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# Novel Circulating Exosomal microRNAs for Early Diagnosis and Prognosis of HCV-Associated Liver Diseases

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**SUMMARY** Liver cancers are a major global burden accounting for approximately 782,000 deaths annually. Amongst the various risk factors, hepatitis C virus (HCV) is thought to be responsible for 25% of liver cancers resulting in 350-500,000 deaths annually. Irreversible liver damage resulting in hepatocellular carcinoma (HCC) is often associated with late diagnosis and prognosis. With the current field of research moving towards non-invasive liquid biopsies as opposed to solid biopsies, circulating exosomal miRNAs have received a lot of attention over the years as promising biomarkers for detecting HCV and HCV-associated liver diseases. Although studies in finding novel circulating exosomal miRNAs to improve diagnosis have been successful, a number of key questions still need to be addressed. This paper will explore **(i)** the current methods for detecting HCV and HCV-associated liver diseases and address their benefits as well as limitations; second, **(ii)** the promising miRNAs that have been implicated in such diseases and are currently being studied will be examined with supporting literature; and finally, **(iii)** the obstacles that have slowed the development of using miRNAs as a non-invasive biomarker for HCV-associated liver diseases in a clinical setting will be addressed. This paper will focus on two candidate miRNAs that may be used in clinical settings to detect these diseases. MiRNA-122 is a liver-specific miRNA that is significantly downregulated in HCC and can discriminate between HCC and chronic HCV infection. In contrast, miRNA-500a is significantly upregulated in HCC and has been implicated in preventing the anti-apoptotic activity of Bcl-2 via activation of the BID protein, promoting cancer progression. In the future, the field may move towards developing anti-miRNAs as anticancer agents for disease treatment.

## INTRODUCTION

Liver cancer is the sixth most common cancer and the second leading cause of cancer-related deaths globally [1,2]. With an incidence of approximately 850,000 new cases per year and 782,000 deaths annually, this cancer is considered to be a major health problem. Hepatocellular carcinoma (HCC), a malignant form of primary liver cancer often detected at developed stages, accounts for approximately 90% of all cases [2]. To better understand the molecular pathogenesis of liver diseases, researchers have been focusing on the various associated risk factors which include infection with the Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and alcohol abuse [2].

HCV is a single-stranded, positive-sense RNA virus that replicates in the cytoplasm of hepatocytes [3]. Its methods of transmission include blood-blood contact and, although less common, vertical transmission, in which it has been found that approximately 5% of infants born to mothers with chronic HCV are infected [4]. HCV has a genome size of 9.6 kb which encodes a single polyprotein that is cleaved into structural (S) and non-structural (NS) proteins, the latter of which support viral genome replication and particle assembly [5]. The seroprevalence of HCV varies around the world, however it has been found to be highly prevalent in Central Asia, East Asia, North Africa, and the Middle East [6]. In North America, Europe, and Japan, HCV is known to be the leading cause of HCC [2].

Often called the “silent killer”, HCV is thought to be responsible for 25% of liver cancers whereby infection follows a clinical course from acute to chronic and finally to

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HCC and end-stage liver disease if left undiagnosed [3]. Acute HCV infection is often asymptomatic with approximately 15% of cases showing symptoms such as fatigue, nausea, jaundice, and increased liver enzymes. These symptoms are similar to that of the flu and other common illnesses and thus are often left untreated. As such, approximately 55-85% of cases develop into chronic infection within 6 months if the patient does not receive treatment. If this is the case, the course of infection may then develop into liver fibrosis, followed by cirrhosis, and finally to HCC over several decades (Fig. 1) [3]. The more advanced the cancer becomes, the lower the curative rates and treatment options. As inferred from the approximate 350-500,000 deaths due to HCV-associated liver cirrhosis and HCC, HCV infection is often detected too late and irreversible liver damage results from late diagnosis [3].

