: SCR_018249) for all interactions[19]. The step-by-step methodological approach that we followed is depicted in Figure 2.

Table 1 Binding energy (ΔG–kcal/mol) and standard deviation of the interaction between the severe acute respiratory syndrome-coronavirus 2 proteins and resin composite

Chain	Binding energy (ΔG–kcal/mol)				
	6LU7[1]	6M2N[1]	6W9C[1]	6VYB[1]	3BLP+
3-monomer-chain	-4.95 ± 0.53	-5.28 ± 0.65	-5.46 ± 0.83	-5.15 ± 0.49	-5.63 ± 0.48
2-monomer-chain	-4.71 ± 0.72	-4.93 ± 0.71	-4.78 ± 0.83	-4.85 ± 0.56	-5.17 ± 1.30
Monomers	-5.05 ± 0.66	-5.28 ± 0.47	-5.25 ± 1.10	-5.03 ± 0.53	-5.05 ± 0.76
Hydroxyapatite	-4.2	-4.6	-5.1	-4.9	-4.8
PO_4	-3.3	-3.2	-3.6	-3.6	-3.7

[1]Severe acute respiratory syndrome-coronavirus 2.
6LU7: Main protease; 6M2N: 3C-like protease; 6W9C: Papain-like protease; 6VYB: Glycoprotein spike; 3BLP: Salivary amylase.

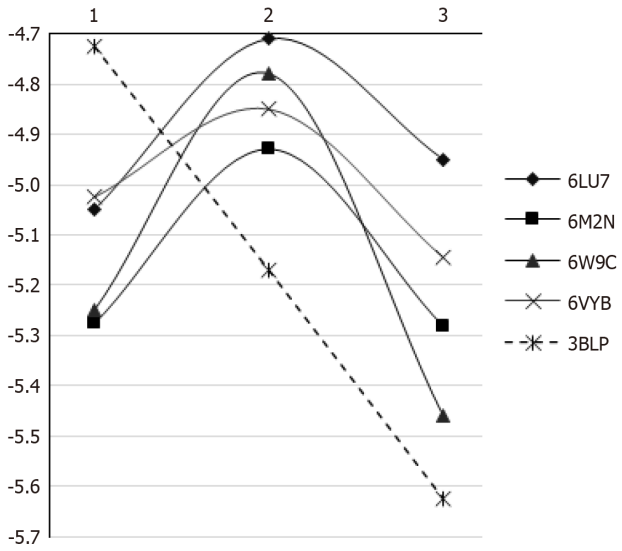

Figure 2 Binding energy between severe acute respiratory syndrome-coronavirus 2 proteins and resin composite chains. 1: Monomer; 2: Two-monomer chain; 3: Three-monomer chain.

RESULTS

Binding energy evaluation

Binding energies ranging from -3.1 kcal/moL to -8.0 kcal/moL were found. The 3-monomer chain had the lowest binding energy (*i.e.* highest affinity) to PLpro, the glycoprotein spike, and salivary amylase (Table 1). For all tested proteins, the 2-monomer chain demonstrated the highest binding energy.

Interaction analyses

To observe the specific interactions between monomers and proteins, we used LigPlot+. The central oxygen and nitrogen atoms from monomers were involved in hydrogen bonds with amino acid residues, and some alkene groups of monomers presented hydrophobic interactions with the residues. Those interactions were also observed in the polymerised chains.

Non-polymerised monomers and polymerised chains interacted with SARS-CoV-2 proteins *via* hydrogen bonds and hydrophobic interactions (Figure 3). Beyond that, any SARS-CoV-2 protein may have interacted with many non-polymerised and polymerised chains simultaneously (Figure 4).

DISCUSSION

The null hypothesis tested in our study-that an interaction between the SARS-CoV-2 proteins and the monomers and polymers would not occur-was rejected because the binding affinity between all monomers and polymers and all proteins (*i.e.* Mpro, 3CLpro, PLpro, and the glycoprotein spike) was observed.

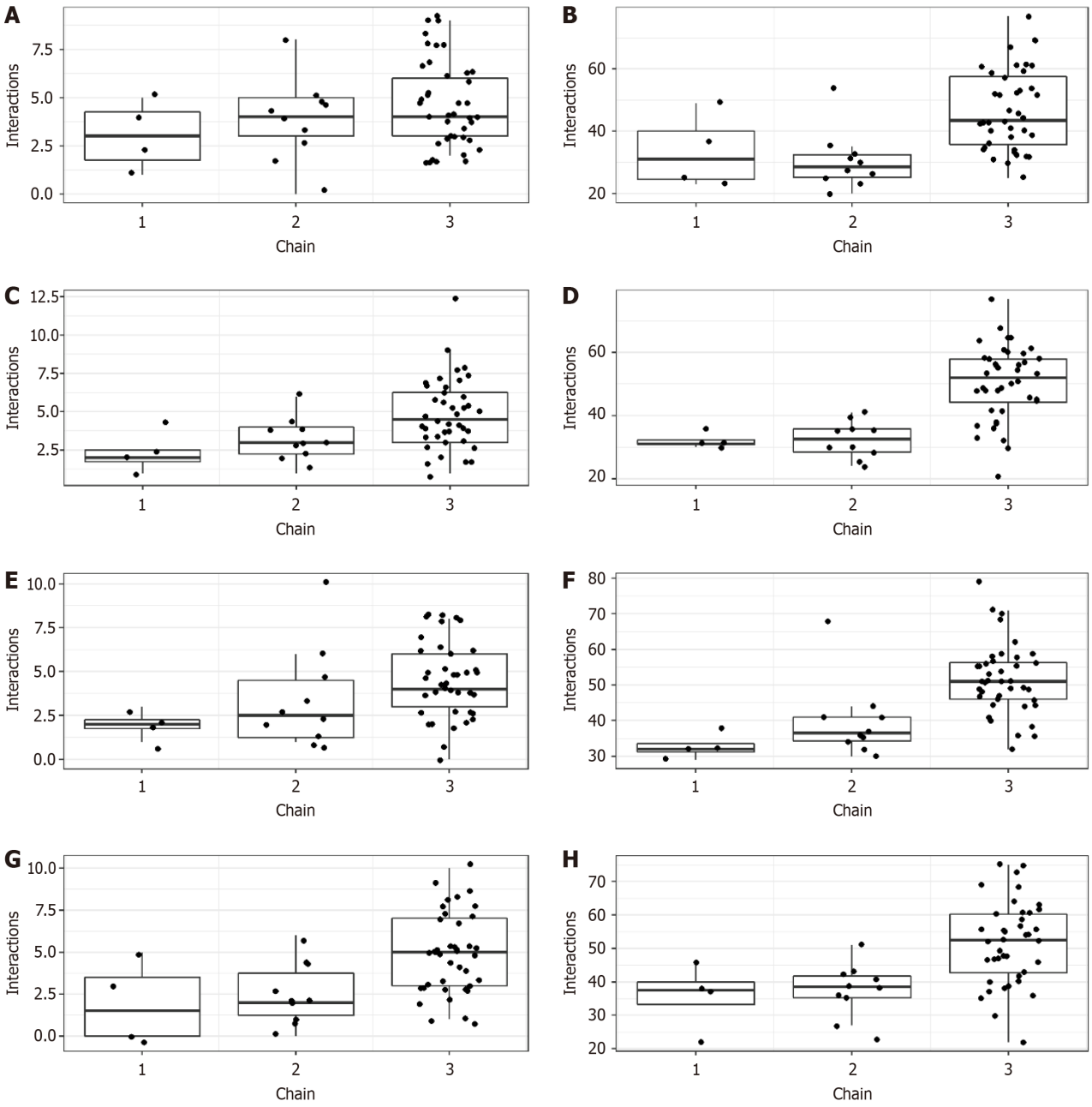

Figure 3 Interactions between severe acute respiratory syndrome-coronavirus 2 proteins and resin composite. A: Hydrogen bond between 6LU7 and resin composite; B: Hydrophobic interactions between 6LU7 and resin composite; C: Hydrogen bond between 6M2N and resin composite; D: Hydrophobic interactions between 6M2N and resin composite; E: Hydrogen bond between 6VYB and resin composite; F: Hydrophobic interactions between 6VYB and resin composite; G: Hydrogen bond between 6W9C and resin composite; H: Hydrophobic interactions between 6W9C and resin composite.