To combat poor prognosis and diagnosis, researchers have investigated this issue using circulating exosome-associated micro-RNAs (miRNAs) as non-invasive biomarkers for the early detection of HCV-associated liver diseases. MiRNAs are a family of small non-protein encoding RNAs that recognize target sequences in mRNAs and either destabilize them or inhibit their translation [7]. MiRNAs play an important role in a variety of cellular functions and have been implicated in multiple cancers including liver cancers [8]. A number of miRNAs associated with exosomes show extreme stability in bodily fluids such as saliva, urine, and serum samples [8]. Exosomes are a form of extracellular vesicles (EVs) that are released by most cell types and can carry specific miRNAs associated with a cancer signaling network [9]. They play a role in cell to cell communication by delivering chemical cargo to recipient cells. They also play a role in intercellular communication by carrying proteins and RNAs between neighbouring cells or distant organs [9]. MiRNAs associated with exosomes are protected against RNA degrading enzymes (RNases) and can therefore be stably acquired and stored for research purposes. Due to the ease of access, storing, and stability, exosome-associated miRNAs serve as a promising biomarker to be used for non-invasive cancer diagnosis purposes [10].

## RESEARCH QUESTIONS

Due to the high prevalence of HCV-associated liver diseases, the field of research is moving towards discovering new methods for the early diagnosis of HCV and prognosis of HCV-associated liver diseases. Although data on HCV incidence are not available for most countries, several trends have been inferred from the data that has been reported that has allowed research to advance in this field. Some researchers have looked to the use of exosome-associated miRNAs as a promising tool for a new breakthrough. Although significant research has been done in this field, a number of key questions need to be addressed.

This paper will **(i)** explore the current methods used for detecting HCV and HCV-associated liver diseases and address their benefits as well as limitations. Second, **(ii)** the promising miRNAs that have been implicated in such diseases and are currently being studied will be examined with supporting literature. Finally, **(iii)** the obstacles that have slowed the progression in using miRNAs as a non-invasive biomarker for HCV-associated liver diseases will be addressed.

## PROJECT NARRATIVE

**What are the current methods used for detecting HCV and HCV-associated liver diseases?** HCV is often referred to as the "silent killer" because during the course of infection, it may take decades for the infected individual to develop noticeable symptoms. The hepatotropic virus causes progressive liver damage which might ultimately result in irreversible damage if the infection remains undiagnosed and untreated [11]. The functionality of the liver may never be reestablished, and HCCs often result from severe liver fibrosis and cirrhosis caused by chronic liver inflammation. In Japan, HCV is thought to be responsible for approximately 70% of HCCs whereas in France or Belgium, HCV accounts for 25-30% of HCC cases [12]. The main goal for researchers is to find a method for the early prognosis and diagnosis of liver diseases. In addition, they are working to discover effective treatments to eliminate the infection and decrease the rate of transmission

to other individuals and to reduce the risk of development of HCC and end-stage liver disease.

The primary mode of transmission for HCV is blood-blood contact, primarily via unsafe needle exchange in impoverished areas as well as unsanitary tattoos and piercings [6]. Before the identification of HCV, the main mode of transmission was via blood transfusions and organ transplants. Today, routine anti-HCV antibody screening is done for blood transfusions to eliminate the risk of transmission. Not all countries do this screening, and so it is estimated that 90% of infected individuals are not aware that they are infected [6]. For advanced countries that have multiple testing methods, there are benefits as well as limitations that must be addressed (Table 1). Globally, between 64 and 103 million people are chronically infected, thus indicating the strong need for improved diagnostic tools [11].

Today, risk-based screening is recommended for individuals who are at risk for HCV infection. Two common types of screening currently used are universal screening and selective screening, where the former screens all individuals in a certain category and the latter screens individuals who are at a high risk for becoming infected. Screening for HCV involves measuring antibody to HCV (anti-HCV) in a person's serum using an enzyme immunoassay (EIA) [13]. A positive test for the screening test has a sensitivity of at least 97% and a specificity of 100%. A sensitivity of 97% indicates that the screening test detects at least 97% of individuals exposed to the HCV virus and a specificity of 100% indicates that 100% of those tested without being infected with HCV test negative with no false-positive results [14]. Although this screening test presents both high sensitivity and specificity, the diagnostic accuracy is not optimal. A "positive" test could mean that the individual is a chronic carrier of HCV or is acutely infected, or they have resolved the infection but still have antibodies against the virus. In addition, it takes 6-8 weeks to develop antibodies against HCV, and so an infected individual who tests "negative" may transmit the virus without their knowledge [15]. A positive test for anti-HCV antibodies is therefore not a complete diagnosis of the disease. An additional screening test involves the detection of HCV RNA via polymerase chain reaction (PCR). If HCV RNA is present for at least 6 months, the HCV infection is considered chronic and the patient will require additional tests and examinations to determine the extent of liver damage. Although the

**TABLE. 1 Current methods used to diagnose HCV-associated liver diseases.** At-risk individuals are recommended to undergo an anti-HCV antibody screening test annually. Blood tests, radiographic imaging, and solid biopsies are performed for increasing confidence in diagnoses. Although there are advantages to these methods, there are limitations that highlight the need for new non-invasive diagnostic tests.

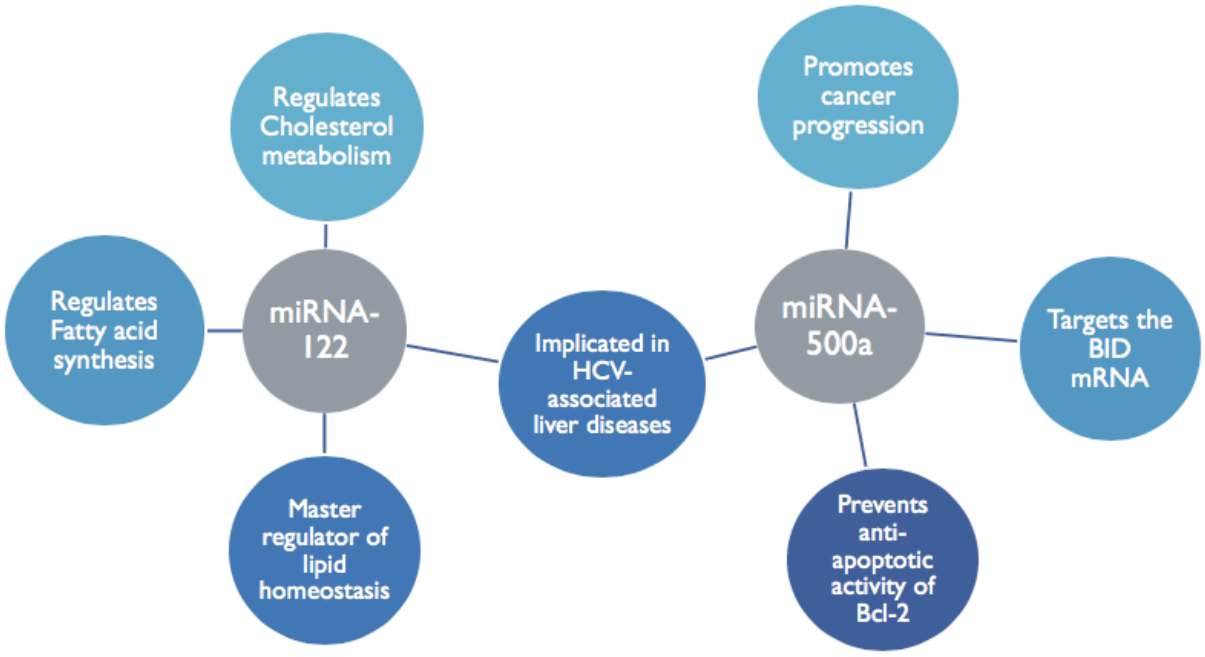
| Methods | Advantages | Limitations |
|---|---|---|
| Anti-HCV Antibody Screening | 97% sensitivity and 100% specificity. Beneficial for at-risk individuals. | Not a definitive test. Need further testing to confirm diagnosis. |
| Blood Test | Accurate. Several blood markers provide information on liver functionality. | Not a definitive test. Need further testing to confirm diagnosis. |
| Radiographic Imaging | Accurate. Can show changes in liver morphology. | High radiation and difficulty in early diagnosis of liver cirrhosis. |
| Solid Biopsies | Can confirm the extent and severity of liver damage. | Can lead to complications including bleeding, tumour seeding, and rarely, mortality. |

HCV screening test is informative, a limitation is that the results are not definitive and the patient will often require additional testing.