Among other results, Bis-GMA and Bis-EMA showed remarkable binding energy with all tested proteins, with ΔG values equal to or less than -5.0 kcal/moL. Such affinity relates to the number of interactions presented (*i.e.* hydrogen bonds and hydrophobic interactions) such that hydrogen bonds are more potent than hydrophobic interactions[20]. A higher number of oxygen atoms and a central, highly hydrophobic group present in Bis-GMA and Bis-EMA formed hydrogen bonds with hydroxyl radicals of polar amino acid residues and hydrophobic interactions with nonpolar amino acid residues. The fact that Mpro showed the highest binding energy to UDMA can be due to many interactions, primarily hydrogen bonds. A highly hydrophobic central area of Bis-EMA promoted many hydrophobic interactions with residues of PLpro, which was responsible for promoting the highest binding energy. Meanwhile, the highest binding energy (*i.e.* lowest affinity) obtained between TEGDMA and all proteins tested related to its having the smallest area of the monomers, which decreased the number of interactions with amino acid residues.

We evaluated binding energy values and interactions between SARS-CoV-2 proteins and non-polymerised methacrylate monomers and polymerised chains of resin composites. The growth of polymeric chains occurs when monomers are linearly connected by converting double C = C bonds into C–C bonds from different terminal methacrylate groups [21]. Monomers, especially Bis-GMA, may also be cross-linked *via* hydrogen bonds between hydroxyl groups and nitrogen or oxygen[22]. Thus, because hydrogen bonds and hydrophobic interactions between each non-polymerised