The serological and virologic screening tests are often done for those that are at a high risk for infection. For individuals that get infected with HCV but do not fit the criteria for being at a high risk, the diagnosis of HCV infection is often too late and is associated with irreversible liver damage [15]. In these cases, patients that present with symptoms of fatigue, fever, mild abdominal pain, and jaundice will see their doctor, who may recommend a series of tests. A routine blood test will take into account several blood or clinical markers including albumin, bilirubin, as well as alanine transaminase (ALT) and aspartate aminotransferase (AST), which are two enzymes that can be used to indicate liver functionality [16]. To increase diagnostic certainty, radiographic imaging may follow a blood test to observe morphological changes in the liver. Although ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI) are accurate non-invasive diagnostic methods, high radiation and difficulty in early diagnosis of liver cirrhosis are two limitations that researchers are working to address [17]. Finally, a solid liver biopsy may then be done by removing liver tissue to determine the extent and severity of liver damage. For decades, liver biopsies had been regarded as the gold standard for the diagnosis of liver cirrhosis, however there are several limitations including subjective interpretation, invasiveness, bleeding, tumour seeding, and rarely, mortality [17]. In recent years, non-invasive methods for early prognosis and diagnosis of HCV-associated liver diseases have been extensively researched. Researchers have turned to liquid biopsies by using circulating exosomal miRNAs as a non-invasive biomarker that can be stably found in serum, urine, and saliva. The following research question will explore two miRNAs that have gained attention over the years as potential candidate biomarkers of HCV infection and its associated liver diseases.

**Which circulating exosomal miRNAs can be used to detect HCV-associated liver diseases?** MiRNAs are a class of small non-encoding RNAs of about 20-24 nucleotides that regulate gene expression at the post-transcriptional level by inhibiting translation or accelerating mRNA decay [18]. They play an important role in a variety of cellular

**FIG. 2 Respective roles of miRNA-122 and miRNA-500a.** MiRNA-122 and miRNA-500a have both been implicated in HCV-associated liver diseases. MiRNA-122 was found to be significantly downregulated in HCC and miRNA-500a was found to be significantly upregulated in HCC.



functions and have been implicated in multiple cancers [18]. Some MiRNAs are packaged within circulating exosomes, a form of extracellular vesicle (EV) that allows for intercellular transport and delivery [9]. In addition to providing a means of targeting their content to neighbouring cells and distant organs, exosomes protect their cargo from the environment. In the case of miRNAs, exosomes protect them against RNases, allowing for these miRNAs to be acquired and stored for research purposes [9]. Additionally, exosomal miRNAs have been extensively studied because of their remarkable stability in serum, saliva, and urine samples and thus serve as a promising non-invasive biomarker for detecting cancer or other diseases [19]. Two miRNAs that have been implicated in HCV-associated liver diseases are miRNA-122 and miRNA-500a (Fig. 2). Both miRNAs have a set of targets and can regulate multiple signaling pathways involved in cancer development.

MiRNA-122 has been found to play an important role in HCV replication. It is a liver-specific miRNA and is highly abundant in hepatocytes, with over 60,000 copies per cell [20]. It is a master regulator of lipid metabolism whereby it inhibits proliferation and migration, and promotes hepatocyte death [8]. MiRNA-122 directly downregulates cyclin G1 expression, and since cyclin G1 regulates p53 protein stability, this consequently results in the elevation of p53 and its transcriptional activities [21]. Kutay *et al.* found that the downregulation of miRNA-122 is associated with hepatocarcinogenesis and can therefore be a potential biomarker for liver cancers. The researchers analyzed the RNA from human HCC samples and found that miRNA-122 was significantly downregulated in 50% of the tumours compared to non-malignant liver tissue from the same individuals [22]. Follow-up studies found that the loss of miRNA-122 contributes to the malignant phenotype of HCC cells since miRNA-122 plays a central role in the regulation of these cells by suppressing hallmark HCC oncogenic genes [23, 24]. Moreover, miRNA-122 can discriminate between HCC patients and patients with chronic HCV infection as it was found that miRNA-122 was significantly increased in chronically HCV-infected livers [24]. Although more research needs to be done to confirm whether miRNA-122 can be used as a diagnostic marker, results indicate that it serves as a potential tool for the differential diagnosis of HCV-associated liver diseases.

Another miRNA that researchers have been focusing on is miRNA-500a. Although the underlying mechanisms of its actions are not well understood, miRNA-500a serves as a promising biomarker because of its role in targeting the 3′ untranslated region of the BH3-interacting death agonist (BID) mRNA [25]. Upon the activation of the BID protein by Caspase-8, truncated BID (tBID) is transported to the outer mitochondrial membrane to combine with Bcl-2 and inhibits its anti-apoptotic effect via the tBID-mitochondrial apoptosis pathway. Through these mechanisms, miRNA-500a expression was found to be upregulated in HCC tissues, with high expression correlating with poor prognosis of the

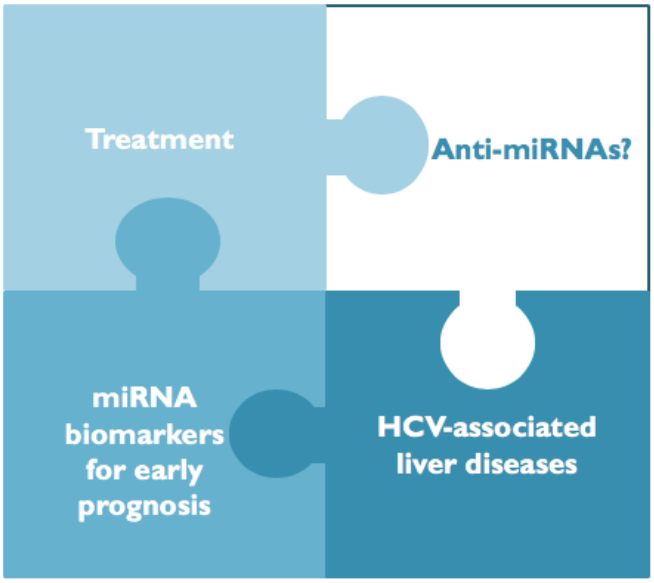



**FIG. 3 Future directions for the use of miRNAs and anti-miRNAs for disease treatment.** The use of anti-miRNAs as antitumor therapeutic molecules has attracted a lot of attention over the years. Future studies will need to confirm the efficacy of candidate anti-miRNAs as anticancer agents for the treatment of HCV-associated liver diseases.

cancer. The targeting of miRNA-500a to BID promotes cancer progression *in vivo* and *in vitro*, and thus miRNA-500a serves as both a potential diagnostic and prognostic biomarker and potential therapeutic target for HCC patients [25]. By investigating all of the targeting pathways miRNA-500a is involved in, future studies may identify additional roles that can help elucidate whether this miRNA can serve as a biomarker.

**What are the obstacles in using miRNAs as a non-invasive biomarker for HCV-associated liver diseases?** Although the use of miRNAs as a non-invasive biomarker for detecting HCV-associated liver diseases is promising, there are obstacles that have made their implementation in clinical settings a challenge. There is currently no effective biomarker for the early diagnosis of the primary form of liver cancer, HCC. One of the most important reasons for using miRNAs as a non-invasive biomarker is to detect HCV-associated liver diseases before they cause irreversible damage to the liver. Non-invasive biomarkers are preferable because invasive approaches often lead to complications including tumour seeding and bleeding. An obstacle in finding the ideal miRNA for diagnosing advanced liver diseases such as HCC is the limited knowledge of their pathophysiology due to their heterogeneity. One of the biggest challenges is finding novel miRNAs that have the potential to be used precisely and effectively in detecting HCV-associated liver diseases [8].

Multiple miRNAs are dysregulated in cancer cells and a single miRNA can target multiple mRNAs. Identifying the dysregulated miRNAs in liver cancer and uncovering their roles in cancer development is an ongoing process. There are 20,000 genes in the human genome and approximately 2,500 miRNAs. This implies that a single miRNA has multiple mRNA targets and likely plays a variety of roles in a number of cellular functions and diseases. This has been a challenge for researchers when it comes to deciding which miRNAs they should choose as a biomarker. For example, miRNA-21 is one of the most frequently upregulated miRNAs in multiple cancers including breast cancer, cervical cancer, colon cancer, and liver cancer [26]. Upon finding an attractive candidate miRNA, researchers need to determine whether the miRNA is highly specific to liver diseases only, or whether it is implicated in different disease pathways. If a patient has their blood tested and the results show an upregulation of miRNA-21, this may implicate a number of cancers and so they will need to undergo further tests. As such, using miRNA-21 alone to detect liver cancer would not be considered ideal. Rather, it can be used with a number of other biomarkers for increasing the specificity of the diagnosis. This leads to the question of whether there are other biomarkers that also play a role in multiple cancers and if so, should they be used for diagnostic testing? With the many roles that a single miRNA may have in a variety of diseases, researchers need to determine whether chosen miRNAs are specific biomarkers of the disease in question.