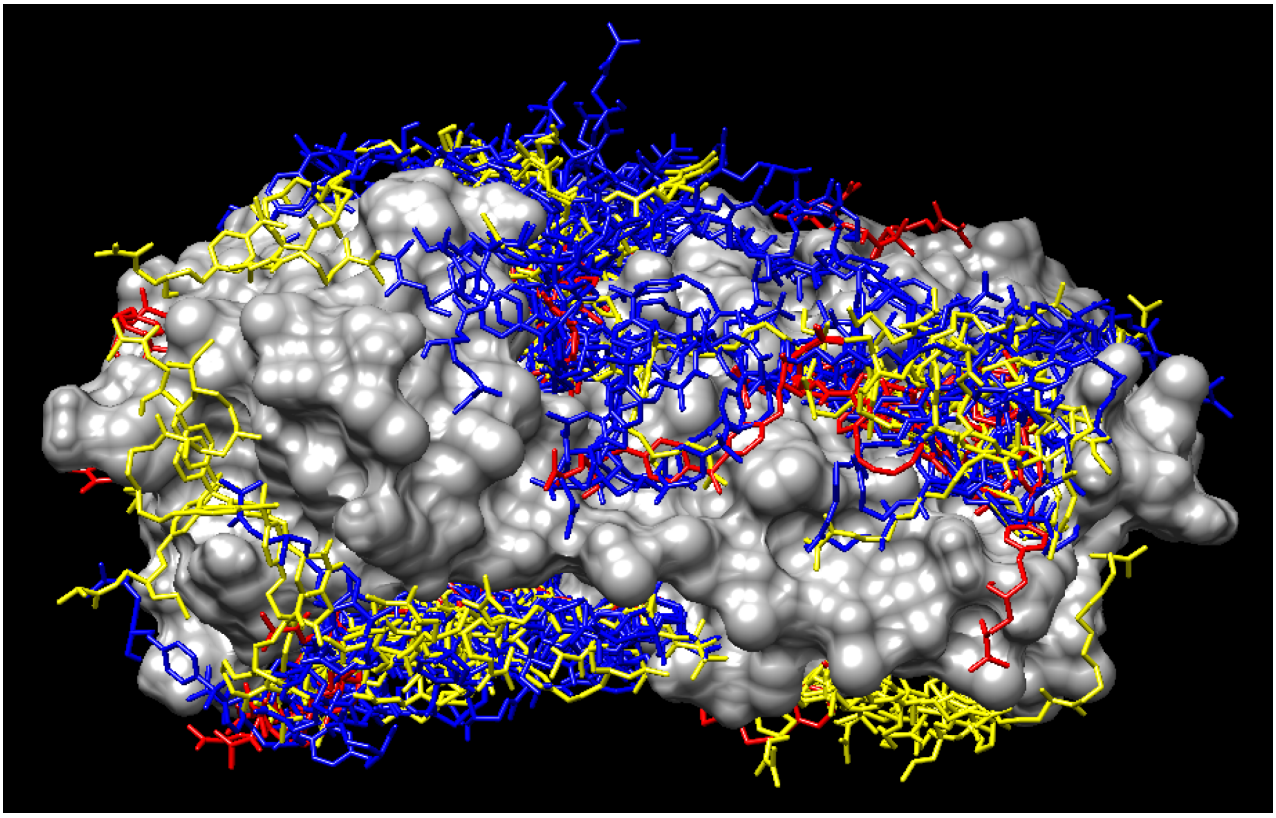

Figure 4 Graphical interaction between unpolymerised chain (red), 2-monomer chain (yellow), 3-monomer polymerised chain (blue), and the severe acute respiratory syndrome-coronavirus 2 protein.

monomer and SARS-CoV-2 proteins did not involve terminal methacrylate groups or hydroxyl groups in Bis-GMA, it was probable that similar binding between SARS-CoV-2 proteins and a polymerised chain would occur. Our results validate that assumption.

In a tooth preparation restored with resin composite, polymer chains of polymerised monomers and filler particles are likely present[21]. In general, resin composite restorations are polished in clinical conditions in order to achieve adequate smoothness and aesthetic properties and expose filler particles[23]. Thus, further investigations should evaluate the binding affinity of SARS-CoV-2 proteins to different filler particles. If a high binding affinity between them were found, then an increase the number of microorganisms might increase in resin composite restorations, and all implications highlighted by our results might increase. At the same time, another study[10] has shown that salivary amylase interacts with resin composites and collaborates in AP formation in filled resin composites. Thus, given our results, we believe that SARS-CoV-2 may also collaborate in AP formation.

In our computational study of a new microorganism, evidence to compare and corroborate our findings was inadequate. In response, *in vitro* and *in vivo* analyses need to be performed to validate our findings. Further research should also be conducted to clarify the mechanisms of interaction observed in our study. Despite those limitations, the chief strength of our work lies in its being the first to provide data about a possible interaction between resin composites and SARS-CoV-2. Besides that, due to concerns about the degradation of the resin–dentin interface[24], further studies could be performed to determine whether the virus will adhere to resin and collaborate in the resin–dentin degradation in dental adhesive systems.

CONCLUSION

SARS-CoV-2 proteins (*i.e.* Mpro, 3CLpro, PLpro, and the glycoprotein spike) showed an affinity to non-polymerised and polymerised resin composite chains.

FOOTNOTES

Author contributions: Sette-de-Souza PH, Fernandes Costa MJ and Dutra Borges BC designed, performed the experiments, acquired, analyzed, and interpreted the data; all of the authors wrote the manuscript and approved the final version of the article.

Supported by The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES), No. 001.

Conflict-of-interest statement: All authors declare no conflict of interest in publishing the manuscript.

Data sharing statement: Request to the corresponding author.

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S-Editor: Luo ML
L-Editor: A
P-Editor: Zheng XM

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World J Exp Med 2025 March 20; 15(1): 98575

DOI: 10.5493/wjem.v15.i1.98575

ISSN 2220-315x (online)

LETTER TO THE EDITOR

What would Hippocrates have sworn upon witnessing the COVID-19 mandates and mortality paradox

Mina Thabet Kelleni

Specialty type: Medicine, research and experimental

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's classification
Scientific Quality: Grade C
Novelty: Grade C
Creativity or Innovation: Grade B
Scientific Significance: Grade B

P-Reviewer: Dharmshaktu GS

Received: June 29, 2024
Revised: November 16, 2024
Accepted: December 9, 2024
Published online: March 20, 2025
Processing time: 179 Days and 9.3 Hours

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Abstract

For the first time in human history, hundreds of millions of people all over the world have been subjected to compulsory vaccination with a new type of nucleic acid based vaccines in order to keep their jobs or be able to travel due to some notorious coronavirus disease 2019 (COVID-19) mandates. The vast majority of African countries were either initially deprived of these vaccines, or later, a majority of the population was too skeptical to receive them and preferred a safe early treatment pharmacological approach. Yet, Africa had the lowest COVID-19 mortality rate compared to those countries that adopted mass vaccination. This letter to the editor adds African insights that should be helpful in future pandemics to save millions of precious lives.