Another obstacle that researchers have faced is the fact that most of the potential miRNA biomarkers exist in both healthy individuals and diseased patients [27]. In finding a single ideal biomarker or a panel of biomarkers, researchers will need to determine whether the miRNAs are significantly dysregulated in diseased patients. MiRNA-122 and miRNA-500a were found to be significantly downregulated and upregulated in HCC, respectively [22,25]. Future candidate miRNAs will have to be extensively studied to elucidate the significance of their dysregulation.

## CONCLUSIONS

The global burden of liver cancers is increasing, and our attempts at understanding and treating this complex disease have met with significant obstacles [2]. Global efforts to eradicate HCV are underway, however other etiologies continue to cause liver cancers, which remain the second leading cause of cancer-related deaths globally [28]. Up until the mid-20$^{th}$ century, chronic liver diseases could often be diagnosed with certainty only at an advanced stage in which irreversible damage to the liver had already been done [29]. HCV is thought to be responsible for 25% of liver cancers [3]. A malignant form of primary liver cancer, HCC, is often detected at advanced stages and accounts for approximately 90% of all cases of liver cancers [2]. With no effective biomarkers for the early diagnosis and prognosis of HCV-associated liver diseases, there is a global need to expand HCV

diagnostic testing. Current screening methods require multiple tests to increase confidence in diagnosis and are often not reliable. In the case of solid liver biopsies, severe complications such as bleeding, tumour seeding, and death may arise [17]. To combat the poor prognosis of HCV-associated liver diseases and complications arising with solid biopsies, the field of research has moved towards the use of liquid biopsies for diagnosis. Researchers have looked to using circulating exosomal miRNAs as a non-invasive biomarker due to their role in a variety of diseases, including liver cancers [8].

Although a lot of information about the replicative mechanism of HCV remains to be confirmed, research shows that its replication may be regulated by miRNAs. MiRNAs are stable when associated with exosomes and can be accessed via blood, urine, or saliva samples. As such, researchers have been studying how miRNAs can be used as potential biomarkers for detecting HCV-associated liver diseases. Two miRNAs, miRNA-122 and miRNA-500a, are currently being studied as potential biomarkers due to their role in regulating signaling pathways involved in liver cancer development. MiRNA-122, a liver-specific miRNA, is an attractive miRNA that has received a lot of attention over the years [19, 20, 28]. It plays an important role in HCV replication and is associated with hepatocarcinogenesis [21]. It is found to be significantly reduced in patients with HCC and, in contrast, significantly increased in patients with chronic HCV infection. Because of its ability to discriminate between two HCV-associated liver diseases, it serves as an attractive biomarker that has the potential to be implemented in clinics.

Although miRNAs are attractive biomarkers that can be used for improved prognosis and diagnosis, there have been challenges in implementing them in a clinical setting. With 20,000 genes in the human genome and 2,500 miRNAs, a single miRNA can target multiple mRNAs. As such, one challenge is finding a tissue-specific miRNA that can accurately discriminate between multiple diseases. Further studies will need to investigate all targeting pathways associated with the candidate miRNA in order to elucidate its potential as a biomarker.

In addition to being used as diagnostic biomarkers, the use of anti-miRNAs as antitumor therapeutic molecules has attracted a lot of attention over the years (Fig. 3). One study found that one dose of anti-miRNA-122 leads to a significant decrease in HCV RNA by preventing viral spread [31]. Still an active area of research, the efficacy of anti-miRNAs as anticancer agents for disease treatment remains to be fully confirmed. Current findings implicate a breakthrough for the therapeutic potential of miRNAs and anti-miRNAs, and with advancing research in the field, combating the global burden of HCV-associated liver diseases is becoming more feasible.

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