Key Words: Hippocrates; COVID-19; Nucleic acid based vaccines; COVID mandates; COVID mortality paradox; Kelleni's protocol

Core Tip: From an African perspective, we feel fortunate that we were able to avoid the compulsory nucleic acid-based coronavirus disease 2019 (COVID-19) vaccination and most COVID mandates. This letter to the editor aims to call for a fair assessment of the damage induced by those mandates compared to our African early treatment approach that saved the lives of the African people who were too skeptical to adopt the early global propaganda claiming "perfectly safe and perfectly effective vaccines". This propaganda was later revealed to not be as safe or effective, at least as shown by societies of COVID-19 vaccine victims all over the world, as well as by a COVID-19 mortality paradox that favored Africa over wealthy, heavily COVID-19 vaccinated countries.

Citation: Kelleni MT. What would Hippocrates have sworn upon witnessing the COVID-19 mandates and mortality paradox. *World J Exp Med* 2025; 15(1): 98575
URL: https://www.wjgnet.com/2220-315x/full/v15/i1/98575.htm
DOI: https://dx.doi.org/10.5493/wjem.v15.i1.98575

TO THE EDITOR

I have read with gratitude a quality report that commented on my recently published article in your esteemed *World Journal of Experimental Medicine*[1]. The reviewer graciously acknowledged that the article contained important information regarding the African approach to managing coronavirus disease 2019 (COVID-19) that was supported by numerous bibliographical entries. However, he/she criticized my strong tone and requested more clarification, including numerical data about the patients treated with our approach.

MY VIEWPOINT

I acknowledge that my tone may have been perceived as strong by some reviewers, but I respectfully argue that it should be more appropriately viewed as passionate. Since March 2020 to April 2020, I have witnessed the tragic loss of young friends and colleagues who were treated using Western protocols, whether as I suggested in my previously mentioned article[1], through improper pharmacotherapy or nucleic acid based vaccines that were rapidly approved despite potential data manipulation regarding their safety and efficacy[2]. For over four years, I have been in direct contact with numerous families of these victims who have experienced ongoing sorrow.

I am guilty as charged if you consider me passionate in this published article, but again I respectfully argue that my tone is considered not strong enough when considering the perspective of those who suffered serious adverse effects when forced to be vaccinated by nucleic acid based vaccines and later deemed as a necessary sacrifice or collateral damage until safer vaccines are developed[3,4].

Furthermore, our African approach, particularly the early immune-modulation as best revealed by Kelleni's protocol has proven to be safe, effective and highly adaptive throughout the pandemic compared to the ongoing published data revealing potential serious adverse effects associated with nucleic acid based vaccines[5-7]. Additionally, some reports have suggested that the severe acute respiratory syndrome-coronavirus 2 spike protein, which is also expressed or its antigenic receptor binding domain through nucleic acid-based vaccines in human cells, plays a role in the survival of cancer cells[8], induces lung cancer migration, invasion and progression[9] and may contribute to oncogenesis and tumor growth through DNA damage and induction of chronic inflammation[10].

Regarding the number of patients, in my clinic, I have treated hundreds of patients, especially those at high risk with various co-morbidities as discussed and cited[1]. Moreover, my protocol has been widely adopted by Egyptian colleagues [11], and I have cited in my recently published article in your esteemed journal, as well as in previous peer-reviewed and published articles, supporting academic and clinical data from other countries.

Importantly, I suggest that any fair assessment comparing mortality rates in countries that adopted the Western approach with Africa and other countries that have adopted early immune-modulation should consider our early immune-modulation approach as a significant factor contributing to this COVID mortality paradox.

CONCLUSION

Finally, two decades ago, when I graduated from college of medicine, I recited the well-known Hippocratic Oath with great passion. However, I believe that if Hippocrates were alive today and witnessed the COVID-19 mandates and mortality paradox, he would have added to his famous quote "I will do no harm or injustice to patients" another statement declaring: "I will not be intimidated into staying silent while harm or injustice is being done to innocent patients even if it was committed by my own teachers".

FOOTNOTES

Author contributions: Kelleni MT wrote the content of the manuscript; the author read and approved the final version of the manuscript to be published.

Conflict-of-interest statement: The author declares that there are no competing interests associated with this manuscript.

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S-Editor: Luo ML
L-Editor: A
P-Editor: Zhang XD

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